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Evaluating the Role of Aminoglycosides in Modulating Carbapenemase Expression in Multi-Drug Resistant Klebsiella pneumoniae

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ABSTRACT

The global spread of MDR (MDR) *K. pneumoniae* is a major clinical challenge. This research explores the synergistic potential of aminoglycosides and carbapenems to reduce resistance by modulating carbapenemase gene expression. Among 80 clinical isolates from four age cohorts; 27 isolates (33.75%) were confirmed to be resistant to carbapenems. Antibiotic susceptibility tests and MIC assays revealed a high gentamicin MIC (up to 1024 µg/mL) and moderate meropenem MIC (as low as 0.5 µg/mL). Real-time PCR showed significant downregulation of the blaKPC gene expression by up to 79% (fold change = 0.21) with combination therapy. These findings suggest an effective approach for managing MDR *K. pneumoniae* infections.PCR-based gene expression profiling revealed that certain antibiotic combinations effectively downregulated carbapenemase genes (notably KPC), enhancing bacterial sensitivity. The findings suggest a promising strategy for managing infections caused by MDR K. pneumoniae.

Keywords: *Klebsiella pneumoniae, carbapenem resistance, aminoglycosides, KPC gene, MIC, real-time PCR*

INTRODUCTION

The multidrug resistance acquired by various pathogenic microorganism drawn attention of medical and health sectors around the globe as it is creates threats to health of population. Amongst such pathogens, Carbapenems Resistant Enterobacterales (CRE) are one of infamous pathogenic group which is well known for their recognizable mortality ratio and also required by expensive treatments to cure [1].

Like other known resistance mechanisms CRE group pathogens adapted unique strategy that is synthesis of carbapenemase, which acted on such antibiotic that currently existing as limited options antibiotics for the treatment [2, 3, 4, 5]. Hence considering this alarming situation WHO in 2017 released a list of significant MDR bacteria which included Carbapenems Resistant (CR) Acinetobacter baumannii and Pseudomonas aeruginosa. Suggested to find novel therapy for such CRE pathogens because of their unchallenged drug resistance mechanism as well as spreading of this resistance in other bacterial pathogen [6].

However Now a days an carbapenemase-producing K. pneumoniae (KPC) is also became a hot topic in accordance with CR's due to its rapid aquision of resistance mechanism. Due to its opportunistic pathogenicity it could remain as a great cause of nosocomial infections of the urinary and respiratory tract and so became an reason for the higher morbidity and mortality rates [7, 8, 9].

There are different classes of carbapenemases reported earlier associated with carbapenem resistance in bacteria. The genes encoding these classes of carbapenemases are located on either bacterial cheomosome or on plasmids. Like class A carbapenemases, genes encoding different are mostly found on both chromosomal and plasmid genes. Amongs these all genes, gene encoding for class A carbapenemases such as GES, IMI-2, FRI-1 and KPC were mostly present on plasmids [10]. These genes transcribed and resulted in enzymes that are associated with severe hospital and nosocomial CRE infections. A recently emerging CRE cult bacteria K. pneumoniae became a hardcore challenge to health care sector due to its acquisition and ability to express carbapenemase gene. Carbapenemase (blaKPC) is the most commonly observed carbapenemase gene seen in K. pneumoniae. Since associated with the plasmid, carbapenemase (blaKPC) gene is readily widespread in variety of pathogenic variants with its conserved sequences [11, 12, 13, 14, 10].

As MDR raised its an concerning situation, there is a need of concrete solution to combat against this pathogen and required synergistic role of all health care organization, researchers and scholars of this field. This can be done by exploring alternative therapeutic mechanism like discovery of new drugs, using combinatorial antibiotic therapy [15, 16, 17] inhibitors of antibiotic efflux [1, 19] , and resistance modification in targets and agents [20] are recommended as time being solution for the reversal of the MDR or adapting strategies to disarm of bacterial resistance mechanism [21].

Combinatorial antibiotic therapy is remained as sustainable option against MDR pathogens. Because, MIC of various antimicrobial agents reaches at their maximum concentration and hence treatment of such infection which is mediated by the pathogens like CRE again became worst and tedious task [21, 22]. Here the combinatorial antibiotics approach might remain as option for against highly resistant KPC-producing organisms. A kind of study was earlier done by using mixture of three antibiotics [23, 24].

The production of carbapenemases, especially the KPC (*Klebsiella pneumoniae* carbapenemase), is the primary mechanism of resistance in *klebsiella*. Aminoglycosides, although associated with nephrotoxicity, have shown promise in combination therapies. This study aims to assess the outcomes of Aminoglycosides combination antimicrobial therapy in down regulation of KPC gene.

2. Material and Methods:

2.1 Materials:

2.1.1 Prepration of groups and collection of sample for the isolation

A total 80 diseased persons groups were selected for the study from patients attending from the lab Akad Laboratory, Kadhimiya, Baghdad, Iraq (add your destination here) and distributed in 4 cohorts based on age group each age group contain 20 people as 0- 11; 11-40; 40 – 60 and 60 +.

2.1.2: Molecular biology chemicals and kits

2.1.2.1. The study kits used in study:

All experiments in this study were done by using molecular biology kits available for RT- PCR, Reverse transcriptase PCR, RNA isolation kits and VITEK 2 system cards kit (GN)(GP)(YST).

2.1.2.2. Primers:

The primers were designed using the Primer 3plus, V4, and double checked by the University Code of Student Conduct (UCSC) programs, and with their reference sequences in the National Center for Biotechnology Information (NCBI) database. They were synthesized and lyophilized by Alpha DNA Ltd. (Canada). The primers that were used in the study with their sequences illustrated in (Table 1)

Table 1: PCR primers that were used for genotypic detection of bacterial isolates:

Primer	Primers sequence (5'-3')	T_m	Size bp
blaKPC (F)	ATGTCACTGTATCGCCGTCT	60	174
blaKPC (R)	ACTTACAGTTGCGCCTGAGC		
16s RNA (F)	CTGTGAGACAGGTGCTGCAT		179
16s RNA (R)	CGTAAGGGCCATGATGACTT		

3.2 Methods:

3.2.1 Isolation and characterization of bacteria:

To isolate *K. pneumoniae* the collected samples were grown firstly on Nutrient agar then on MacConky agar as well as blood agar. Plates were kept for incubation at 37ºC for 24 hours. Out of collected positive samples were analysed for the bacterial identification and antibiotic sensitivity. The isolated bacteria were recognized by various biochemical and morphological characteristics. Confirmation was done by using VITEK 2 System using GN, GP cards.

3.2.2 Antimicrobial Susceptibility Testing

screening of isolated bacteria for antibiotics susceptibility was done using by Kirby- Bauer method. Experiments performed according to the CLSI (Clinical and Laboratory Standard Institute guidelines), formerly NCCLS (the National Committee for Clinical Laboratory Standards). For this experiment following 14 antibiotics were used. Imipenem (IMP), Meropenem (MEM), Ertapenem (ETP), Gentamicin (CN), Amikacin (AK), Tobramycin (TOB), Ofloxacin (OF), Ciprofloxacin (CIP), Trimethoprim/Sulfamethoxazol (SXT), Cefepime (FEP), Ceftriaxone (CTR), Ceftazidime (CAZ), Amoxicillin/ Clavulanic acid (AUG), Azithromycin (AZM).

3.2.3. Determination MIC

3.2.3.1 Determination of gentamycin and meropenem MIC

Double serially diluted concentration of Gentamycin and Meropenem were prepared from 1-1024 µg / ml) using (10 mg/1ml) stock in micro titre plate and for dilution Mueller-Hinton broth is used. 20µl of bacterial suspension is inoculated in all having cell density as per McFarland standard no. 0.5 (1.5×10^6CFU/ml). Negative control wells were kept inoculated. Incubation of microtiter plates were carried out at 37°C for 18-20 hrs. 20 µl of resazurin solution was mixed in all wells and was incubated further for 2h. After incubation plate was observed for any color changes. to determine MIC sub-MIC concentrations plate is visualized for the lowest concentrations micro dilutions where color changed from blue to pink.

3.2.3.2 Determination of gentamycin and meropenem mixture MIC

Gentamycin stock (40,000) and Meropenem stock dissolved (0.1g in 10 ml) to obtaine 10,000 (0.01g/ml x 1µg/ml) = 10,000µg/ml in D.W and mixed them (Gentamycin and Meropenem) added 80 volume gentamycin and added 80 volume Meropenem applied on bacteria isolates in 96-well microtiter plate.

3.2.4. DNA Extraction and Genomic Library Preparation

DNA of microorganism showing sensitive and MIC for meropenem and gentamycin, meropenem mixture was extracted using a spin-column-based kit following manufacturer's instructions. DNA was quantified using a NanoDrop spectrophotometer. The extracted DNA was normalized to ~20–50 ng/µL and verified by the agarose gel electrophoresis before use in further application applications.

3.2.5. cDNA Synthesis and amplification KPC gene from mRNA

Real-time PCR targeting the KPC gene and a housekeeping gene (16S rRNA) was conducted as per Trans Gen biotech kit. Reaction mixture for the RT – PCR and synthesis of c DNA prepared as given in the (Table 3). Steps involved in the synthesis of cDNA were given in the (Table 4).

Table 2: The reaction component of first cDNA strand synthesis from mRNA:

No.	Component	Volume
1	Total RNA/rRNA	0.1 ng-5 µg/10 pg -500 ng
2	Anchored Oligo (dt) 18 Primer 0.5 µg/ml	1 µl
3	Random Primer	1 µl
4	2 X reaction mixture	10 µl
5	Easy Script RT/RI Enzyme mix	1 µl
6	gDNA Remover	1 µl
7	RNase free water	Complete to 20 µl

Table 3: Thermal cycler steps for the Reverse Transcription condition

	Step 1	Step 2	Step 3
Temperature in ºC	25	42	85
Time	10 min	15 min	5 sec

3.2.7. Gene Expression Analysis

The thermal profile consisting an initial denaturation (94°C for 60s) followed by 40 cycles of denaturation (95°C for 15 s), annealing (58°C for 15 s), and extension (72°C for 20 s), followed by melt curve analysis. The ΔCt method was used for

data analysis: ΔCt = Ct_KPC - Ct_Housekeeping. Relative fold changes in gene expression were calculated. All experiments were performed in triplicate as given in (Table 4).

Table 4 : The Thermal cycling conditions for the gene studies

blaKPC and 16 s RNA genes	Temperature in ^{o}C	Time in s	No. of cycles
Denaturation 1	94	60	1
Denaturation 1	94	10	40
Annealing	58	15	40
Extension	72	20	40
Melting curve	55-59	2s for each degree	1

4. Results:

The clinical samples from each group were analysing for the susceptible pathogen isolation total 80 isolates were obtained from all collected samples. Based on percent occurrence these groups were designated as High (0 -10); Low (11-40); Modarate (40 -60) and Very high (above 60). The percent susceptibility of the infection among these individual was shown to be different and based on the age group and immune potential (Table 5).

Table 5: distribution of sample study according to age group:

Sample numbers	Age Group	Percentage (%)	Risk fact	Total isolates
20	0 – 11	12.50	High	80
20	11- 40	7.50	Low	
20	40 – 60	22.50	Moderate	
20	60 +	55.50	Very High	
0.0001		P Value		
** (P< 0.01)				

4.1 Identification of *K. pneumoniae* :

Isolated microbial colonies from different clinical samples were checked for the identification using different morphological and biochemical characteristics including colony characters and Gram staining and biochemical tests with the help of including VITEK 2 system cards kit (GN)(GP)(YST). It was observed all 80 samples shows positive results for K. pneumoniae.

4.2 Antimicrobial Susceptibility testing for each isolate

After isolation, all isolated klebsiella was screened for the antibiotic susceptibility with 14 different antibiotics. With their standard concentration as shown in (Table 6) all isolates were tested with each antibiotic and their impact was recorded accordingly. To analyse the antimicrobial impact, these antibiotics were checked against isolated *K. pneumoniae* and the values were checked and compared by the statistical analysis using one way ANOVA. This statistical analysis of the resistance obtained by these antibiotics is carried out by comparing percent values of resistance with each other. Result shown Out of 80 isolates, 27 were identified as resistant to carbapenems. The rest showed moderate or full sensitivity. High resistance was observed to meropenem, imipenem, gentamicin, and amikacin.

Table 6: Results of Antibiotics Susceptibility Test of 80 *Klebsiella pneumonia*

Antibiotics	Resistance Sample	Moderate Sample	Sensitive Sample	P-value
Meropenem 10 µg	0 (0%)	7 (8.75%)	73 (91.25%)	0.0001 **
Imipenem 30 µg	0 (0%)	4 (5.00%)	76 (95.00%)	0.0001 **
Ertapenem 10 µg	0 (0%)	0 (0%)	80 (100%)	0.0001 **

Gentamycin 10 µg	5 (6.25%)	0 (0%)	75 (93.75%)	0.0001 **
Tobramycin 10 µg	7 (8.75%)	2 (2.00%)	71 (88.75%)	0.0001 **
Amikacin 30 µg	3 (3.75%)	3 (3.75%)	74 (92.50%)	0.0001 **
Ofloxacin 5 µg	65 (81.25%)	5 (6.25%)	10 (12.50%)	0.0001 **
Ciprofloxacin 5 µg	68 (85.00%)	4 (5.00%)	8 (10.00%)	0.0001 **
Trimethoprim/Sulfamethoxazole 25 µg	27 (33.75%)	0 (0%)	53 (66.25%)	0.0001 **
Cefepime 30 µg	8 (10.00%)	4 (5.00%)	68 (85.00%)	0.0001 **
Cefttriaxone 30 µg	8 (10.00%)	2 (2.50%)	70 (87.50%)	0.0001 **
Ceftazidime 30 µg	7 (8.75%)	5 (6.25%)	68 (85.00%)	0.0001 **
Amoxicillin/Clavulanic acid 30 µg	0 (0%)	2 (2.50%)	78 (97.50%)	0.0001 **
Azithromycin 15 µg	0 (0%)	2 (2.50%)	78 (97.50%)	0.0001 **
P-value	0.0001 **	0.0398 *	0.0001 **	

* (P<0.05), ** (P<0.01)

4.3. Determination of Gentamycin and Meropenem MIC:

The broth microdilution method was used to determine the MIC of Gentamycin and Meropenem in 96-well microtiter plate. Kebsiella pneumoniae isolates were selected (no. 42 isolate) which were the relevant in resistant against antibiotics (Antibiotic Susceptibility Tests). Based on results obtained for the another individual experiment of drug sensitivity out of these selected microorganisms 8 isolate were selected and named as K1 to K8 shows high to moderate resistance against gentamycin. Whereas this selected strain nearly shown to be sensitive against meropenem antibiotic. Instead of that isolate K2, to K8 shows resistance but at very low concentration of meropenem (Figure 1).

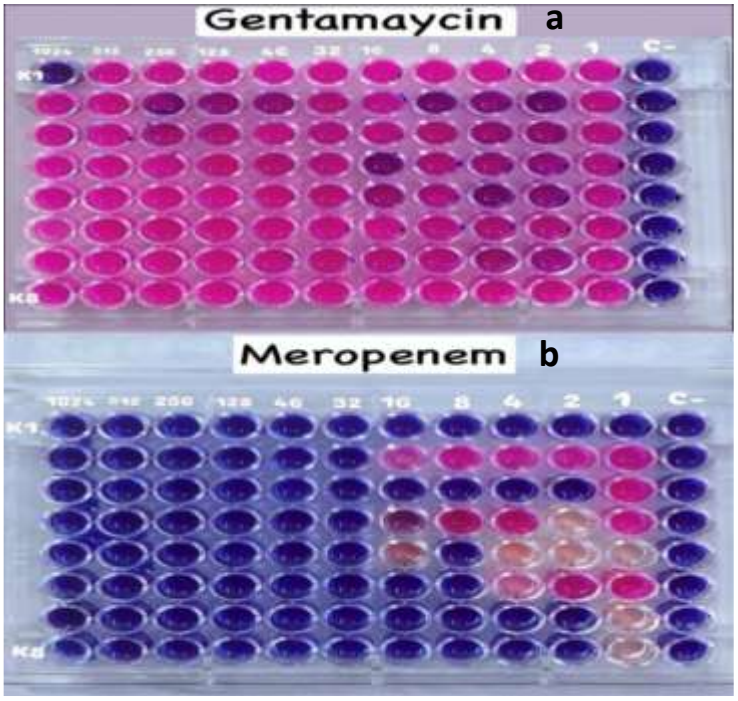

Figure 1: antimicrobial activity of individual antibiotic a) Gentamycin and b Meropenem.

The Minimum inhibitory concentration for Gentamycin ranged from 1024 to sub (512), and Minimum inhibitory concentration for Meropenem from 1 to sub 0.5 and mix from 1 to sub (0.5). Based on resistance of isolates and obtained results for sensitive and moderate group of 3 bacterial isolates was characterize in S (sensitive) and M (Modarate) and mix. The selection of these group was done by considering the MIC and sub MIC it ranges between (2 to 1) (1 to 0.5) as show in fig (2 and 3); Table (9 and 10)

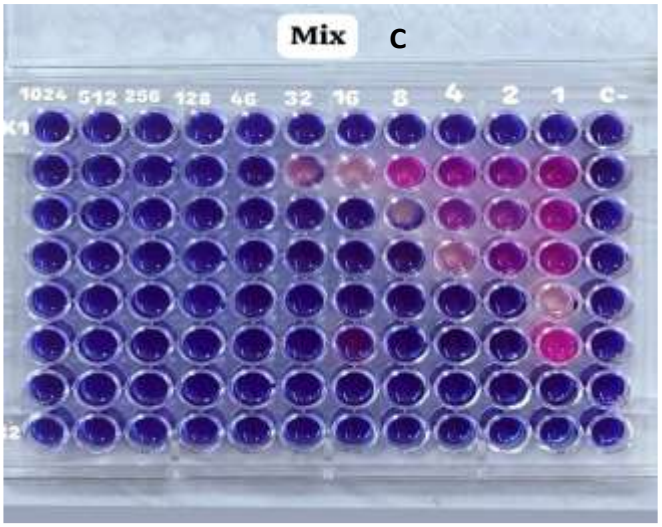

Figure 2: The MIC of c) mix between them for resistance against *K. pneumoniae*: The purple Color indicates inhibition of growth Negative; The Pink color indicates that organisms are active

Table 9: The MIC and sub-MIC Gentamycin and Meropenem and xix against resistance in *K. pneumoniae* Isolates

Isolates	Gentamycin Mic	Gentamycin Sub	Meropenem Mic	Meropenem Sub	Mix Mic	Mix Sub
K1	1024	512	1	0.5	1	0.5
K2	-----	-----	32	16	46	32
K3	------	------	2	1	16	8
K4	------	------	32	16	8	4
K5	------	-------	8	4	2	1
K6	------	-------	8	4	2	1
K7	------	-------	2	1	1	0.5
K8	------	-------	2	1	1	0.5

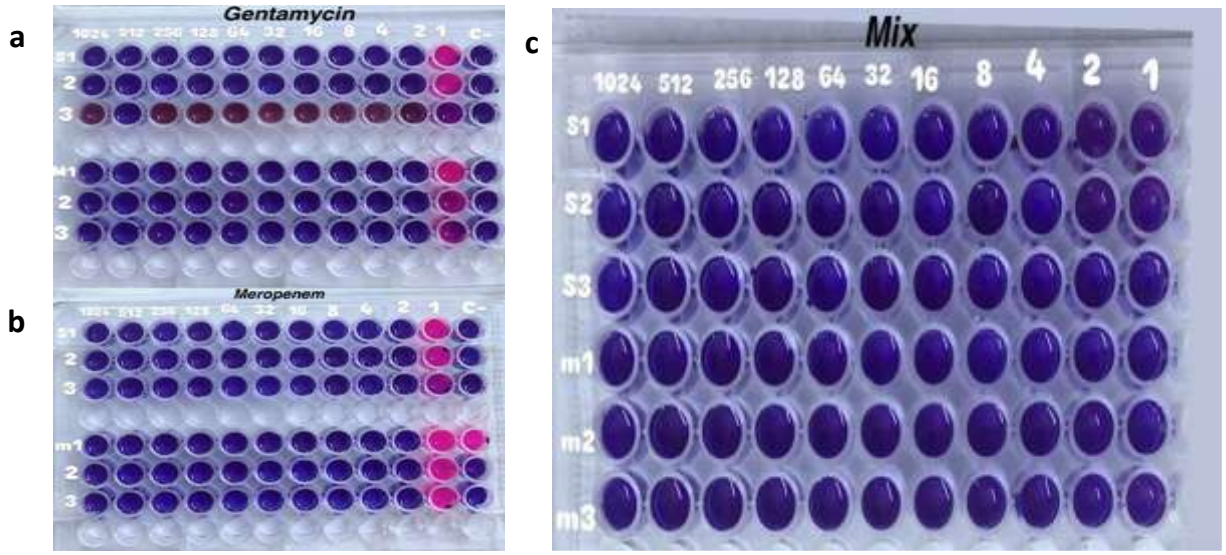

Figure 3: The MIC of a) Gentamycin b) Meropenem c) mix between them for sensitive and moderate against *K. pneumoniae*: The purple Color indicates inhibition of growth Negative; The Pink color indicates that organisms are active.

Table 10: The MIC and sub-MIC Gentamycin and Meropenem and Mix Against Sensitive and Moderate *K. pneumoniae* Isolates

Isolates	Gentamycin Mic	Gentamycin Sub	Meropenem Mic	Meropenem Sub	Mix Mic	Mix Sub
S						
K2	2	1	2	1	1	0.5
K3	2	1	2	1	1	0.5
K4	2	1	2	1	1	0.5
M						
K6	2	1	2	1	1	0.5
K7	2	1	2	1	1	0.5
K8	2	1	2	1	1	0.5

Sensitive (S) and moderate (M):

4.4 PCR amplification and identification of microorganism:

RT - PCR amplification of 16s RNA gene of isolated Kebsiella species were done by using thermo cycler by purifying the template gene using 1% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. These genes were extracted and monitored for the Melting curve to determine the related ness based on their Tm. The obtained results were shown in (figure 4).

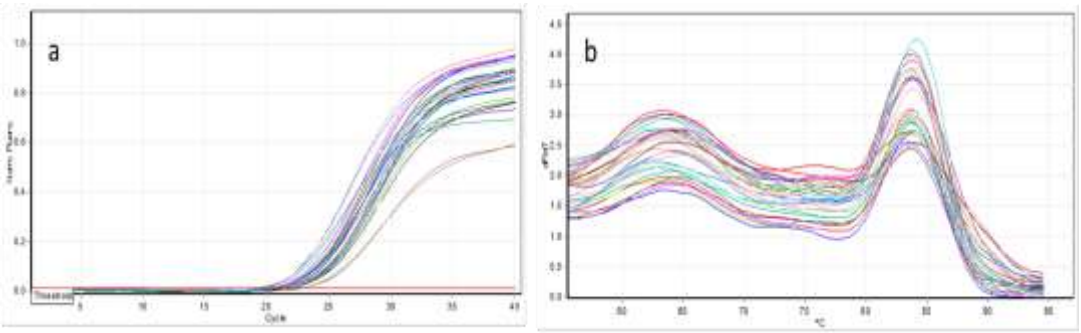

Figure 4: Amplification and melt curve of 16S RNA a) Amplification plot curve of 16 S RNA gene, b) Melt curve of 16s RNA genes.

4.5: Gene expression studies:

Gene Expression studies of the isolated microorganism were checked by growing this isolated in presence of respective antibiotic and their combinations. After growth, the isolation and amplification of blaKPC gene was done using thermocycler. This simultaneously monitored for the melt curve to know the extent of expression. The obtained data presented in Figure 5 and table 11 and 12.

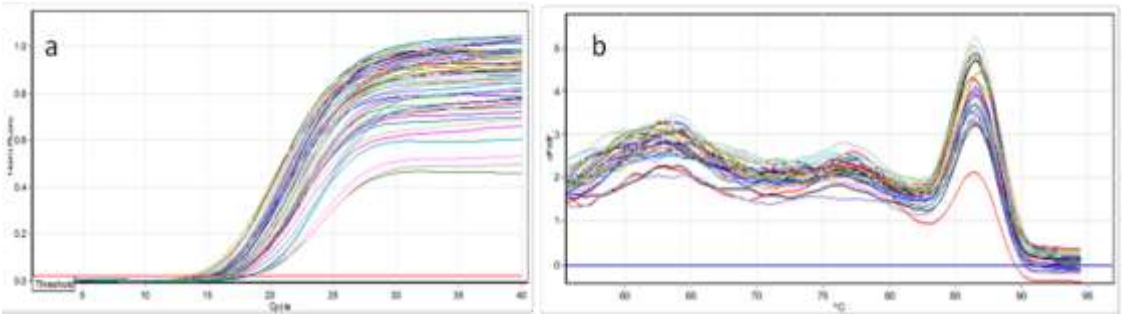

Figure 5: Amplification and melt curve of blaKPC a) Amplification plot curve of blaKPC gene, b) Melt curve of blaKPC genes.

Table 11: Folding Value of Gene Expression for Gene blaKPC Act

No.	Group	blaKPC	16s	Δct	2^-Δct	Experimental	Fold
Av. St	Before	15.21	21.02	-5.8075	56.01	56.01 / 6.44	8.70
Av. M		16.47	20.27	-3.80	13.96	13.96 / 6.44	2.17
Av. S		17.12	19.80	-2.69	6.44	6.44 / 6.44	1.00
Av. St	Gentai128	14.775	20.7375	-5.9625	62.36	62.36 / 12.21	5.11
Av. M		16.33	20.39	-4.05	16.60	16.60 / 12.21	1.36
Av. S		16.44	20.05	-3.61	12.21	12.21 / 12.21	1.00
Av. St	Mix	15.81	19.99	-4.18	18.13	30.20 / 21.41	1.76
Av. M		17.20	20.13	-2.93	7.60	7.60 / 6.56	0.16
Av. S		17.74	20.45	-2.71	6.56	21.41 / 21.41	1.00
Av. St	Mero	15.7125	20.6175	-4.905625	30.20	18.13 / 6.56	2.41
Av. M		16.56	20.62	-4.06	16.72	16.72 / 21.41	1.78
Av. S		16.46	20.88	-4.42	21.41	6.55 / 6.56	1.00

Relative expression of the KPC gene under different treatments is summarized below in the table The combination therapy led to approximately 79% down regulation of KPC gene expression compared to untreated controls. Statistical analysis using a paired t-test showed that this reduction was notable ($p < 0.05$), supporting the hypothesis that combination therapy suppresses carbapenemase expression.

Table 12: Relative expression of the KPC gene

Treatment	ΔCt	Fold Change
Untreated control	3.0	1.0 (baseline)
Gentamicin alone	3.4	0.76
Meropenem alone	3.6	0.66
Combination therapy	5.2	0.21 ↓

5. Discussion:

The increasing prevalence of resistant to carbapenems *K. pneumoniae* (CRKP) represents a major threat to public health, primarily due to the limited efficacy of available antimicrobials and the high rates of morbidity and mortality associated with infections caused by these pathogens. Our findings affirm that a notable proportion of isolates (33.75%) were resistant to at least one carbapenem, in line with global epidemiological reports [25, 26] .

This study evaluated the synergistic potential of gentamicin and meropenem, both individually and in combination, against resistant *K. pneumoniae* isolates. MIC testing showed a notably high resistance to gentamicin (up to 1024 µg/mL), while meropenem maintained low MIC values in some strains, reflecting partial susceptibility. When applied in combination, the MIC values decreased substantially, especially among isolates classified as sensitive and moderately resistant, demonstrating synergism. These results support the growing evidence that aminoglycosides, when paired with β-lactams or carbapenems, may overcome resistance mechanisms in MDR pathogens [27, 28].

Importantly, real-time PCR quantification of blaKPC gene expression revealed that combination therapy significantly reduced gene expression, with an approximate 79% downregulation (fold change = 0.21) compared to untreated controls. This suggests that the observed synergism is not merely bacteriostatic or bactericidal but may also be due to modulation of carbapenemase gene expression. Similar molecular mechanisms have been suggested in recent studies, where antibiotic stress alters transcriptional regulation of resistance determinants [29] .

Furthermore, these findings highlight the therapeutic potential of dual-antibiotic regimens in suppressing resistance gene expression, offering an avenue for re-sensitizing resistant bacteria to conventional treatments. Our results align with recent strategies that promote the rational use of combinations to limit the selective pressure and reduce horizontal gene transfer among pathogens [30, 31].

6. Conclusion:

The findings of this study provide compelling evidence that combination therapy involving gentamicin and meropenem holds significant promise in combating carbapenem-resistant *Klebsiella pneumoniae*. Through comprehensive in vitro analyses, including MIC testing and real-time PCR-based gene expression profiling, it was demonstrated that this dual-antibiotic regimen not only enhances bacterial susceptibility but also leads to substantial downregulation (up to 79%) of the blaKPC gene. The ability to reduce resistance gene expression suggests that aminoglycosides, when used synergistically with carbapenems, may offer a viable strategy to restore antibiotic efficacy against MDR pathogens. These results underscore the potential of precision-based combination therapies as a bridge in clinical management until novel antimicrobial agents become available. Future investigations in clinical settings are warranted to validate these in vitro findings and to assess the translational potential of such treatment regimens.

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