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EVALUATION EFFECT OF COMBINED ANTIBIOTICS GENTAMICIN AND MEROPENEM MIXTURE ON THE EXPRESSION OF VIRULENCE -RELATED GENES IN *KLEBSIELLA PNEUMONIA* ISOLATED FROM HOSPITALIZED PATIENTS

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Abstract: The advent of Klebsiella pneumoniae that is resistant to multiple drugs (MDR) poses a significant threat to public health, particularly in regions with high antibiotic misuse. This study aims to evaluate the synergistic effects of gentamicin and meropenem on the expression of virulence-related genes in K. pneumoniae isolates biofilm-associated genes (fimH, and wabG). Understanding the impact of antibiotic combinations on gene expression may offer insights into novel therapeutic strategies against MDR pathogens.

The increasing prevalence of multidrug-resistant (MDR) K. pneumoniae strains has rendered many conventional antibiotics ineffective, leading to limited treatment options and increased mortality rates. Efflux pumps and biofilm formation are critical mechanisms contributing to antibiotic resistance and virulence in K. pneumoniae. Combining antibiotics, such as gentamicin and meropenem, may exhibit synergistic effects that suppress these resistance mechanisms. This study aims to fill the knowledge gap regarding the impact of gentamicin and meropenem combination on the expression of these critical genes in *K. pneumoniae* isolates from Iraq. This study demonstrates that gentamicin and meropenem elicit distinct transcriptional responses in *K. pneumoniae*, particularly in genes encoding adhesion and LPS synthesis. The downregulation of fimH by gentamicin and wabG by meropenem highlights the need for a nuanced understanding of how antibiotics affect not only resistance phenotypes but also virulence gene regulation. These findings have implications for designing targeted antimicrobial therapies and inform strategies to mitigate the clinical impact of multidrug-resistant *K. pneumoniae* strains.

Keywords: Antibiotics, Biofilm, genes expression, PCR

Introduction

Numerous nosocomial infections are caused by the Gram-negative bacteria *Klebsiella* pneumoniae. The emergence of MDR strains has been ascribed to processes like biofilm development and efflux pumps. Antibiotics are actively expelled by efflux pumps such as OqxAB, which lowers their intracellular concentrations and effectiveness (Huang *et al.*, 2013). Persistent infections are a result of biofilms shielding bacterial colonies from harsh conditions, such as antibiotic treatments (Shadkam *et al.*, 2019).

Antibiotic combination have been investigated to fight MDR the microbes. Gentamicin's antibacterial effectiveness can be increased by mixing it with other antibiotics, according to studies. For example, gentamicin and plumbagin worked in concert to combat *K. pneumoniae* that was resistant to carbapenem (Zhang et al., 2020). Similarly, in mouse studies, meropenem plus ceftazidime/avibactam showed greater efficacy (Zhou *et al.*, 2024). It is essential to comprehend how K. pneumoniae responds genetically to different antibiotic combinations. Increased resistance has been associated with overexpression of the efflux pump genes (oqxA, oqxB) (Li *et al.*, 2018). Resistance mechanisms are modulated by regulatory genes such as marK (Wang *et al.*, 2020). According to Johnson *et al.* (2017), biofilm-associated genes like fimH enhance adhesion and colonization, two crucial stages in the formation of an infection. Antibiotic resistance is increasingly acknowledged as a global health concern in contemporary medicine, following more than 70 years of widespread use of antibiotics to treat infectious infections (Watkins, Smith, and Bonomo 2016). As common natural occupants of our microbiome, the sharp rise in the prevalence of infections caused by Enterobacteriaceae-related multidrug-resistant (MDR) and exceptionally drug-resistant (XDR) pathogens is cause for serious concern. Additionally, infections brought on by these genotypes are frequently linked to ,with high mortality rates, prolonged hospitalization and costs (Giske et al., 2008). The process of antibiotic resistance is complex and multifaceted (Watkins et al. 2016). From a bacterial standpoint, however, it represents evolution in action, as repeated exposure to antibiotics results in the emergence of numerous genetic processes due to selective pressure (Davies and Davies 2010). The rise of MDR and XDR Enterobacteriaceae strains (Magiorakos *et al.* 2012) that are resistant to almost all antibiotics and have no viable treatment options is the result of this ongoing evolution throughout time (Hersh *et al.* 2012). For example, recent antibiotic use, catheter carriage, and extended hospitalization are risk factors for acquiring a *K. pneumoniae* infection (Boyle and Zembower 2015). As a result, *K. pneumoniae* is considered a common and even dominant nosocomial pathogen (Pitout, Nordmann, and Poirel 2015). K. pneumoniae transmits from person to person in hospitals. It's interesting to note that various carriers have different capacities for spreading CRKP; only a small percentage of them—likely those with greater rectal *K. pneumoniae* concentrations—are largely to blame for environmental contamination (Lerner *et al.* 2015). According to Conlan *et al.* (2016), colonization may continue for two to four years. *K. pneumoniae* carriers do not necessarily get sick. The spread of *K. pneumoniae* is facilitated by its capacity to live on hospital equipment surfaces. One striking instance of KPC-2 ST11 K. pneumoniae successfully spreading without patient-to-patient contact is an outbreak linked to ventilators in China (Hu *et al.* 2016).

Materials and Methods

1. Sample Collection:

Clinical isolates of *K. pneumoniae* will be obtained from patients in Iraqi hospitals such as Al-Yarmouk Teaching Hospital, Al-Habibiya Hospital, and Al-Kindi Hospital. During a period between January to March 2025, 200 samples have been collected from clinical sources (including urine, sputum, burn wounds, blood, and soft tissue sites) to evaluate the prevalence of *Klebsiella pneumoniae*.. Samples were collected from patients who visited hospitales clinical by both sex. All these specimens were collected with sterile condition, then placed into collection tubes and were swirled in buffer solution and transported to laboratory for isolation and identification. All colonies from primary cultures were purified by subculture on different media ,then identified (Aqel et al., 2017). Antibiotic Susceptibility Testing: Minimum inhibitory concentrations (MICs) for gentamicin and meropenem will be determined using the broth microdilution method. Synergy Testing: Checkerboard assays will assess the synergistic effects of the antibiotic combination. Gene Expression Analysis: Quantitative real-time PCR (qRT-PCR) will measure the expression levels of target genes before and after treatment.

2. Experimental Design:

Isolation and Identification: Use common microbiological methods to gather and validate K. pneumoniae isolates. Bacterial isolation Standard bacteriological methods were used to isolate and identify facultative anaerobic, anaerobic, and aerobic microorganisms (Ghanizadeh et al., 2021). Samples were spread out on Blood and MacConkey agar to isolate the bacteria. All plates were then incubated aerobically and anaerobically for the strict anaerobic bacteria using an anaerobic jar with a Gas-Pak, and facultative and microaerophilic bacteria using an anaerobic jar with a candle. All plates were then incubated at 37°C for 1–5 days. The chosen species were purified by sub-culturing on the same media used for primary isolation following the conclusion of the incubation period. After being cultivated on Brain Heart Infusion Agar, each colony with a variety of morphologies was separated, purified, and identified; K. pneumoniae was one of the thirty bacteria that were extracted. The physical characteristics on culture media, microscopic analysis, and biochemical tests were used to identify the species (Forbes et al., 2007; Brooks et al., 2013).

Antibiotic Treatment: Expose isolates to gentamicin, meropenem, and their combination at sub-inhibitory concentrations.

RNA Extraction: Extract total RNA from treated and untreated bacterial cultures cDNA Synthesis and qRT-PCR: Synthesize cDNA and perform qRT-PCR to quantify gene expression levels.

Data Analysis: Analyze gene expression changes using the 2-ΔΔCt method, with appropriate statistical tests to determine significance.

Important note: Due to the high costs of testing genes, we will only select two of the genes

3. Genotyping Detection

According to Ali et al. (2018), a boiling process was used to create the DNA template. In short, five separate colonies of overnight growth bacteria were completely suspended in two milliliters of distilled water and then heated for ten minutes in a water bath. Following centrifugation, the supernatant served as the PCR's template DNA.

4. PCR Amplification

The PCR amplification procedure for the genetic level to detecting Klebsiella spp local isolates table (1) by follows step: Final volume for PCR mixture was 25 µl (12.5 of Master Mix 2x, 5 µl template DNA, 1 µl primers for each forward and reverse primer, finally, 5.5 µl nuclease free water) in uniplex PCR Eppendorf tubes but amount changed in multiplex PCR, mixed briefly via vortex then been placed in thermocycler polymerase chain reaction. Annealing were set, the program used for each PCR mixture was illustrated in the table. (2).

Table 1. Primers Oligonucleotide Sequences Used in This Study.

Primer	Direction	5 → 3	Size bp
16SrRNA	F	TCC TAC GGG AGG CAG CAG T	448
	R	GGA CTA CCA GGG TAT CTA ATC CTG TT	
fimH	F	TGCAGAACGGATAAGCCGTGG	**508**
	R	GCAGTCACCTGCCCTCCGGTA	
wabG	F	GGTTGGGTCAGCAATCGTA	**683**
	R	ACTATTCCGCCAACTTTTGC	

Table 2. Amplification program of primers

Amplified gene	Initial denaturation	No. of cycle	Denaturation	Annealing	Elongation	Final extension
16SrRNA	95°C/ 4min	32	94°C/ 30 sec	55°C/45 sec	72°C/50 sec	72°C/5min
fimH	95°C/ 4min	32	94°C/ 30 sec	63°C/45 sec	72°C/60 sec	72°C/5min
wabG	95°C/ 4min	32	94°C/ 30 sec	58°C/45 sec	72°C/60 sec	72°C/5min

5. Primer dilution

The oligonucleotide primers which in lyophilization status were dissolved and diluted first in free nuclease D.S.D.W (amount according to recommended of manufactured company) to obtains 100 picomol/µl, then this stock was diluted in free nuclease D.D.W to obtain nearly 10 picomol/µl. This technique accorded on all primers in this study, as listed in table (2). The Specifications primers of all genes provided from Alpha DNA Company, USA.

6. Agarose Gel Electrophoresis

The amplified PCR products were detected using gel electrophoresis and observed using a UV transilluminator documentation system and Ethidium bromide dye. where 100 milliliters of 1x TBE buffer were mixed with one gram of agarose. After covering the gel with TBE buffer, the tank was sealed, and the electrophoresis for the DNA extract and PCR products ran for an hour at 5 volts per centimeter of the gel. The agarose gel was taken out of the tank during electrophoresis, examined with a UV transilluminator documentation system, and then captured on camera with a digital camera (Mishra *et al*., 2010). PCR product, 5µl of the monoplex PCR products were placed into each well (Morovat *et al*., 2009). To measure the size of the PCR product, DNA ladders were always run in tandem with each electrophoretic run. The UV transilluminator documentation system was used to visualize the DNA bands.

7. Antibiotics Susceptibility Test

a. Disk Diffusion Method

The antibiotics susceptibility test was carried out by following Kirby-Bauer method described by CLSI ,2020, (Table 2-5).Out of a pure and fresh culture, a few

colonies were transferred to a sterile test tube that has 5 ml of normal saline. Then it was compared with 0.5 McFarland standards (1.5x108 CFU/ml). A portion of bacterial suspension was carefully transferred by use of a sterile cotton swab. Then it was evenly spread on Mueller-Hinton agar medium. After that, plates were left to dry. Antibiotic discs were placed on the inoculated plate by use of a sterile forceps (duplicate was done for each antibiotic). The plates were inverted and incubated at 37 ºC for 18-24 hrs. A metric ruler was used for measuring the inhibition zones around the discs Figure(1).

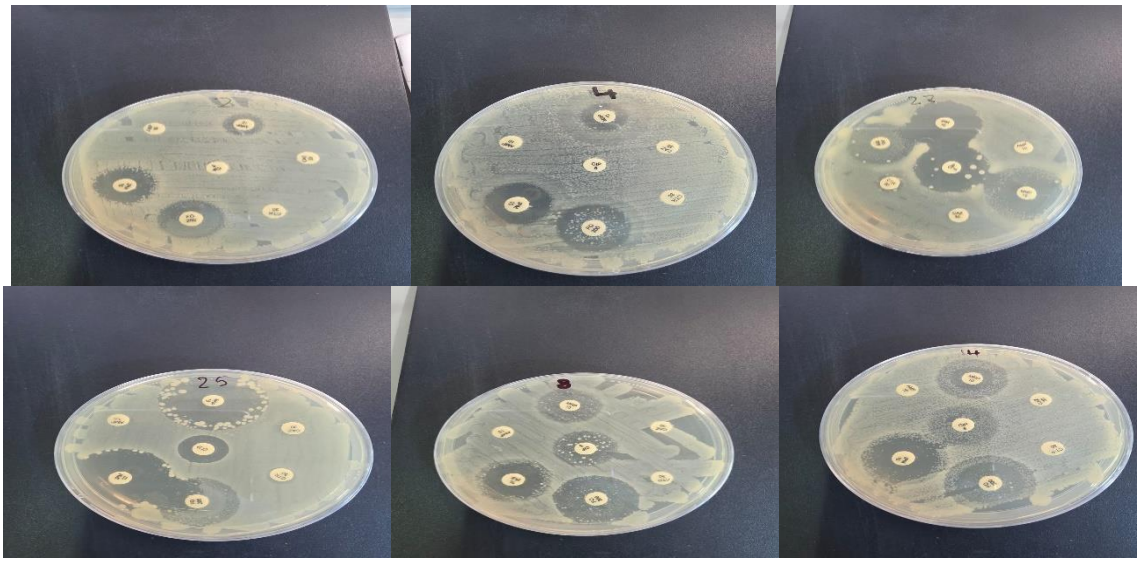


Figure 1. bacterial species growing on specific selective media.

Figure (1) shows bacterial species growing on Mueller-Hinton agar plates with antibiotic discs used in the disk diffusion method. Clear inhibition zones can be observed around certain discs, indicating bacterial susceptibility to those antibiotics, while the absence or reduction of zones reflects resistence. This demonstrates how the Kirby-Bauer method is applied to assess antibiotic sensitivity.

b. cDNA synthesis from RNA

RT-qPCR protocol

This is the main step in the project that is composed of two stages, the first is done by synthesis of cDNA from RNA, therefore, all the component was thawed, mixed and the reaction mixture was prepared and performed in an optimized condition according to the instructions of the manufacturing company, follows:

1- Ten microliters from each extracted total RNA sample were added to a new PCR tube.
2- cDNA Mastermix that contains dNTPs, buffer, and other essential components was added as 4ul for each sample.
3- The volume was completed up to 20ul by adding 6ul dd D.W., mixed briefly by centrifugation.

5. The mixture was amplified and annealed at 25°C for 10 minutes, followed by extension at 42°C for 15 minutes and finally heat inactivation of reverse transcriptase at 85°C for 5 seconds.

The second stage of this process was done by selecting the cDNA sample from bacteria and the control at the same run. Each sample consisted of three PCR tubes, one designated for each gene: oqxA, oqxB, and 16sRNA, which was regarded as a housekeeping gene in this study. Quantitative detection was based on the

fluorescent intensity of SyberGreen. The reaction mixture consists of components with their quantity as mentioned in table (3) below:

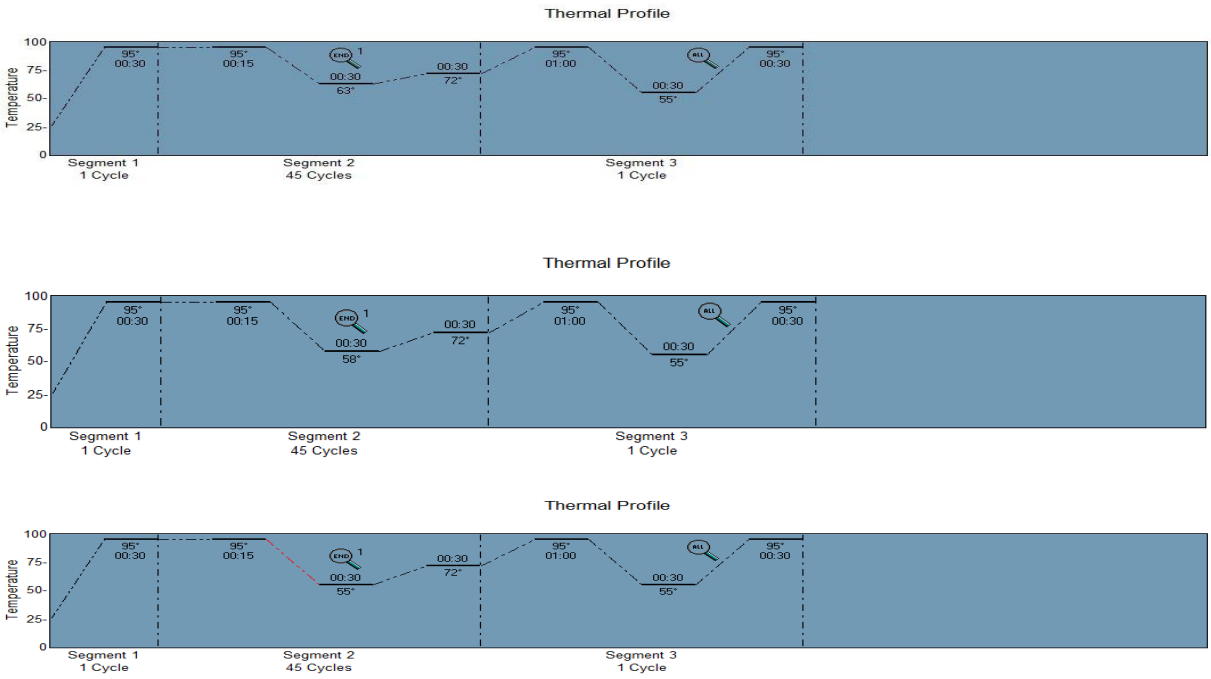
Table 3. components of RT-PCR reaction

Component	20 ul Reaction
2×Universal Super SYBR Master Mix	10 ul
Forward primer (10 μM)	0.5ul
Reverse primer (10 μM)	0.5ul
Template DNA	2ul
Nuclease-free Water	7ul

The PCR tubes were centrifuged rapidly to remove bubbles and collect the liquid (1 minute at 2000xg, then the program for Real-Time PCR was set up with the indicated thermocycling protocol as shown in the table (4).

Table 4. RT-PCR conditions

Cycle step	Temperature	Time	cycles
Initial denaturation	95ºC	30 seconds	1
Denaturation	95ºC	15 seconds	
Annealing	**55**,58,63ºC	30 seconds	45
Extension	72ºC	30 seconds	
	95ºC	1 minute	
Melting curve	55ºC	30 seconds	1
	95ºC	30 seconds	



c. Gene expression analysis

The qRT-PCR data outcomes have been verified based on a direct comparison of the Ct values between the target and reference (housekeeping gene and some efflux pump genes) genes. The genes were assessed using the following equations, which are based on the relative quantification of gene expression levels using Livak formula defined by as illustrated in the equations below:

The Δ G of the target gene to the reference gene was assessed for each sample, including both the calibrator sample and the unknown samples.

Δ G = Ct target gene – Ct reference gene

1. The variance in Δ Ct values between the unknown and the calibrator was determined.

   ΔΔCt= (Ct target gene – Ct reference gene) sample – (Ct target gene – Ct reference gene) control.

2. The sample's normalized target quantity was equalized to $2^{-\Delta\Delta Ct}$. The expression levels of the samples were compared by applying this value:

   Fold change = $2^{-\Delta\Delta Ct}$

3. To compare antibiotic-treated bacterial isolates with untreated ones, the relative changes in mRNA expression levels were measured using the comparative threshold cycle (CT) value method (= 2 –ΔΔCt).

8. Statistical analysis

The obtained data were subjected to one way analysis of variance (ANOVA) test to compare the means of various groups with each other. For the descriptive analysis subjected Chi-square test was used. were expressed as mean ± standard deviation (SD) and values of $p>0.05$ were considered statically non-significant while $p<0.05$ and <0.01,0.001 were considered significantly different, highly significantly different respectively. The statistical analysis was carried out by SPSS (V.20).

Results and Discussion

1. Prevalence of *Klebsiella pneumoniae* in Different Clinical Samples.

A total of 200 bacterial isolates were collected from various clinical sources, including urine, sputum, burn wounds, blood, and soft tissue sites, to evaluate the prevalence of *Klebsiella pneumoniae*. Among these, 30 isolates tested positive for *K. pneumoniae*, representing an overall positivity rate of 15%. The distribution of positive *K. pneumoniae* isolates varied by source. Urine samples showed the highest proportion of positive isolates (18%, 9/50), followed by sputum and burn wound samples, each with 16.67% positivity (10/60 and 5/30, respectively). Blood samples exhibited a positivity rate of 12.5% (5/40), whereas soft tissue sites had the lowest prevalence, with only 5% (1/20) of isolates testing positive (table 5).

Odds ratios (ORs) were calculated to assess the likelihood of detecting *K. pneumoniae* in isolates from each source relative to the others. The OR for urine isolates was 1.36 (95% confidence interval [CI]: 0.55–3.14), indicating a slightly higher, meaning *K. pneumoniae* is 1.36 times as likely to be found in urine isolates compared to the baseline, but not statistically significant, ,likelihood of *K. pneumoniae* presence. Similarly, sputum (OR = 1.21; 95% CI: 0.50–2.73) and burn wound isolates (OR = 1.18; 95% CI: 0.36–3.20) showed elevated odds without statistical significance. Blood (OR = 0.79; 95% CI: 0.25–2.08) and soft tissue isolates (OR = 0.31; 95% CI: 0.01–1.60) had lower odds, with soft tissue showing the lowest likelihood of *K. pneumoniae* detection.

Fisher's Exact Test p-values for all sources exceeded 0.05, confirming that the observed differences in *K. pneumoniae* prevalence across sample types were not statistically significant. These findings suggest that the presence of *K. pneumoniae* is relatively uniform across clinical sources in this study population.

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Table (5). The total number of samples

Source	Total number of isolates	No. of positive *K. pneumoniae* Isolates	Percentage of positive *K. pneumoniae* isolates	Odds Ratios (OR) with 95% CI and p-values
Urine	**50**	9	18 %	1.3571315 (0.54711138 3.141555) 0.4978344*
Sputum	60	10	16.67 %	1.2066390 (0.50494909 2.728206) 0.6697663*
Burn	**30**	5	16.67 %	1.1827231(0.36443442 3.198875) 0.7831146*
Blood	**40**	5	12.50 %	0.7891236 (0.24716311 2.078708) 0.8054262*
soft tissue sites	**20**	1	5 %	0.3109918(0.01262185 1.600273) 0.3204145**
Total	**200**	30	15 %	p-values 0.71

*** Chi-square test p-values ; ** Fisher's Exact Test p-values**

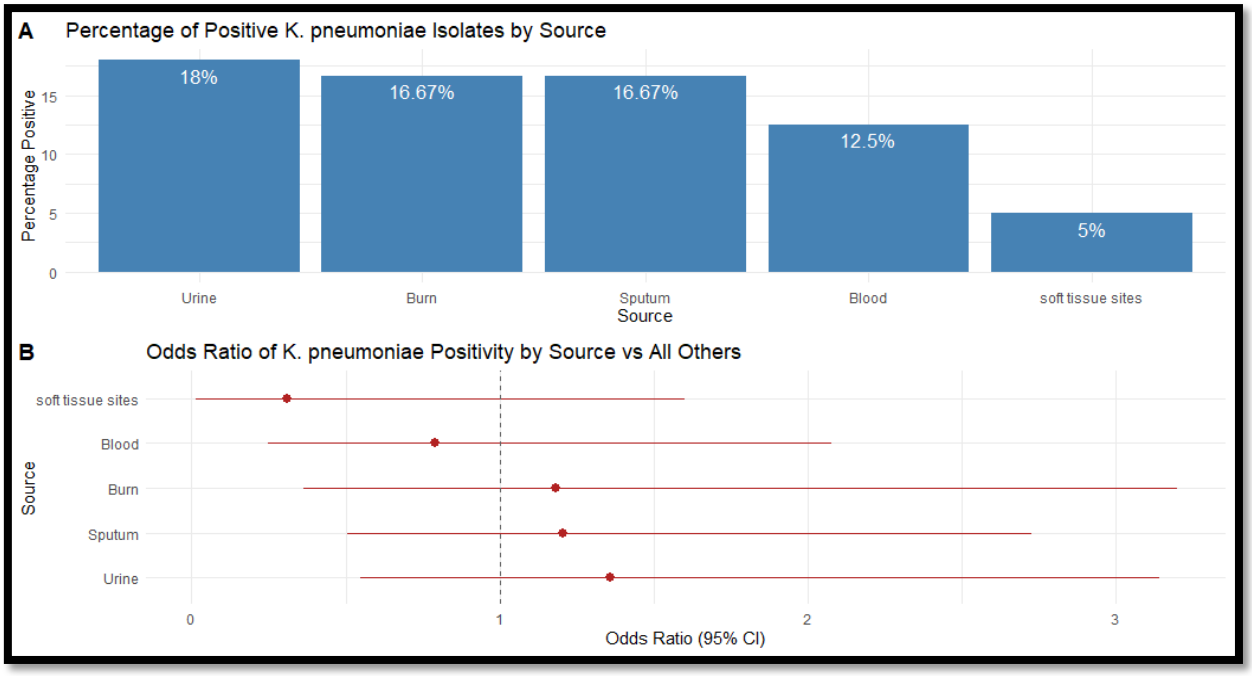

Figure 2. Percentage of positive growth

Figure (2) shows that the highest positivity of *K. pneumoniae* was in urine samples (18%), followed by burn and sputum (16.7%), The odds ratios indicate no statistically significant differences between source (P= 0.71).

2. Distribution of Bacterial Growth Types

The analysis of bacterial growth types revealed three distinct outcomes: *Klebsiella pneumoniae* (15%), negative growth (10%), and other bacterial species

(75%). As shown in Figure ---, the majority of the isolates demonstrated growth of bacterial species other than *K. pneumoniae*, indicating a broad diversity of bacterial presence (table 6).

Statistical analysis was conducted to compare the distribution among the three categories. A significant difference was observed between *K. pneumoniae* and negative growth ($p < 0.05$), as well as a highly significant difference between *K. pneumoniae* and other bacterial species ($p < 0.01$). However, no statistically significant difference was found between the negative growth group and the other bacterial group ($p > 0.05$), suggesting that the rate of negative growth does not differ substantially from that of other bacterial growth in this dataset.

These findings highlight the relatively low isolation rate of *K. pneumoniae* in comparison to other bacterial species, while also emphasizing the presence of a considerable proportion of samples that showed no bacterial growth.

Table 6. The positive and negative growth of total number od samples.

Number of sample	Number	Percentage	Statistic
No. of positive *K. pneumoniae* Isolates	30	15	X-squared = 157, df = 2, p-value < 2.2e-16
No. of positive growth other bacteria	**150**	75	
No. of negative growth	**20**	10	

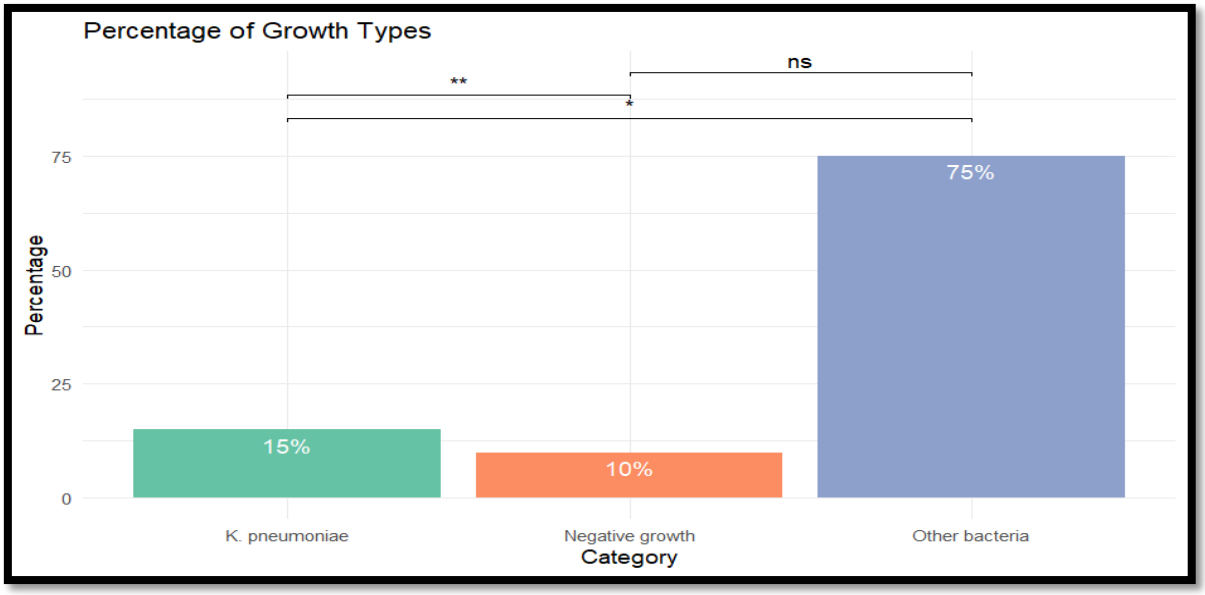

Figure 3. percentage of growth type □ * indicates $p < 0.05$ (significant); □ ** indicates $p < 0.01$ (highly significant); □ ns means *not significant*

Figure (3) shows the distribution of growth types among the clinical isolates: *K.pneumoniae* accounted for 15%, negative growth for 10%, and other bacteria for the majority (75%). Statistical analysis indicates a highly significant difference between K.pneumoniae and other bacteria ($P< 0.01$), while the comparison between negative growth and other bacteria was not significant (ns).

3. Gender Distribution of *Klebsiella pneumoniae* Isolates

Out of 200 total clinical isolates, 90 were obtained from male patients and 110 from female patients. Among males, 10 isolates (11.11%) were positive for *Klebsiella pneumoniae*, whereas among females, 20 isolates (18.18%) tested positive (table 7).

To evaluate the association between gender and *K. pneumoniae* positivity, an odds ratio (OR) was calculated. The OR for male patients was 0.57 (95% confidence interval: 0.24–1.27), indicating a lower likelihood of *K. pneumoniae* isolation compared to females. However, this association was not statistically significant, as indicated by a Fisher's Exact Test p-value of 0.232.

Although the data show a numerically higher prevalence in females, with a male-to-female ratio of approximately 1:2 among *K. pneumoniae* positive cases, the findings do not support a statistically significant difference in isolation rates between genders. Therefore, gender does not appear to be a strong predictor of *K. pneumoniae* presence in this study.

Table 7. Number of of positive *K. pneumonia* Isolates in male and femal samples

Gender	No. of isolate	No. of positive *K. pneumonia* Isolates	%	*P-value / Odds Ratio (95% CI)*
Male	**90**	10	11.11 %	0.232 / 0.57 (0.24-1.27)
Female	**110**	20	18.18 %	
Total	**200**	30		Male to Female ratio: 1:2

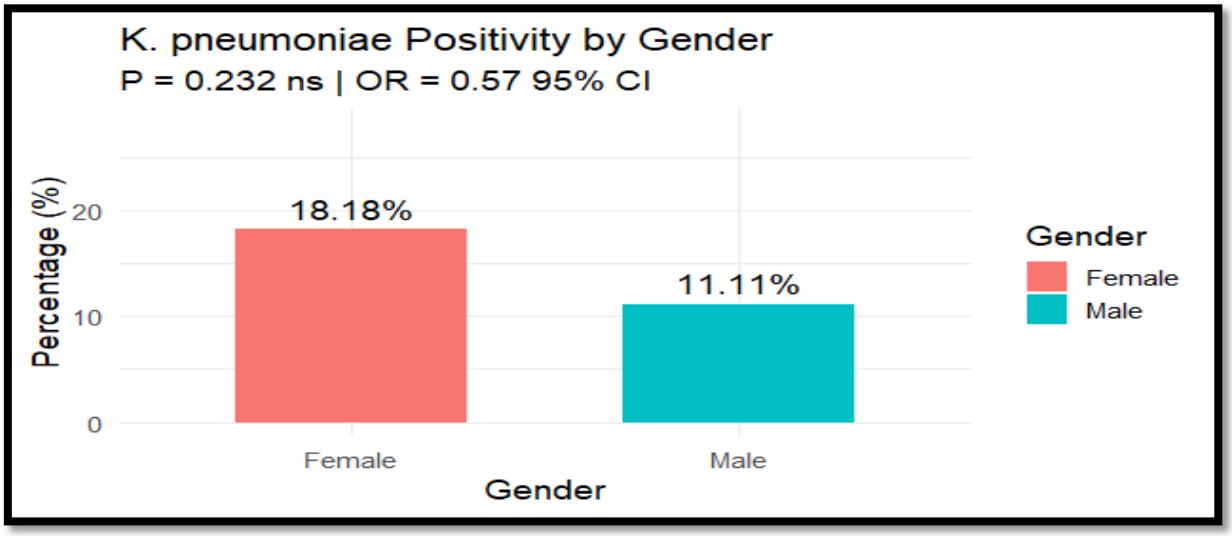

Figure 4. K.Pneumoniae Positivity by gender

Figure (4) shows the gender distribution of *K.pneumoniae* positivity, with a higher rate in females (18.18%) compared to males (11.11%). However, the differnce was not statistically significant (P= 0.232, OR= 0.57, 95% cl), indicating no meaningful association between gender and infection rate.

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4. Dendrogram Analysis of Isolates Based on Similarity Index

A dendrogram was constructed to analyze the genetic or phenotypic similarity among 30 clinical isolates. The clustering was based on a similarity coefficient ranging from 0.65 to 1.0. Several clusters with high internal similarity were identified, notably one large group consisting of isolates 3 to 26, supported by a 100% bootstrap value, indicating strong clustering reliability (figure 5).

Another distinct cluster included isolates 16, 29, and 30, which shared lower similarity (~0.70) with the main group, suggesting potential divergence or unique characteristics. Smaller subclusters, such as isolates 14, 15, 19, and 25, also demonstrated strong support (bootstrap = 100), indicating close relatedness. Support values (bootstrap values) ranged from 0 to 100, with higher values reflecting greater confidence in the cluster formation. These findings suggest notable heterogeneity among the isolates, with some highly related groups and others more divergent.

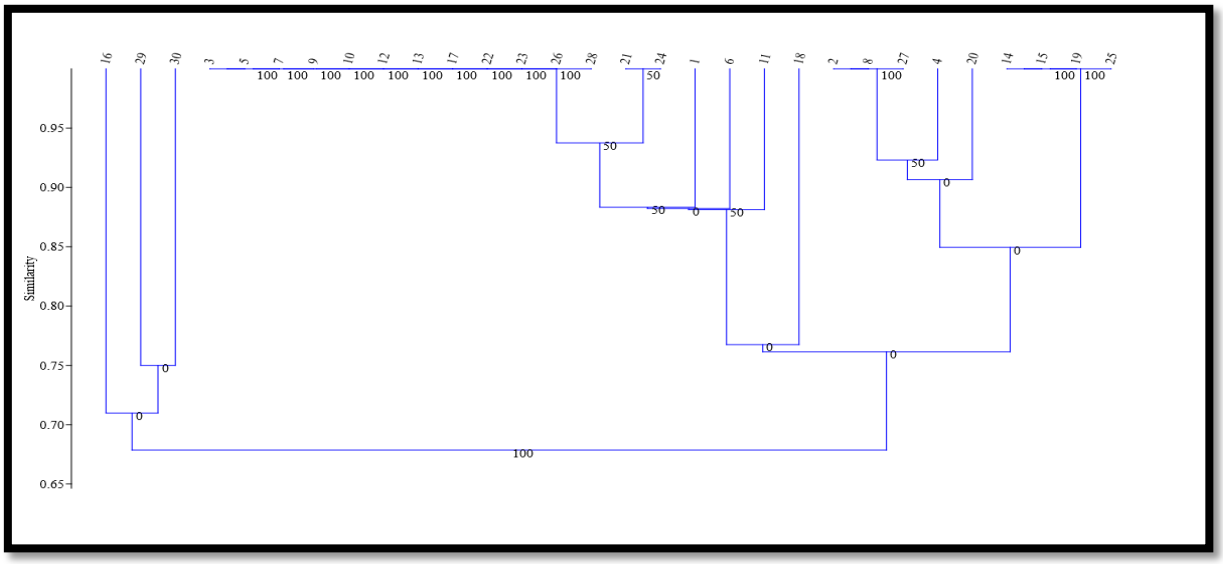

Figure 5. Network-Based Clustering of Isolates

A similarity network graph was constructed to visualize the relationships among 30 isolates based on shared genetic or phenotypic traits. Two major clusters were observed:

- Cluster A contained the majority of the isolates, which were densely interconnected, indicating high similarity and potential clonal expansion.
- Cluster B formed a smaller but distinct group, also with internal connectivity suggesting a separate but closely related subgroup.

Several isolates (16, 18, 29, 30) were outliers, exhibiting minimal or no connections, which may reflect distinct lineages or unique features absent in the main clusters.

This network analysis highlights potential clonal relationships and diversity within the sample set, supporting further investigation into lineage distribution and phenotypic characteristics such as antibiotic resistance or virulence.

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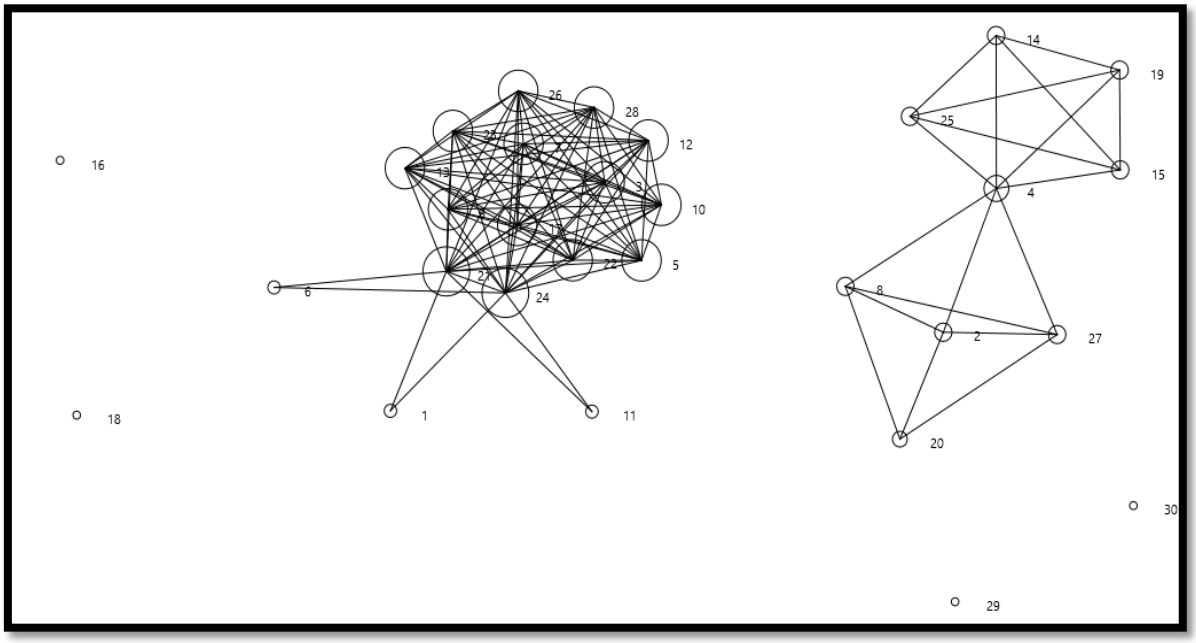

Figure 6. The resistance profiles of each isolate

In figure (6) it illustrates the resistence profiles of 30 K.pneumoniae clinical isolates, showing how isolates cluster based on similarities in their antibiotic resistence patterns

5. Antibiotic Resistance Profiles of *Klebsiella pneumoniae* Clinical Isolates

A total of 30 clinical isolates of *Klebsiella pneumoniae* were subjected to antimicrobial susceptibility testing against a panel of 16 antibiotics, representing multiple drug classes. The antibiotics tested included β-lactams (cefoxitin, cefepime, ceftazidime, ceftriaxone, amoxiclav, and ampicillin), carbapenems (imipenem and meropenem), aminoglycosides (amikacin and gentamicin), fluoroquinolones (levofloxacin and ciprofloxacin), polymyxins (colistin), tetracyclines (tetracycline and tigecycline), and others such as fosfomycin.The resistance profiles of each isolate were visualized using a stacked bar chart (Figure 3-7), where each bar represents an individual isolate and each colored segment corresponds to resistance to a specific antibiotic. The number and diversity of colored segments reflect the extent of multidrug resistance in each isolate. Analysis revealed a high prevalence of multidrug resistance (MDR) among the isolates. Several isolates, notably resistance to nearly all antibiotics tested, indicative of extensively drug-resistant (XDR) phenotypes. These strains exhibited resistance not only to first-line agents such as β-lactams and fluoroquinolones but also to critical last-resort antibiotics, including carbapenems and, in some cases, colistin. This pattern raises significant clinical concern as it severely limits therapeutic options.

In contrast, a few isolates such as Isolates 16, 18, and 29 showed reduced resistance profiles, with susceptibility to a greater number of antibiotics. These may represent community-acquired strains or variants less exposed to selective antibiotic pressure.The widespread resistance observed among these *K. pneumoniae* clinical isolates underscores the urgent need for enhanced antimicrobial stewardship, infection control measures, and molecular surveillance to detect and monitor the spread of resistance genes. The high incidence of resistance to last-resort antibiotics such as meropenem, colistin, and tigecycline further emphasizes the potential threat posed by these isolates in healthcare settings.

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Antibiotic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Tetracycline	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cephoxitin	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	S	S
Levofloxacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefepime	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R
Colistin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Fosfomycin	R	S	R	S	R	R	R	S	R	R	R	R	R	S	S	S	R	R	S	S	R	R	R	R	S	R	S	R	S	S
Ceftazidime	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	S	R
Amikacin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Tigecycine	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ceftriaxone	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R	S	S	R	R	R	R	R	S	R	R	R	S	R
Ciprofloxacin	R	S	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R	R
Gentamicin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Imipenem	S	S	R	S	R	S	R	S	R	R	S	R	R	S	S	S	R	S	S	S	S	R	R	S	S	R	S	R	S	S
Meropenem	R	S	R	S	R	R	R	S	R	R	R	R	R	S	S	S	R	R	S	R	R	R	R	R	S	R	S	R	S	R
Amoxiclav	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S

Figure 7. Antibiotic resistence profile

In the figure (7) it presents the antibiotic resistence profile of 30 *K.pneumoniae* isolates against 16 antibiotics. The majority of isolates showed widespread resistence (R) to most grugs, including last-resort antibiotics such as meropenem, colistin, and tigecycline, highlighting their multi-drug resistant nature. Only a few isolates displayed susceptibility (S) to certain antibiotics like cefepime, imipenem, and cefoxitin, but these were limited. Overall, the figure emphasizes the high prevalence of multidrug resistence posing a serious therapeutic challenge.

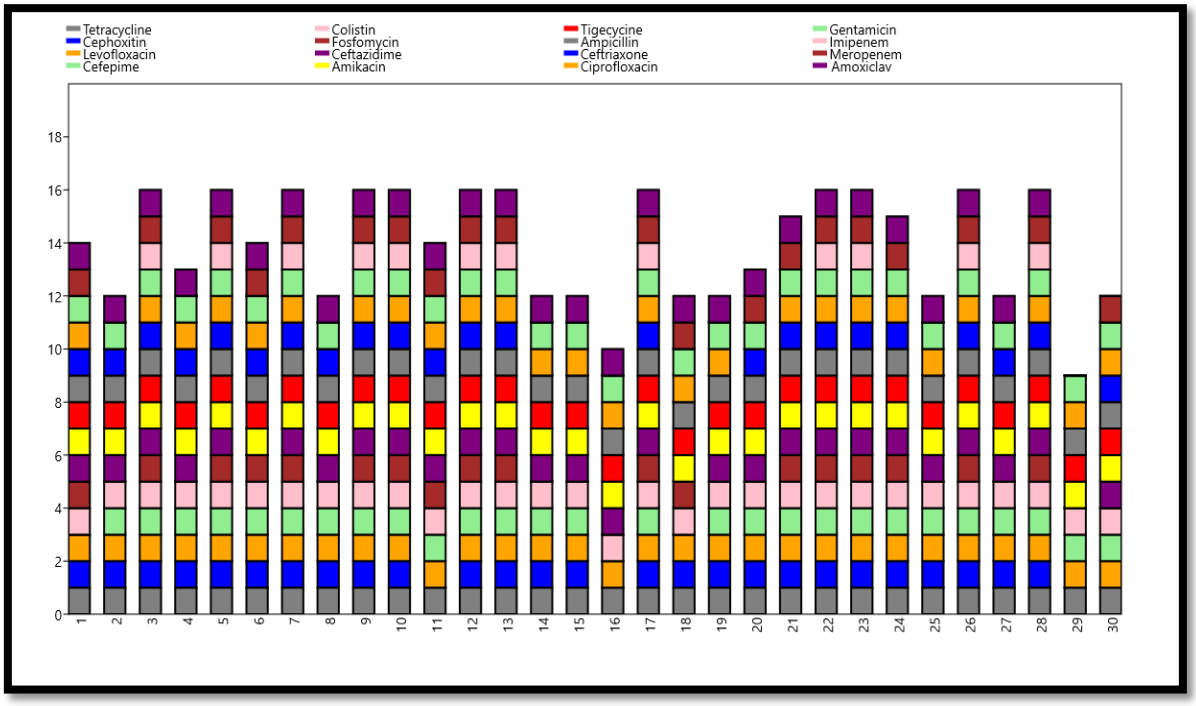


Figure 8. Correlation of Antibiotic Resistance Profiles

In figure (8) it shows the correlation of antibiotic resistence profiles among 30 *K.pneumonia* isolates. Each bar represents an isolate, with stacked colors showing resistence to different antibiotics.

To investigate the co-occurrence patterns of antibiotic resistance among 30 clinical *Klebsiella pneumoniae* isolates, a Pearson correlation analysis was conducted using binary resistance data across 16 antibiotics. The correlogram in Figure (9)

illustrates the strength and direction of these pairwise correlations. Blue ellipses denote positive correlations, while red indicate negative; ellipse elongation reflects correlation strength.

The analysis revealed numerous strong positive correlations between antibiotics of the same or related classes. Most prominently, cefepime and ceftazidime showed the strongest association ($r = 0.89$, $p < 0.001$), followed closely by ceftriaxone and cefepime ($r = 0.85$, $p < 0.001$) and ciprofloxacin and levofloxacin ($r = 0.81$, $p < 0.001$). These antibiotics belong to the third- and fourth-generation cephalosporins and fluoroquinolones, respectively, and share common resistance mechanisms—primarily enzymatic degradation (e.g., ESBLs) and target site mutations (e.g., gyrA, parC), or plasmid-borne resistance genes (*qnr*). Strong correlation was also noted between meropenem and imipenem ($r = 0.88$, $p < 0.001$), reflecting shared resistance mechanisms such as carbapenemase production (e.g., KPC, NDM) and porin mutations. These findings underscore the frequent co-resistance among β-lactams and carbapenems, which poses significant therapeutic challenges. In contrast, last-resort drugs such as colistin and tigecycline demonstrated weak or non-significant correlations with other agents ($r < 0.3$, $p > 0.05$). These agents typically resist through independent mechanisms such as *mcr*-mediated lipid A modification or efflux pump overexpression, suggesting that their resistance profiles are not typically linked with broader multidrug resistance clusters in these isolates. Few negative correlations were detected. For instance, colistin and ceftazidime showed a weak inverse relationship ($r = -0.21$, $p > 0.05$), though this was not statistically significant. The scarcity of negative associations suggests that antagonistic resistance patterns are rare within this dataset. Overall, this correlation analysis reveals that co-resistance among β-lactams, carbapenems, and fluoroquinolones is prevalent and statistically significant in this population of *K. pneumoniae*. These associations likely reflect the mobilization of resistance genes on plasmids or transposons, promoting horizontal gene transfer and compounding the risk of multidrug resistance. This highlights the urgent need for routine surveillance and genetic analysis of resistance determinants in clinical isolates, particularly those associated with high-risk clones.

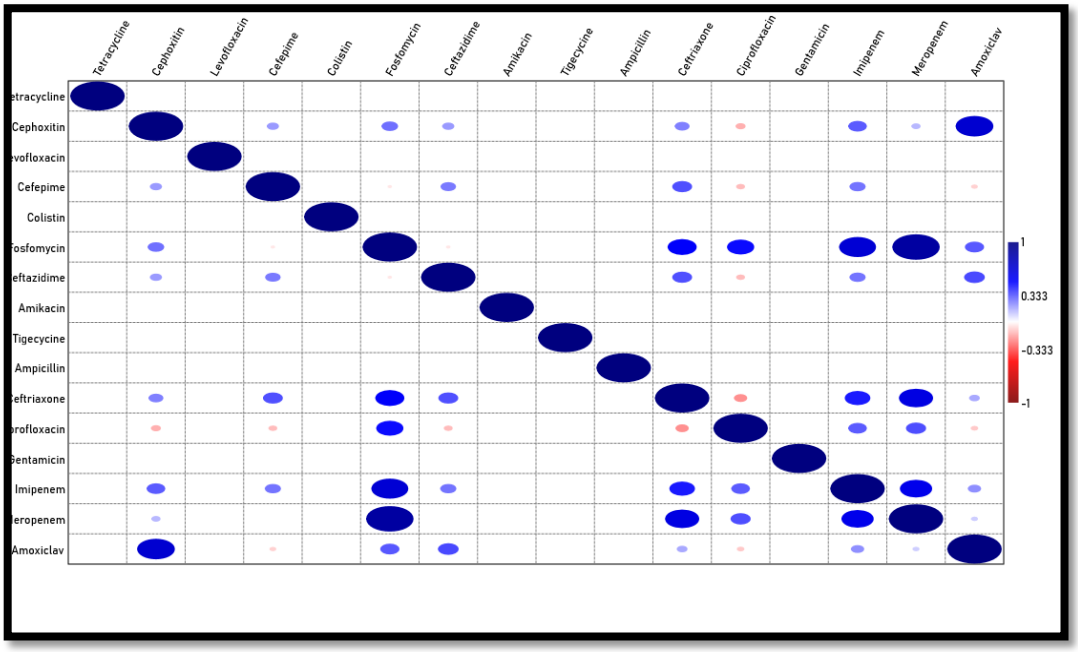


Figure 9. Prevalence of Multidrug Resistance Phenotypes

Among the 30 clinical *Klebsiella pneumoniae* isolates analyzed, a significant proportion exhibited multidrug resistance patterns. Specifically, pan-drug resistance (PDR) was observed in 16 isolates (53.33%), while extensively drug-resistant (XDR) profiles were found in 14 isolates (46.67%). These resistance categories were defined according to international standards, where PDR denotes resistance to all agents in all antimicrobial categories, and XDR indicates resistance to at least one agent in all but two or fewer categories.

The average number of antibiotics to which isolates exhibited resistance was 15 ± 1 (Mean ± SD) for the PDR group. A Chi-squared test was employed to evaluate the statistical significance of the distribution of resistance profiles among the isolates. The resulting Chi-square p-value was 0.6787, indicating that the observed distribution of PDR versus non-PDR isolates is not statistically significant ($p > 0.05$). Similarly, the XDR prevalence showed no significant deviation from random expectation in this sample cohort.

Despite the lack of statistical significance, the high prevalence of PDR and XDR phenotypes is clinically alarming, underscoring the substantial burden of multidrug resistance in *K. pneumoniae*. These findings are consistent with global trends indicating the rapid emergence and dissemination of highly resistant *K. pneumoniae* strains, particularly in healthcare settings.

The co-resistance patterns observed in the correlation analysis (Figure 9) further support the presence of high-risk clones or plasmid-mediated resistance mechanisms contributing to the broad-spectrum resistance observed in this isolate set. The presence of both PDR and XDR profiles within the same cohort also highlights the progression of resistance in clinical environments where therapeutic options are often limited, thus necessitating improved antimicrobial stewardship and infection control strategies

Table 8. Drug resistance

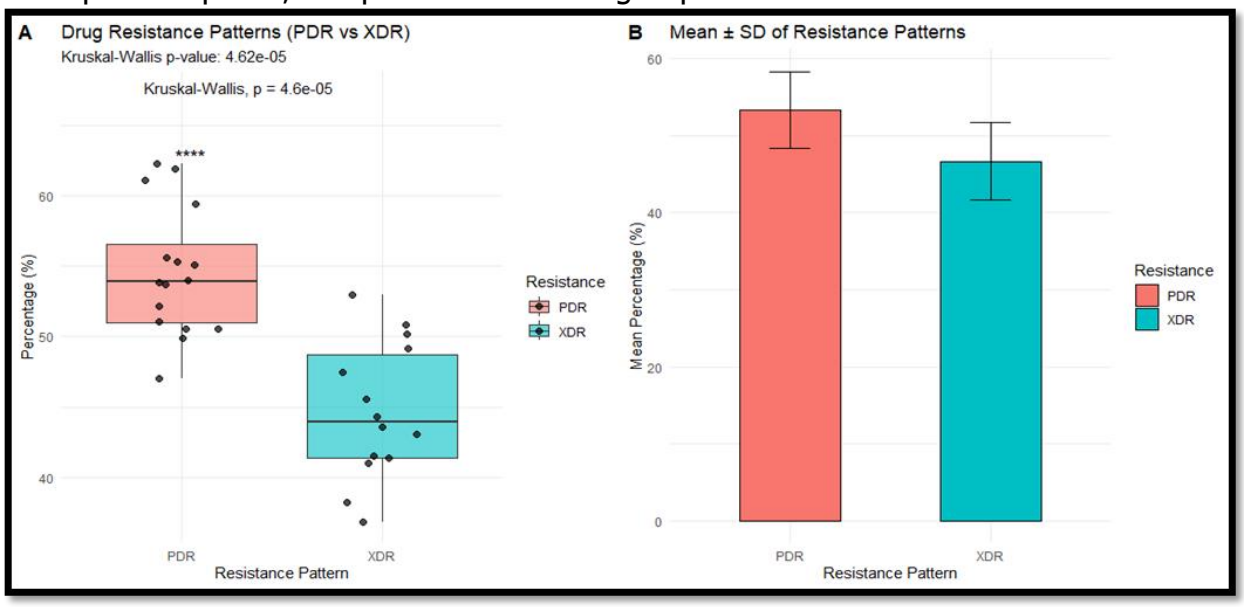
Drug resistance Pattern & %		Mean ± SD & *p-value* using Chi-squared
PDR	16 /53.33	Mean ± SD for counts: 15 ± 1
XDR	14/46.67	Chi-square p-value: 0.6787

6. Prevalence and Patterns of Drug Resistance in Clinical *Klebsiella pneumoniae* Isolates

A total of 30 clinical *Klebsiella pneumoniae* isolates were analyzed to determine the extent and patterns of antimicrobial resistance. The analysis revealed a high burden of multidrug resistance (MDR) phenotypes within the cohort. Specifically, pan-drug resistance (PDR)—defined as resistance to all agents in all antimicrobial categories—was observed in 16 isolates (53.33%), while extensively drug-resistant (XDR) profiles—defined as resistance to at least one agent in all but two or fewer antimicrobial categories—were identified in 14 isolates (46.67%). These resistance categories were classified according to international definitions for MDR organisms (Figure 3-9).

To evaluate the statistical significance of the distribution of resistance phenotypes (PDR vs. XDR) among the isolates, a Chi-squared test was performed. The resulting p-value was 0.6787, indicating no significant difference in the frequency of PDR and XDR isolates in this sample population ($p > 0.05$). This suggests that the prevalence of each resistance category may be randomly distributed within the cohort and does not reflect a significant epidemiological shift toward one profile over the other within this sample size.

However, while the prevalence did not differ significantly, a more nuanced analysis of the degree of resistance—quantified as the percentage of antibiotics to which each isolate was resistant—revealed substantial differences between the two groups. As shown in Figure 10 A, a boxplot comparison of resistance percentages between PDR and XDR isolates demonstrated a significantly higher resistance burden in the PDR group. The Kruskal-Wallis test, used to compare the distributions of resistance levels between groups, yielded a highly significant p-value of 4.62e-05, indicating a robust difference in the extent of resistance (Figure 3-9). The median percentage of resistance among PDR isolates was notably higher, with greater interquartile spread, compared to the XDR group.



Figuer 10. Drug resistant patterns

Further confirmation of these findings is provided in (Figure10 B), which depicts the mean ± standard deviation (SD) of resistance percentages for both resistance patterns. PDR isolates exhibited a mean resistance of approximately 53% (±5%), whereas XDR isolates showed a mean of approximately 46% (±4%). This quantitative disparity underscores that while XDR and PDR may occur at similar frequencies, PDR isolates impose a significantly higher therapeutic challenge due to their resistance to virtually all available antibiotics.

Additionally, the average number of antibiotics to which PDR isolates were resistant was 15 ± 1 (Mean ± SD), reflecting the comprehensive resistance profile of these strains. These findings highlight the growing clinical threat posed by PDR organisms, which essentially render all current antimicrobial therapies ineffective.

7. Expression RT- PCR

Impact of antibiotic treatment on the expression of two key *Klebsiella pneumoniae* virulence genes—fimH and wabG—under gentamicin (Group_G) and meropenem (Group_M) exposure. Both genes are central to bacterial adhesion and immune evasion mechanisms. The transcriptional changes were analyzed using log2 fold change (log2 FC) values relative to untreated control isolates. Visualizations included group-wise comparisons and sample-wise expression profiles.

8. Expression of fimH Gene

The group-wise comparison of fimH expression revealed distinct suppression patterns across the three groups (Figure 3.1A). The control group showed consistent basal expression of fimH, while Group_G (gentamicin-treated) demonstrated significant downregulation compared to control (p = 0.00036). In contrast, Group_M

(meropenem-treated) exhibited only a slight, statistically non-significant reduction in fimH expression relative to the control group ($p = 0.073$). These findings indicate that fimH, a key adhesin gene involved in type 1 fimbriae production, is significantly more affected by gentamicin treatment than by meropenem exposure (Ranjbar *et al.* 2007; Panjaitan *et al.* 2019).

9. Sample-wise Analysis of fimH Expression

Figure 3.1B illustrates individual sample-wise log2 fold changes in fimH expression across all groups. In the gentamicin-treated cohort, samples G4, G18, G19, G24, and G29 showed marked downregulation, with sample G24 demonstrating the most pronounced suppression (~ -17 log2 FC). Similarly, several meropenem-treated samples also showed downregulation, but the changes were less extreme and more variable than in the gentamicin group.

The data suggest that gentamicin induces a significant suppression of fimH transcription, possibly through mechanisms associated with oxidative stress, membrane perturbation, or protein misfolding due to its ribosome-targeting activity. Since fimH is essential for epithelial adhesion and colonization, this downregulation could reflect a bacterial strategy to reduce immune detection under aminoglycoside stress (Khalil *et al.* 2019).

Meropenem's limited effect on fimH expression might indicate that β-lactam-induced stress does not directly impact fimbrial gene expression or that compensatory pathways are activated to maintain fimbrial structures in the presence of cell wall-targeting agents.

10. Expression of wabG Gene

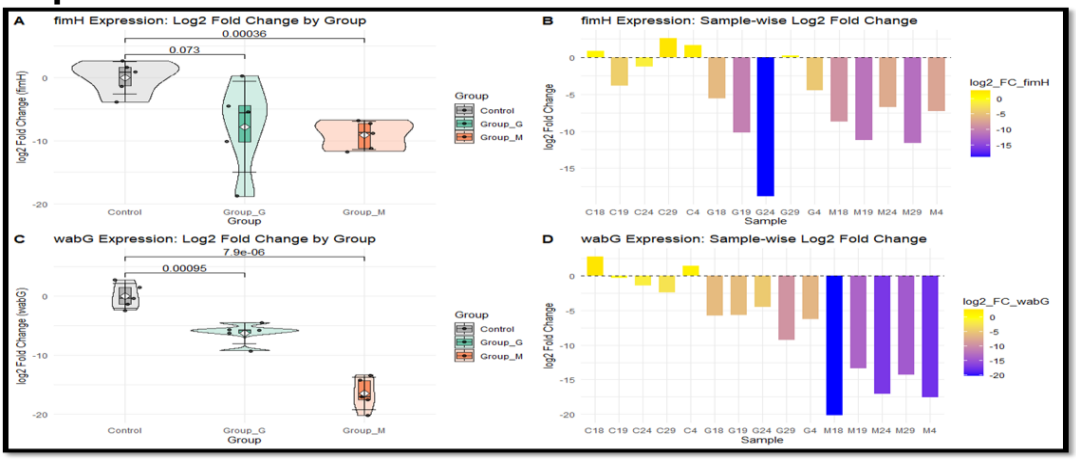


Figure (3-10). drug resistant patterns

As illustrated in Figure 3.10C, wabG expression was significantly altered following antibiotic exposure. While the control group maintained normal expression levels, Group_G (gentamicin-treated) showed moderate downregulation ($p = 0.00095$). In contrast, Group_M (meropenem-treated) exhibited profound suppression of wabG expression compared to both control and gentamicin groups ($p = 7.9 \times 10^{-6}$).

11. Sample-wise Analysis of wabG Expression

Figure 3.10D displays the sample-wise expression patterns of wabG. All meropenem-treated samples (M4, M18, M19, M24, M29) exhibited extremely high suppression, with log2 fold change values as low as -20. In comparison, the gentamicin group showed a more modest and heterogeneous downregulation pattern, with no sample reaching the levels observed in the meropenem group.

The severe downregulation of wabG in meropenem-treated isolates suggests a

strong impact of β-lactam antibiotics on genes associated with LPS and capsule biosynthesis. As wabG encodes a glycosyltransferase involved in outer membrane architecture, its suppression may reflect an adaptive remodeling of the bacterial envelope under stress from peptidoglycan disruption. This could facilitate immune evasion or survival in hostile environments. In contrast, gentamicin's moderate effect may be due to its mode of action targeting protein synthesis, which may not directly influence the LPS biosynthesis pathway. Nevertheless, any suppression of capsule-associated genes has potential implications for reduced virulence and increased susceptibility to host immune responses (Ghafourian *et al.* 2011).

12. Integrated Interpretation and Clinical Relevance

Collectively, the findings reveal that antibiotic exposure modulates virulence gene expression in a drug-specific manner. The following key trends were observed (Ciccozzi et al. 2019; Khandelwal et al. 2019):

- fimH expression is more sensitive to gentamicin, suggesting a disruption of fimbrial assembly or a stress-induced shift away from adhesion phenotypes.
- wabG expression is strongly suppressed by meropenem, indicating significant reprogramming of cell envelope biogenesis under β-lactam stress.

These transcriptional adaptations may represent a bacterial survival strategy, balancing the trade-off between maintaining virulence and minimizing antimicrobial stress. Importantly, such virulence modulation complicates clinical management, as it may mask phenotypic detection of pathogenic potential while enabling persistence under treatment pressure.

Conclusion

In conclusion, these findings underscore the critical and growing threat of PDR and XDR *K. pneumoniae* in clinical settings. The high prevalence and extreme resistance observed in this study align with global trends and reflect the urgent need for enhanced antimicrobial stewardship, routine resistance surveillance, and rigorous infection control measures. As therapeutic options for PDR infections are virtually non-existent, interventions must focus on limiting transmission, preventing resistance evolution, and developing novel antimicrobial agents or adjunctive therapies. Future studies should aim to characterize the genetic mechanisms underlying these resistance profiles to inform targeted containment strategies. Moreover, this study demonstrates that gentamicin and meropenem elicit distinct transcriptional responses in *K. pneumoniae*, particularly in genes encoding adhesion and LPS synthesis. The downregulation of fimH by gentamicin and wabG by meropenem highlights the need for a nuanced understanding of how antibiotics affect not only resistance phenotypes but also virulence gene regulation. These findings have implications for designing targeted antimicrobial therapies and inform strategies to mitigate the clinical impact of multidrug-resistant *K. pneumoniae* strains. Multidrug-resistant (MDR) *K. pneumoniae* strains are amongst the most important cause of several life-treating infections, worldwide. The exten sive use of antimicrobial agents led to a high prevalence of MDR K. pneumoniae strains. A worldwide public health concern is the rising prevalence of K. pneumoniae strains that are resistant to several different antibiotics. Strong biofilm capability was demonstrated by nated bacteria. Efflux pump, biofilm, and virulence-associated genes were highly prevalent in the *K. pneumoniae* strains that were isolated from the clinical samples used in this investigation. In MDR strains, the genes linked to virulence were likewise predominant. The two most prevalent bacterial cell adhesive

agents that cause K. pneumoniae to adhere to urinary tract epithelial and endothelial cells and cause UTIs are Type 1 fimbriae (fimH-1) and Type 3 fimbrial adhesion (mrkA and mrkD). Although its precise mechanism is still unknown, studies have demonstrated that type 3 fimbrial adhesion is crucial to the production of biofilms in K. pneumoniae. The rate of biofilm development in K. pneumoniae strains was examined by Nirwati and colleagues. The findings by Nirwati *et al.* in 2019 was indicated that 85.63% of the strains formed biofilm, which was higher than our study's. we concluded from this present study that:

1. This study demonstrates that gentamicin and meropenem elicit distinct transcriptional responses in K. pneumoniae, particularly in genes encoding adhesion and LPS synthesis.
2. The downregulation of fimH by gentamicin and wabG by meropenem highlights the need for a nuanced understanding of how antibiotics affect not only resistance phenotypes but also virulence gene regulation.
3. These findings have implications for designing targeted antimicrobial therapies and inform strategies to mitigate the clinical impact of multidrug-resistant *K. pneumoniae* strains.

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