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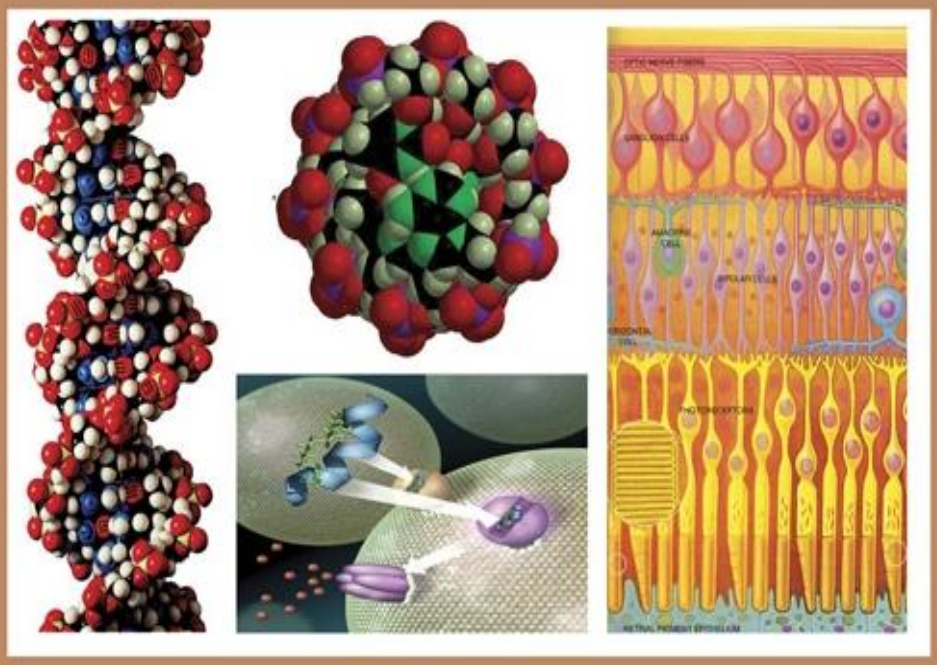

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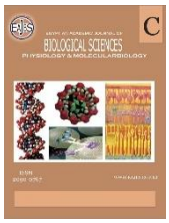

Efficacy of Amikacin and Cefotaxime Synergy Against CTX-M-15-Resistant *Klebsiella pneumoniae* in Neonatal Sepsis

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ABSTRACT

Background: Neonatal sepsis is a significant cause of neonatal mortality, particularly in developing countries, and *Klebsiella pneumoniae* is a significant contributor to this problem in some Egyptian hospitals. The aim of this study was to investigate the association between CTXM-15 and *Klebsiella pneumoniae* resistance in neonates.

Results: Five hundred and nine positive samples were collected from newborns at some Egyptian hospitals between March 2019 and March 2021, and 101 isolates of *Klebsiella pneumoniae* were tested for antimicrobial susceptibility using VITEK® 2. The majority of the isolates were from late-onset infections and showed high levels of resistance to several antibiotics, including Ampicillin, Ampicillin/sulbactam, Ceftazidime, and Cefotaxime. The $bla_{\text{CTX-M-15}}$ gene was found to be highly expressed in 66% of the multidrug-resistant *Klebsiella pneumoniae* isolates, indicating the high level of resistance conferred by this gene. Double combination therapy was evaluated, and the combination of cefotaxime and amikacin showed the most promising results, with synergistic effects against the tested isolates. The addition of magnesium was suggested to enhance cell wall integrity, allowing cefotaxime to diffuse more easily into the cells, and the Cefotaxime- Ethylenediaminetetraacetic acid combined disc and double disc synergy test confirmed the absence of metallo-beta-lactamase CTXM-15.

Conclusion: The study highlights the high prevalence of antibiotic resistance in *K. pneumoniae* isolates in neonatal sepsis in Egypt and demonstrates that the combination of cefotaxime and amikacin may be an effective treatment option for multidrug-resistant isolates with high CTXM-15 expression.

INTRODUCTION

In recent years, there has been a marked increase in the prevalence of Klebsiella pneumoniae in hospital settings, which has been attributed to the bacterium's ability to disrupt the hospital microbiota and develop drug resistance (Mohsen *et al.,* 2017). This resistance has led to Klebsiella becoming the bacterium most frequently associated with hospital-acquired infections (Martin & Bachman, 2018).

Of particular concern is neonatal sepsis caused by Klebsiella, which is often resistant to penicillin derivatives and third-generation cephalosporins, which are commonly used as first-line treatments (Hammoud *et al.,* 2017; Hassuna *et al.,* 2020). This resistance has been linked to the presence of the $bla_{\text{CTX-M-15}}$ gene, which encodes for an extended-spectrum beta-lactamase, on a plasmid found in all Klebsiella species (Ramdani Bouguessa *et al.,* 2006; Chong *et al.,* 2013; Hassan & Abdalhamid, 2014; Awosile *et al.,* 2022). Recent studies have also highlighted the expanding range of detection for extended-spectrum beta-lactamases through real-time PCR (Yang *et al.,* 2021; Harbaoui *et al.,* 2022; Hasan & Swedberg, 2022).

In a study conducted by Sands *et al.* (2021), 258 Klebsiella pneumoniae isolates from seven low- and middle-income countries were found to cause neonatal sepsis and were resistant to several antibiotics, including ampicillin, cefotaxime, ceftriaxone, and ceftazidime. Bielicki *et al.* (2020) also found that third-generation cephalosporins were not more effective than benzylpenicillin and gentamicin in treating neonatal sepsis caused by Klebsiella.

Given the widespread resistance of Klebsiella to commonly used antibiotics, the identification of suitable combination therapies is crucial to improve treatment outcomes. Studies have shown that the combination of amikacin and cefotaxime, as well as piperacillin/tazobactam and cefotaxime, can significantly increase Klebsiella susceptibility, particularly the amikacin combination (Hrbacek *et al.,* 2021). These combinations work by chelating calcium and magnesium, which are essential for cell wall integrity, thereby enhancing the action of third-generation cephalosporins (Pot, M. *et al.* (2021); Rocha *et al.,* 2022).

Therefore, the objectives of this study are: 1) to evaluate the expression of the $bla_{\text{CTX-M-15}}$ gene in multidrug-resistant *Klebsiella pneumoniae* in Egyptian neonatal intensive care units, where it is the primary cause of newborn sepsis, and 2) to identify suitable combination therapies to overcome the resistance acquired by Klebsiella towards cefotaxime and other commonly used antibiotics. By identifying effective combination therapies, additional clinical research can be conducted to address bacterial resistance and improve treatment outcomes, ultimately reducing infant mortality.

MATERIALS AND METHODS

Study Design and Clinical Specimen Collection:

The study was designed to identify neonates exhibiting clinical signs and symptoms of sepsis at the time of admission or those who were diagnosed with sepsis during their hospital stay. A sepsis screening tool developed by the Egyptian Neonatal Network (EGNN) was utilized to identify eligible participants. The study period covered a span of two years, from 2019 to 2021, during which 509 clinical specimens were collected from neonates admitted to Al Demerdash and Ain-Shams Specialized Hospital in Egypt. The specimens were submitted for bacteriological investigation, and infants were categorized into two groups based on when they were diagnosed with sepsis. Early onset sepsis (EOS) was diagnosed within the first 72 hours of life, while late-onset sepsis (LOS) was detected after the first 72 hours of life. A blood culture was performed for all clinical samples before the initiation of antibiotic therapy. A volume of 1 milliliter of blood sample was plated on a culture medium for 72 hours and was considered sterile if no growth was detected. In addition, BACTEC was used to detect

bacterial growth at a concentration of 1-2 CFU/ml within 12-24 hours.

Bacterial Culture and Growth:

In order to isolate and identify bacterial strains, various culture media were used, including nutrient agar, MacConkey's agar, MacConkey's Broth, and Brain Heart infusion agar. Blood samples collected from neonates suspected of having sepsis were cultured on these media, and the plates and slants were then incubated at 37 ºC for a period of 24-48 hours. During this time, the bacterial colonies that developed on the media were monitored, as well as the growth turbidity of the bacterial cultures. The aim of this process was to identify the bacterial strains present in the clinical specimens submitted for investigation and to determine their susceptibility to antimicrobial agents. Furthermore, all bacterial cultures were performed in accordance with standard microbiological techniques, which have been previously described in the literature (Forbes *et al.,* 2019; Winn *et al.,* 2006). Specifically, the samples were processed in a sterile environment, and all laboratory personnel followed strict aseptic techniques to prevent contamination. The bacterial colonies were then identified based on their morphological characteristics, as well as their biochemical and metabolic properties, using standard methods such as the Gram stain and various biochemical tests (Murray *et al.,* 2020). The susceptibility of the bacterial strains to antimicrobial agents was determined using the Kirby-Bauer disk diffusion method, as described by the Clinical and Laboratory Standards Institute (CLSI, 2020).

Bacterial Identification Using VITEK 2:

For bacterial identification, 5 ml of positive bacterial culture was pelleted by centrifugation at 6,000 xg for 5 minutes. The bacterial suspension was then adjusted to the McFarland 0.5 standard in a 0.45% sodium chloride solution using VITEK Densichek bioMe'rieux to achieve the desired turbidity. Next, the VITEK 2 ID-GN card, the AST-GN ID card, and the bacterial solution were manually loaded into the VITEK 2 system. With software release 2.01, the VITEK 2 system automatically reported the results, allowing for efficient and accurate bacterial identification. The VITEK 2 system has been shown to have high sensitivity and specificity in identifying a broad range of bacterial species, making it a reliable tool for bacterial identification in clinical settings (García-Sánchez *et al.,* 2017; Kim *et al.,* 2017).

Antimicrobial Susceptibility Testing:

In this study, the susceptibility of Klebsiella pneumoniae isolates to various antibiotics was evaluated using the VITEK 2 system. To ensure the accuracy and reliability of results, a reference strain, *K. pneumoniae* ATCC 13883, was used. Antibiotic susceptibility testing was performed using the VITEK 2 direct susceptibility technique in accordance with NCCLS recommendations. The results obtained from the VITEK 2 system were compared to those obtained using the broth microdilution method (MIC-2000 System; Dynatech, McLean, VA) using pure cultures. A total of 16 antibiotics were examined, including ampicillin, ampicillin/sulbactam, meropenem, cefepime, ceftazidime, ciprofloxacin, gentamicin, trimethoprim/sulfamethoxazole, piperacillin-tazobactam, tobramycin, levofloxacin, and nitrofurantoin. Discrepancies in susceptibility were classified as either extremely large disparities or minor discrepancies. Extremely large disparities were recorded when the VITEK 2 system was resistant, but the reference method was sensitive. Minor discrepancies occurred when the VITEK 2 system was susceptible or resistant, but the reference test was intermediate, or vice versa. In case of any inconsistencies, pure cultures were retested using the reference procedures to confirm the results.

Detection of *bla*$_{\text{CTX-M-15}}$ gene Using Real-time RT-PCR:

RNA Isolation:

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany, GmbH, Cat. no. 74104), following the manufacturer's instructions with minor modifications. Briefly, an overnight culture of bacteria was subcultured in Mueller Hinton broth (10 mL), and grown to an optical density (OD600) of

0.5-0.6 in the mid-log phase. To avoid RNA degradation, one volume of the broth was mixed with one volume of RNAprotect Bacteria Reagent (Qiagen, Germany, GmbH), and centrifuged at 8000 rpm for 10 minutes. The supernatant was decanted, and the pellets were resuspended in 200 µL of TE buffer containing 1 mg/mL Lysozyme (Biochemica, Applichem). The lysates were cleared by centrifugation at 14,000 rpm for 1 minute, and the supernatant was transferred to RNeasy spin columns set in 2 mL collection tubes. The columns were spun at 14,000 rpm for 1 minute, and the flow-through was discarded. Next, 700 µL of RW1 buffer was added to the columns, which were spun at 10,000 rpm for 1 minute. The flow-through was discarded, and the columns were filled with 500 µL of RPE buffer and spun at 10,000 rpm for 1 minute. The flow-through was discarded, and the RNA was eluted by centrifuging the columns at 10,000 rpm for 1 minute after adding 50 µL of RNase-free water. To eliminate any DNA contamination in the RNA, TURBO DNase (4 U) was added to the mixture, which was then incubated at 37°C for 30 minutes.

Quantitative Real-Time Reverse Transcriptase PCR QRT-PCR:

Quantitative real-time PCR (QRT-PCR) was used to amplify a 281-bp fragment of the 16S-23S ITS and $bla_{\text{CTX-M-15}}$ genes using specific primers. The primers were synthesized commercially at Willow Fort Birmingham Research and Development Park, Birmingham. Table 3 provides the sequences of the primers used in this study. The 16S-23S ITS gene is widely used as a marker for bacterial identification and phylogenetic analysis, while $bla_{\text{CTX-M-15}}$ is a plasmid-mediated extended-spectrum β-lactamase gene commonly found in *K. pneumoniae* strains. qRT-PCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) following the manufacturer's instructions. The PCR reaction mixture contained 1 µL of template cDNA, 5 µL of SYBR Green Master Mix (Bio-Rad, USA), and 0.5 µM of each primer in a total volume of 10 µL. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. A melting curve analysis was performed at the end of the amplification to confirm the specificity of the PCR products. Standard curves were generated using known quantities of plasmid DNA containing the target genes. The relative gene expression levels were calculated using the 2^-ΔΔCt method, with the 16S rRNA gene used as an internal control for normalization.

MIC Testing:

In this study, the antimicrobial susceptibility of *K. pneumonia* isolates was assessed using the broth microdilution technique. The antibiotics used namely, CTX, SAM, TZP, GEN, and AN, were derived from European pharmacopeia reference standards. The experiment involved twofold serial dilutions of the antibiotics in Mueller-Hinton broth using 96-well microtiter plates. The final antibiotic doses ranged from 2048 to 0.5 mg/l, and the final inoculum of each experimental isolate was around 1 x 10^6 cfu/ml. The total volume per well was 200 µl, with negative and positive controls consisting of wells holding 200 µl of uninoculated and inoculated MHB, respectively. The plates were then incubated at 37 °C overnight. For CTX's MIC, the agar dilution technique was used. Mueller-Hinton agar (MHA) plates were prepared and inoculated with 2 µl/spot of each isolate inoculum (1x10^6 cfu/ml) using twofold successive dilutions of CTX (2048-1 mg/l). The inoculation plates were allowed to dry at room temperature before being incubated at 37 °C overnight. The antibiotic's MIC was determined as the lowest antibiotic concentration at which no growth was detected following an overnight incubation at 37 °C, according to the method described by White et al. in 1996. This technique ensured accurate and reliable determination of the minimum inhibitory concentration of the antibiotics against *K. pneumonia* isolates.

Synergy Testing:

The checkerboard microdilution method was used to evaluate the synergistic activity of four different combinations of antibiotics (CTX/SAM, CTX/TZP, CTX/GEN, CTX/AN) against selected *K. pneumonia* isolates. The experiment involved inoculating twofold serial dilutions of the antibiotics in Mueller-Hinton broth (MHB) using 96-well microtiter plates, with final antibiotic concentrations ranging from 64-2048 mg/l and a final inoculum of approximately 1×10^6 cfu/ml. Negative and positive controls were included to ensure accuracy. The fractional inhibitory concentration (FIC) index of the antibiotic combinations was calculated, and the results were interpreted as synergistic (∑FIC<1), indifferent (∑FIC=1), or antagonistic (∑FIC>1). Only combinations where the MICs of each antibiotic met the susceptible levels defined by EUCAST and CLSI were considered valid. The use of this standardized method allowed for an accurate assessment of the synergistic activity of the antibiotic combinations against *K. pneumonia* isolates, providing valuable insights for the development of effective treatment strategies.

Cefotaxime (CTX)-EDTA Combined disc Test:

The CTX-EDTA combined disc test was performed following the methodology outlined by Yong et al in 2002. In accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations, the test organisms were inoculated onto Mueller Hinton agar (MHA) plates. Two 30 g cefotaxime discs from Becton Dickinson were inserted onto the plate, and 10 L of EDTA solution was added to one of the discs to attain a final concentration of 750 µg of EDTA. The plates were then incubated at 35 °C for 16-18 hours, after which the inhibition zones surrounding the cefotaxime and cefotaxime-EDTA discs were assessed. A positive result for MBL was recorded when the increase in the inhibition zone surrounding the cefotaxime and EDTA disc was greater than or equal to 7 mm compared to that observed for the cefotaxime disc alone. This criterion was adopted from previous studies by *Berges et al.*, (2007), Behera *et al.*, (2008), Omair *et al.*, (2012), and Anwar *et al.*, (2016).

Cefotaxime -EDTA Double Disc Synergy Test (DDST):

The double disk synergy test using CTX and EDTA was conducted following the procedure outlined by Lee *et al.* (2003). The CLSI's recommended method for inoculating test organisms on Mueller Hinton agar plates was followed. A blank disc containing 10 µL of 0.5 M EDTA (750 µg) was placed 20 mm from center to center from the cefotaxime (10 µg) disc. A positive result was determined by an increase in the zone of inhibition between the cefotaxime and EDTA discs, as compared to the zone of inhibition on the opposite side of the drug. Similar to other studies (Bergès *et al.*, 2007; Omair *et al.*, 2012), this interpretation was used to classify an isolate as MBL-positive.

Statistical Analysis:

The statistical software package used for data analysis was SPSS Inc., version 16, based in Chicago, Illinois, USA. Descriptive statistics were presented as mean ± standard deviation for quantitative data and as numbers and percentages for qualitative data. The distribution differences were assessed using T-tests and Regression analysis. Statistical significance was considered when the *P*-value was ≤ 0.05. The significance level was defined as $p < 0.05$, and highly significant differences were defined as $P < 0.01$ and $P < 0.001$.

RESULTS

Study Design & Specimen Collections:

A total of 509 blood samples were collected from newborns with sepsis in some Egyptian hospitals, with 39.8% of the cases being male and 60.25% female. Out of the total positive cases, 26 were identified as early-onset EOS and 483 as late-onset LOS, as indicated in Table 1. The occurrence of infection did not differ significantly between males and females at $p \leq 0.05$. The peak infection days were observed in two distinct ranges, namely 16-22 days and 55-60 days.

Table 1: Characteristics of newborns with positive sepsis cases in collected blood samples.

Sex	Total Cases (%)	Early Onset (%)	Late Onset (%)	*P*-value
Male	204 (40)	11(42.3)	191(39.5)	**0.0422**
Female	305(60)	15(57.7)	292(60.5)	
Total	509(100)	26(100)	483(100)	

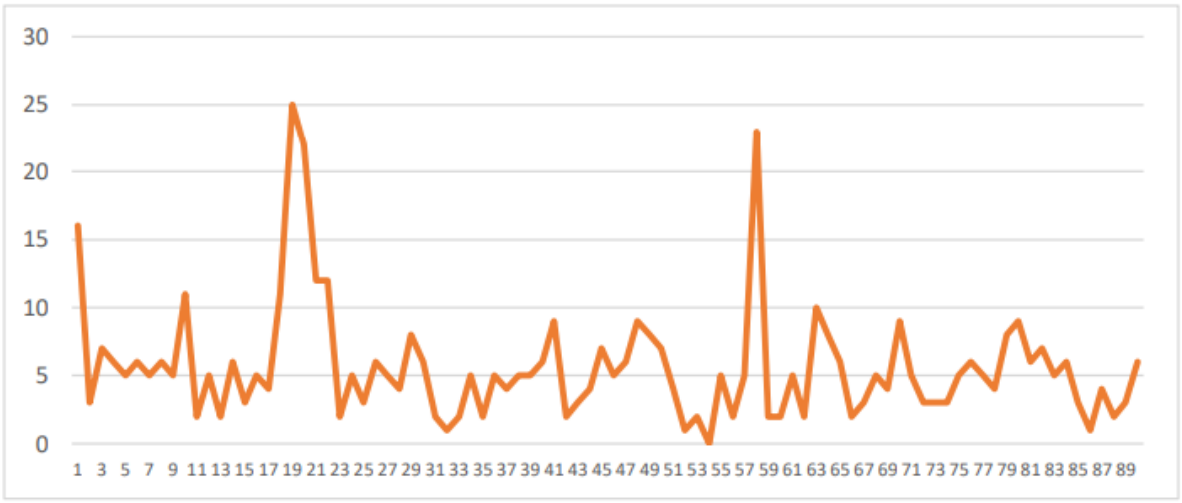

Fig. 1: Frequency of positive sepsis cases based on age early to classify the onset of infections. The x-axis represents the age of the newborns, while the y-axis shows the frequency of positive cases.

Antibiotic susceptibility test (AST)

The antibiotic susceptibility of drug-resistant bacteria isolated from positive cases was determined using the AST-GN73 card BioMérieux VITEK 2. Sixteen commonly used antibiotics, including Ampicillin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefazolin, Cefoxitin, Ceftazidime, Cefotaxime, Cefepime, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantoin, and Trimethoprim + Sulfamethoxazole, were tested. The results showed high resistance rates of *K. pneumoniae* isolates, with 99% resistance to Ampicillin, 90% to Ampicillin/sulbactam, and 91% to Ceftazidime and Cefotaxime (Fig. 2). Furthermore, the majority of K. pneumoniae isolates (66/101, 65%) were found to be multidrug-resistant. AMP=Ampicillin, SAM=Ampicillin/Sulbactam, TZP=Piperacillin/Tazobactam, CZ= Cefazolin, FOX=Cefoxitin, CAZ=Ceftazidime, CTX=Cefotaxime, FEP=Cefepime, MEM=Meropenem, AN=Amikacin, GEN=Gentamicin, TOB=Tobramycin, CIP=Ciprofloxacin, LEV=Levofloxacin, NIT=Nitrofurantoin, SXT= Sulfamethoxazole/Trimethoprim.

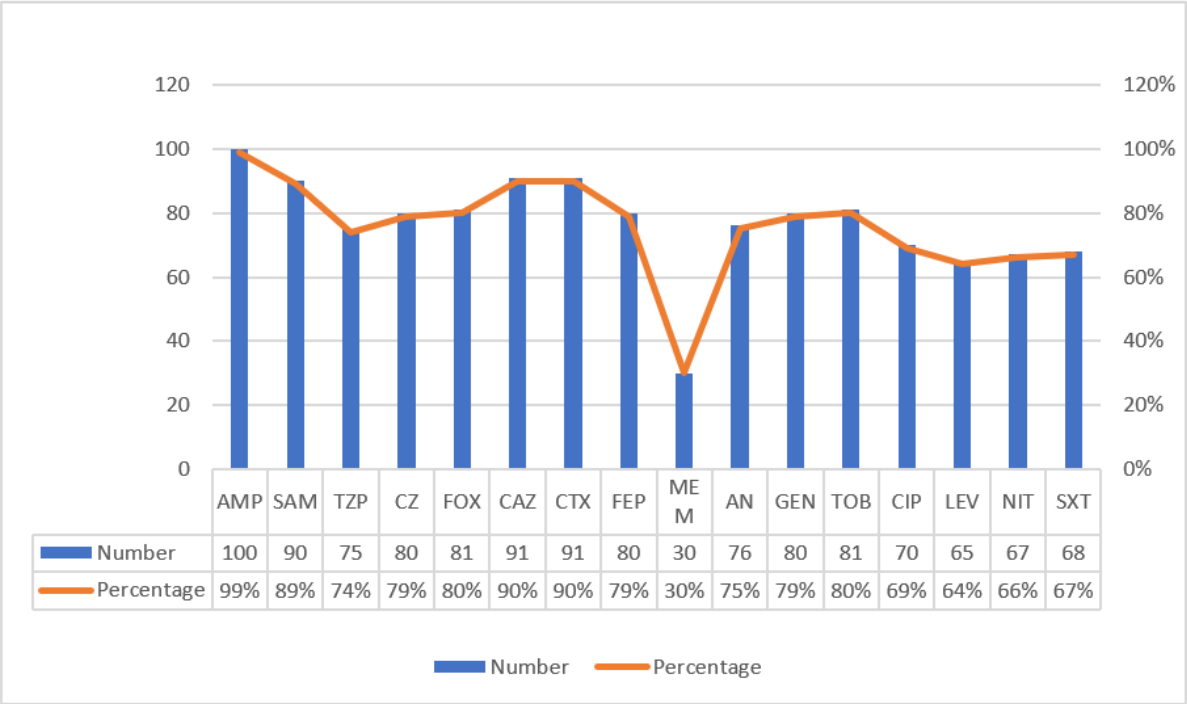

Fig. 2. Antibiotic susceptibilities of *Klebsiella pneumoniae* isolates isolated from neonatal sepsis cases as determined by the VITEK 2 system.

RT-PCR Amplification:

RT-PCR amplification was performed on *K. pneumoniae* isolates to analyze the target gene *bla*$_{\text{CTX-M-15}}$ and the reference gene 16S-23S ITS rDNA. Prior to the Real-time PCR assays, the primers for the selected genes were validated using conventional PCR followed by agarose gel electrophoresis to ensure amplification specificity. Table 2, presents the PCR primer sequences and amplicon sizes for the selected candidate genes that were amplified from *K. pneumoniae* isolates.

Table 2. Primers used for the specific amplification of *K. pneumoniae* and the amplicon sizes.

Name	Gene	Primer sequence 5'-3'	Size of PCR amplicon	Reference
Pf	*16S-23S ITS*	ATTTGAAGAGGTTGCAAACGAT	130 bp	Liu *et al.*, 2008
Pr1		TTCACTCTGAAGTTTTCTTGTGTTC		
CTX-M15	*bla*$_{\text{CTX-M-15}}$	CGGAAAAGCACGTCAATGGG	281 bp	GenBank: DQ302097.1 Liu *et al.*, 2009
CTX-M15'		GCTGTCGCCCAATGCTTTAC		

qRT-PCR:

The real-time PCR assay demonstrated a high degree of confidence in quantification with PCR efficiencies ranging from 95.0% for *bla*$_{\text{CTX-M-15}}$ to 98.00% for the 16S-23S ITS sequence. The standard curves exhibited an acceptable correlation coefficient R2, validating the accuracy of the primer pairs in the RT-qPCR assay. The ΔΔCt method was used to determine the fold change (Fig. 3), which provided a relative estimate of the expression of *bla*$_{\text{CTX-M-15}}$ copies in each sample compared to the housekeeping gene 16S-23S ITS, as described in the materials and methods section. The results revealed that gene expression levels of the *bla*$_{\text{CTX-M-15}}$ gene were significantly higher in resistant *K. pneumoniae* isolates (KR1-KR23) than in susceptible ones (K2-K10) with a mean fold change of 10.0349 vs 1.8646.

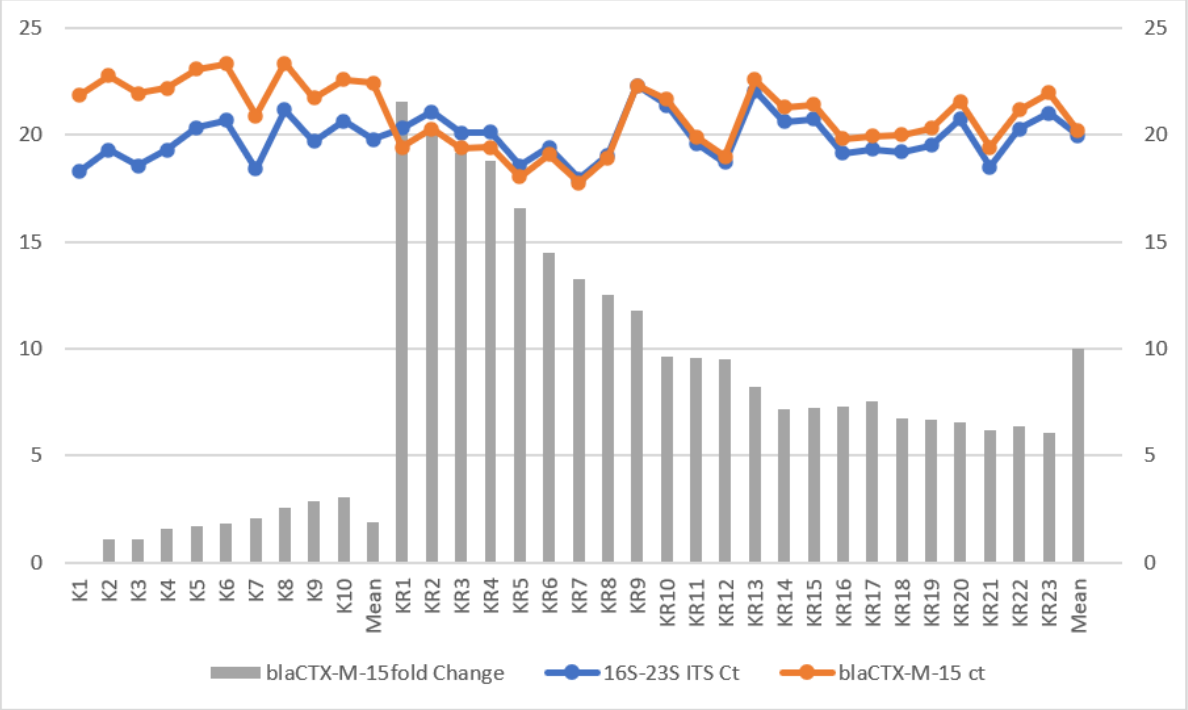

Fig. 3: The outcomes of real-time PCR, including the Ct values and fold changes of *bla*$_{\text{CTX-M-15}}$ in both sensitive and resistant *K. pneumoniae* strains. The fold change was calculated using the ΔΔCt method, which provided a relative assessment of *bla*$_{\text{CTX-M-15}}$ expression levels in each sample compared to the housekeeping gene 16S-23S ITS, as outlined in the materials and methods. The gene expression levels of *bla*$_{\text{CTX-M-15}}$ were significantly higher in resistant *K. pneumoniae* isolates (KR1-KR23) compared to susceptible ones (K2-K10), with a mean fold change of 10.0349 versus 1.8646. Control Isolate= K1, Sensitive Group to cefotaxime represented within K2: K10, Resistant Group to Cefotaxime represented within KR1:KR23. Mean of Sensitive Group CTXM-15-fold change = 1.8646, Mean of Resistant Group CTXM-15-fold change = 10.0349.

Antibiotics MIC and CTX-M15 fold change of *Klebsiella:*

The study revealed that Klebsiella resistance to Cefotaxime and Ceftazidime was associated with a significant increase in the expression levels of the *bla*$_{\text{CTX-M-15}}$ gene, resulting in a rise in the minimum inhibitory concentration (MIC) of the antibiotics. The fold change of *bla*$_{\text{CTX-M-15}}$ ranged from 3.0314 to 21.5557, and the MIC of Cefotaxime increased to <2048 µg/ml. Piperacillin/ tazobactam showed a slower rate of resistance development, with a gradual increase in the fold change of *bla*$_{\text{CTX-M15}}$. The results also demonstrated a positive correlation between the fold change of *bla*$_{\text{CTX-M-15}}$ and the MIC score of Cefotaxime towards Klebsiella. The findings suggest that even a minor change in the fold change could lead to a significant increase in the MIC score, as depicted in Table 3 and Figure 4.

Table (3): Antibiotic susceptibility of *Klebsiella* isolates towards AMP= Ampicillin, SAM= Ampicillin/Sulbactam, TZP= Piperacillin/Tazobactam, CTX= Cefotaxime, CAZ = Ceftazidime Minimum Inhibitory Concentrations (MICs) versus the fold change expression of CTXM-15.

Klebsiella Isolates	*bla*$_{CTX-M-15}$ Fold Change	AMP (MIC) (µg/ml)	Susceptibility	SAM (MIC) (µg/ml)	Susceptibility	TZP (MIC) (µg/ml)	Susceptibility	CTX (MIC) (µg/ml)	Susceptibility	CAZ (MIC) (µg/ml)	Susceptibility
K1	ATCC	>=32	R	>=32	R	<=4/4	S	<=1	S	<=4	S
K2	1.0644	>=32	R	>=32/16	R	<=4/4	S	<=1	S	<=4	S
K3	1.1096	>=32	R	>=32/16	R	<=4/4	S	<=1	S	<=4	S
K4	1.5692	>=32	R	>=32/16	R	<=4/4	S	<=1	S	<=4	S
K5	1.7291	>=32	R	>=32/16	R	<=4/4	S	<=1	S	<=4	S
K6	1.8532	>=32	R	>=32/16	R	<=4/4	S	<=1	S	<=4	S
K7	2.0849	>=32	R	>=32/16	R	<=4/4	S	2	I	8	I
K8	2.5491	>=32	R	>=32/16	R	<=4/4	S	2	I	8	I
K9	2.8481	>=32	R	>=32/16	R	<=4/4	S	2	I	8	I
K10	3.0314	>=32	R	>=32/16	R	<=4/4	S	4	R	16	R
KR23	6.0629	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR22	6.3643	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR21	6.1903	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR20	6.5887	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR19	6.6807	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR18	6.7740	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR17	7.5685	>=32	R	>=32/16	R	16/4	S	4	R	16	R
KR16	7.3107	>=32	R	>=32/16	R	32/4	I	8	R	16	R
KR15	7.2100	>=32	R	>=32/16	R	32/4	I	8	R	16	R
KR14	7.1602	>=32	R	>=32/16	R	32/4	I	8	R	16	R
KR13	8.2249	>=32	R	>=32/16	R	>=128/4	R	8	R	32	R
KR12	9.5137	>=32	R	>=32/16	R	>=128/4	R	16	R	32	R
KR11	9.5798	>=32	R	>=32/16	R	>=128/4	R	16	R	32	R
KR10	9.6465	>=32	R	>=32/16	R	>=128/4	R	32	R	32	R
KR9	11.7942	>=32	R	>=32/16	R	>=128/4	R	64	R	64	R
KR8	12.5533	>=32	R	>=32/16	R	>=128/4	R	128	R	64	R
KR7	13.2691	>=32	R	>=32/16	R	>=128/4	R	128	R	64	R
KR6	14.5203	>=32	R	>=32/16	R	>=128/4	R	256	R	128	R
KR5	16.5642	>=32	R	>=32/16	R	>=128/4	R	512	R	256	R
KR4	18.7654	>=32	R	>=32/16	R	>=128/4	R	1024	R	256	R
KR3	19.1597	>=32	R	>=32/16	R	>=128/4	R	2048	R	512	R
KR2	20.1122	>=32	R	>=32/16	R	>=128/4	R	>2048	R	1024	R
KR1	21.5557	>=32	R	>=32/16	R	>=128/4	R	>2048	R	2048	R

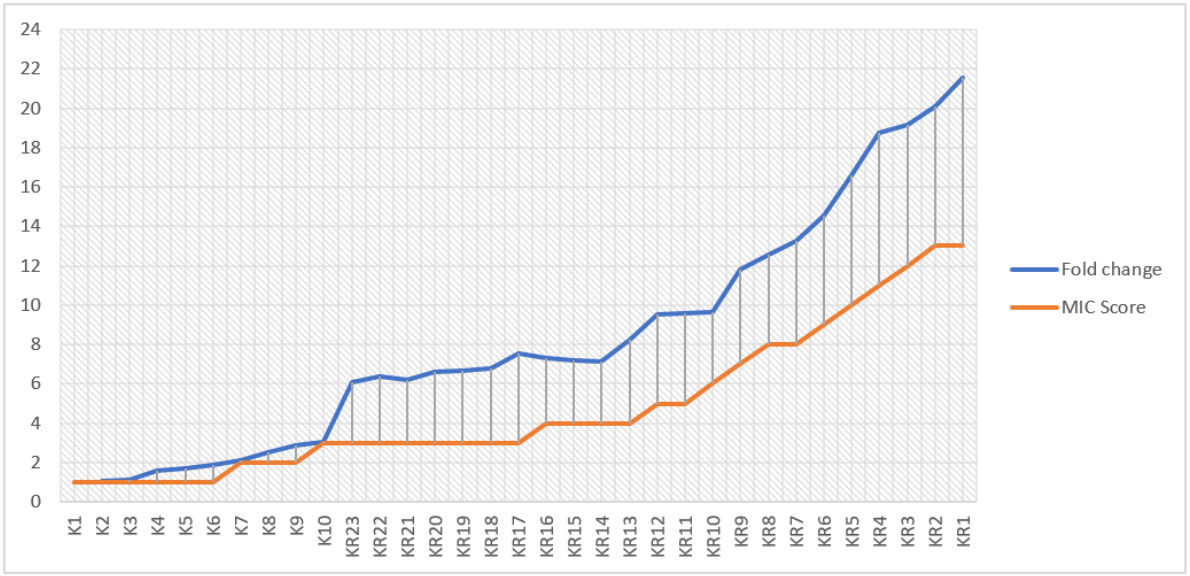

Fig. 4: The correlation between the fold change in expression of blaCTX-M 15 gene and the minimum inhibitory concentration (MIC) of cefotaxime against Klebsiella isolates. The graph demonstrates a positive correlation between the fold change of blaCTX-M 15 and the MIC score of cefotaxime towards Klebsiella, indicating that a minor change in the fold change is associated with a significant change in the MIC score.

MIC Testing of *K. Pneumonia* Isolates:

A total of 66 *Klebsiella pneumonia* isolates were randomly selected using a simple random sampling technique, from which 22 multi-drug resistant isolates were identified. Figure 3 shows that all 22 isolates were resistant to AMP, CTX, NIF, and SXT, while 95% were resistant to CAZ and CIP, and 91% were resistant to SAM, CZ, FOX, TOB, and LVX. Moreover, 68% were resistant to FEP and GEN, and 59% were resistant to TZP, whereas 55% were resistant to MEM and AN. Notably, 12 isolates (55%) exhibited resistance to all antibiotics tested, including BKS-1, BKS-5, BKS-7, BKS-23, BKS-27, BKS-33, BKS-36, BKS-45, BKS-53, BKS-59, BKS-66, and BKS-73, and were chosen for further analysis. Table (4) summarizes the MIC results of five antibiotics individually and in combination against twelve selected *K. pneumonia* isolates. The findings indicate that all twelve isolates were resistant to SAM, TZP, CTX, GEN, and AN, with MICs ranging from 32-2048 mg/l. However, the double combinations of TZP/CTX and AN/CTX exhibited synergistic effects (FIC index <1), leading to a decrease in MICs of CTX, AN, and TZP. This synergism achieved the susceptible breakpoints of CLSI (2021). Conversely, the combination of SAM with CTX and GEN with CTX did not show any synergistic effect (FIC index >1), and their MICs remained above the susceptible breakpoints of CLSI (2021). Checkerboard experiments further validate these findings (Table 5).

Table 4: Shows the percentage of resistance of each *Klebsiella* isolates toward the tested antibiotics.

Isolates	Phenotype of antibiotic resistance	Resistance to antibiotics (%)	Resistance pattern
BKS-1	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-5	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-6	AMP, SAM, CZ, FOX, CAZ, CTX, GEN, TOB, CIP, LVX, NIF, SXT	75%	*MDR*
BKS-7	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-15	AMP, SAM, FOX, *CAZ, CTX*, GEN, TOB, CIP, NIF, SXT	62.5%	*MDR*
BKS-18	AMP, SAM, CZ, FOX, CAZ, CTX, GEN, TOB, CIP, LVX, NIF, SXT	75%	*MDR*
BKS-23	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-27	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-29	AMP, SAM, CZ, FOX, GEN, *CTX*, CIP, LVX, NIF, SXT	62.5%	*MDR*
BKS-31	AMP, SAM, CZ, FOX, CAZ, CTX, GEN, TOB, CIP, LVX, NIF, SXT	75%	*MDR*
BKS-33	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-36	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-41	AMP, SAM, CZ, FOX, CAZ, CTX, FEP, GEN, TOB, CIP, LVX, NIF, SXT	81.25%	*MDR*
BKS-44	AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, GEN, TOB, CIP, LVX, NIF, SXT	87.5%	*MDR*
BKS-45	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-53	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-55	AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, GEN, TOB, CIP, LVX, NIF, SXT	87.5 %	*MDR*
BKS-59	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-65	AMP, TOB, *CAZ, CTX*, LVX, NIF, SXT	43.75%	*MDR*
BKS-66	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***
BKS-69	AMP, GEN, *CTX*, CIP, *CAZ*, NIF, SXT	43.75%	*MDR*
BKS-73	***AMP, SAM, TZP, CZ, FOX, CAZ, CTX, FEP, MEM, AN, GEN, TOB, CIP, LVX, NIF, SXT***	***100%***	***MDR***

Table (5): FIC index calculation of each antibiotic MIC in considering the MIC of each antibiotic in combination with another antibiotic.

MDR isolates	MIC of single antibiotics (mg/l)					MIC of the synergistic antibiotic combinations (mg/l) Double (FIC index)			
	SAM	TZP	CTX	GEN	AN	SAM/CTX	TZP/CTX	GEN/CTX	AN/CTX
BKS-1	1024	128	>2048	>2048	64	1024/2048 (2)	32/8 (0.254)	2048/2048 (2)	8/8 (0.129)
BKS-5	1024	512	>2048	>2048	64	1024/2048 (2)	128/8 (0.254)	2048/2048 (2)	8/8 (0.129)
BKS-7	*1024*	256	>2048	*1024*	*64*	1024/2048 (2)	*64/8 (0.254)*	1024/2048 (2)	8/8 (0.129)
BKS-23	>2048	512	>2048	>2048	64	2048/2048 (2)	128/8 (0.254)	2048/2048 (2)	8/8 (0.129)
BKS-27	1024	512	>2048	>2048	*64*	1024/2048(2)	*128/8* (0.254)	*2048/2048* (2)	8/8 (0.129)
BKS-33	1024	128	1024	1024	32	1024/1024 (2)	32/8 (0.258)	1024/1024 (2)	8/8 (0.258)
BKS-36	2048	128	1024	>2048	32	2048/1024 (2)	32/8 (0.258)	2048/1024 (2)	8/8 (0.258)
BKS-45	2048	128	>2048	>2048	64	2048/2048 (2)	32/8 (0.254)	2048/2048 (2)	8/8 (0.129)
BKS-53	2048	128	1024	>2048	64	2048/1024 (2)	32/8 (0.258)	2048/1024 (2)	8/8 (0.133)
BKS-59	1024	128	>2048	>2048	64	1024/2048 (2)	32/8 (0.254)	2048/2048 (2)	8/8 (0.129)
BKS-66	2048	128	>2048	1024	64	2048/2048 (2)	32/8 (0.254)	1024/2048 (2)	8/8 (0.129)
BKS-73	1024	256	1024	1024	32	1024/1024 (2)	64/8 (0.258)	1024/1024 (2)	8/8 (0.258)

SAM=Ampicillin/Sulbactam, TZP=Piperacillin/Tazobactam, CTX=Cefotaxime, AN=Amikacin, GEN=Gentamicin.

Cefotaxime (CTX)-EDTA Combined Disc and Double Disc Synergy Test:

The presence of Metallo-beta-lactamase (MBL) activity was determined using EDTA with the Combined Disk Test (CDT) and double disc synergy test (DDST). The results showed that all twelve MDR *Klebsiella pneumonia* isolates tested negative for MBL production, as demonstrated in Figures (5 A & B). Figure (5 B) illustrates that the use of the cefotaxime-EDTA disk did not result in an increase in the zone of inhibition ≥7 mm compared to the cefotaxime disk alone. The mean zone of inhibition for CTX-EDTA was 12 ± 2 mm, while the mean zone of inhibition for CTX alone was 12±3 mm (95% confidence interval). Furthermore, there was no enhancement of the inhibition zone between the cefotaxime disc and EDTA disc, confirming the absence of MBL enzyme deficiency as depicted in Figure (5 A).

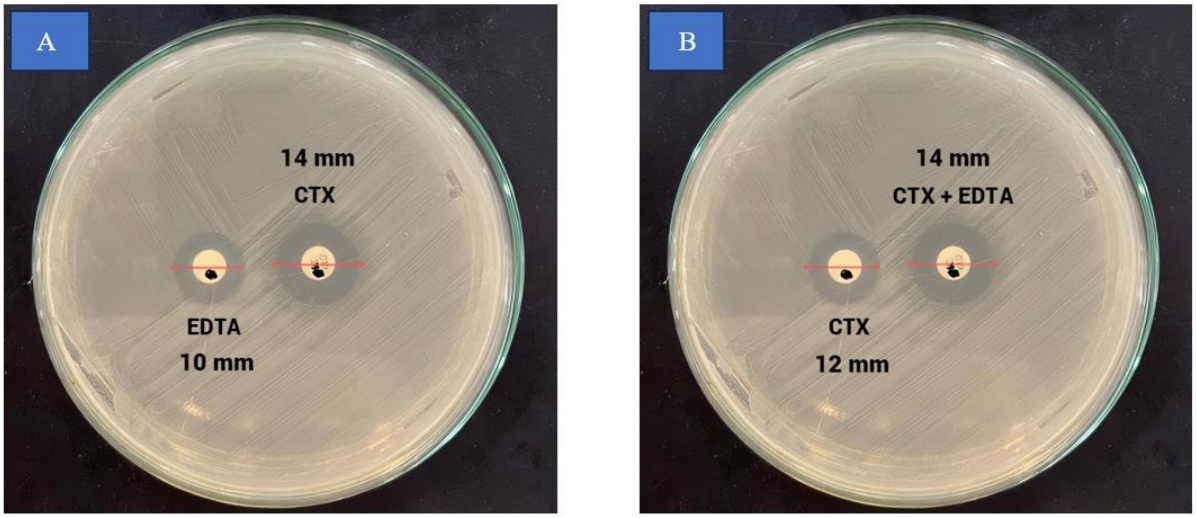

Fig. 5: (A) illustrates that the addition of EDTA to cefotaxime does not result in an increase in the inhibition zone by more than 7 mm, with the extension of the zone of inhibition of cefotaxime being shown. **(B)** depicts the double disc synergy test (DDST) results of cefotaxime and EDTA, indicating no amplification in the area of inhibition between the zones of inhibition of cefotaxime and EDTA.

DISCUSSION

Neonatal sepsis is a serious and life-threatening condition that affects newborn babies, particularly those born prematurely or with low birth weight. It is a major cause of morbidity and mortality worldwide, and early detection and treatment are critical for improving outcomes. The NICU of some Egyptian Hospitals has seen a significant number of cases suspected of neonatal sepsis in the past two years, with 1,500 cases identified. Out of these, 509 cases were clinically diagnosed with neonatal sepsis, indicating that the condition is prevalent in this population. The gender of admitted cases did not show a significant difference, but it was relative to the incidence of the gender of born babies during the study period. This highlights the need for further research to better understand the risk factors associated with neonatal sepsis, including the role of gender and immunity in disease development and progression.

The findings of this study also suggest that females may be more susceptible to neonatal sepsis, with 60% of the total confirmed cases being female. This is contrary to what was reported by Saleem *et al.* (2013), which found that males were more susceptible to the condition. However, it is important to note that other factors, such as the immune system of the baby, may also play a critical role in determining disease incidence and severity. Further research is needed to determine the underlying mechanisms that contribute to the gender differences observed in this study.

Additionally, the study highlights the importance of early detection and treatment of neonatal sepsis. Early Onset Sepsis (EOS) and Late-Onset Sepsis (LOS) are the two categories of neonatal sepsis, with LOS being the more prevalent category, accounting for 95% of confirmed cases. The dominance of LOS may be attributed to the fact that it can occur up to 90 days after labor, providing more time for the infection to develop. Therefore, early screening and diagnosis are critical to identifying and treating neonatal sepsis before it progresses to a more severe stage.

In conclusion, neonatal sepsis is a significant health issue that requires further research and attention to improve outcomes for affected newborns. The findings of this study suggest that gender and immunity may play an important role in disease incidence and severity. The prevalence of LOS also highlights the need for early detection and treatment to prevent the progression of the disease. Ongoing efforts to better understand the underlying mechanisms of neonatal sepsis and to develop effective prevention and treatment strategies are critical to improving outcomes for newborns affected by this condition.

Klebsiella pneumoniae, a bacterium that causes various infections, has been found to be resistant to Ampicillin, a commonly used antibiotic, due to its intrinsic resistance reported by Bernardini *et al.* (2019). Even when beta-lactamase inhibitors are added, the resistance pattern of Ampicillin and Sulbactam remains the same, while Piperacillin and Tazobactam show a decrease in resistance by 26%, with a resistance pattern of 74%. Cephalosporins exhibit a resistance pattern ranging from 79% to 90%, with Cefazolin and Cefepime at 79% and Cefotaxime and Ceftazidime at 90%. Meropenem, a carbapenem antibiotic, showed the lowest resistance pattern of 30%. On the other hand, Aminoglycosides such as Gentamicin, Amikacin, and Tobramycin, exhibit resistance patterns ranging from 75% to 80%. Fluoroquinolones exhibit a resistance pattern of 64% to 78% for Levofloxacin and Ciprofloxacin, respectively. Nitrofurantoin and Trimethoprim-Sulfamethoxazole exhibited a resistance pattern of 66% and 67%, respectively, as reported by Shehab El-Din *et al.* (2015), who found that Klebsiella isolates were 100% resistant to Ampicillin, with a resistance pattern of 90% or more for Cefotaxime and Ceftazidime. These findings indicate the high level of antibiotic resistance that Klebsiella pneumoniae has developed,

making the treatment of infections caused by this bacterium more challenging.

The study found a high prevalence of multi-drug resistant Klebsiella pneumoniae isolates among newborns diagnosed with sepsis. Most of the isolates showed significant resistance to various antibiotics, including Ampicillin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefazolin, Cefoxitin, Ceftazidime, Cefotaxime, Cefepime, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantoin, and Trimethoprim + Sulfamethoxazole, as determined by the AST-VITEK 2. The $bla_{\text{CTX-M-15}}$ gene was detected in 66 out of 101 isolates, consistent with previous studies conducted in Greece and China. Fursova *et al.* (2021) also reported the detection of MDR *Klebsiella pneumoniae* isolates with the $bla_{\text{CTX-M-15}}$ gene in a neuro-ICU. The findings of this study highlight the urgent need for effective antimicrobial stewardship programs to prevent the spread of antibiotic-resistant *Klebsiella pneumoniae* infections in neonatal intensive care units.

The limitations of blood cultures in terms of processing times and sensitivity necessitate the development of a novel, quick, and accurate approach for detecting and identifying *K. pneumoniae* in blood samples from neonates with suspected sepsis. This study highlights the potential of real-time polymerase chain reaction (QRT-PCR) in quantifying the $bla_{\text{CTX-M-15}}$ gene in (23/33, 69%) *K. pneumoniae* isolated from neonates with confirmed sepsis. Our PCR results were similar to those of Jordan *et al.* (2000), who employed a pre-culturing technique to increase the bacterial quantity in clinical samples from septic newborns and facilitate bacterial identification, resulting in PCR investigation with satisfactory sensitivity.

The findings of this investigation highlight that the $bla_{\text{CTX-M-15}}$ gene is commonly present in *K. pneumoniae* clinical isolates causing neonatal sepsis and is associated with multi-drug resistance. The use of quantitative real-time polymerase chain reaction (qRT-PCR) revealed a higher expression level of the $bla_{\text{CTX-M-15}}$ gene in MDR K. pneumoniae isolates compared to susceptible strains. These results are consistent with previous studies from Riyadh, Moghaddam *et al.,* and Eskandari-Nasab *et al.* (2018), which have identified the *blaCTX-M* gene as a common genetic marker of ESBL-producing strains of *K. pneumoniae*. The observed variation in the prevalence of *blaCTX-M* gene expression among different regions in Iran highlights the need for continued monitoring of antimicrobial resistance patterns in clinical isolates of *K. pneumoniae*.

In this study, the expression of the $bla_{\text{CTX-M-15}}$ gene in MDR K. pneumoniae isolates was assessed using a two-step quantitative reverse transcription-PCR method. The data were normalized to the expression level of the 16S-23S ITS rRNA gene that is constitutively expressed in *K. pneumoniae*. The results indicated a significantly higher level of $bla_{\text{CTX-M-15}}$ gene expression in *K. pneumoniae* isolates (KR1-KR23) compared to susceptible isolates ($p < 0.01$). The findings were supported by Ghonaim *et al.* (2021), who reported a significant difference in the presence of the CTX-M gene between ESβL-producing and non-ESβL-producing *K. pneumoniae* isolates. Calibration curves for each gene were produced using serially diluted cDNA prepared from in vitro-obtained RNA standards.

The aim of this study was to investigate the potential impact of CTX-M 15 gene expression on antibiotic resistance patterns in neonatal sepsis caused by Klebsiella pneumoniae. Specifically, we evaluated the minimum inhibitory concentrations (MIC) of selected antibiotics commonly used for treatment, including Ampicillin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefotaxime, and Ceftazidime as mentioned by Abdul-Jabar *et al.* (2020). Our results showed that there was no significant change in the resistance of Klebsiella towards Ampicillin, as it is intrinsically resistant to this antibiotic. However, we did observe changes in the

MICs of other antibiotics tested in relation to the fold change in CTX-M 15 gene expression. This suggests that the acquisition of CTX-M 15 resistance may have an impact on the efficacy of certain antibiotics used for treatment. Overall, our findings highlight the potential significance of CTX-M 15 gene expression in Klebsiella antibiotic resistance and can inform the selection of appropriate treatment options for neonatal sepsis caused by this pathogen.

In an effort to understand the extent of interference of acquired CTX-M 15 resistance on antibiotics resistance patterns in Klebsiella, the minimum inhibitory concentrations (MIC) of various antibiotics were tested in relation to fold changes in CTX-M 15 gene expression. It was found that Klebsiella is intrinsically resistant to Ampicillin, and the addition of sulbactam as a beta-lactamase inhibitor did not affect resistance levels. With regards to piperacillin and tazobactam, Klebsiella isolates showed a range of sensitivity, with some being completely sensitive and others showing intermediate sensitivity with a fold change of up to 7. Resistance began to appear at a fold change of 8.2249 and above, with 40% of the total Klebsiella isolates demonstrating resistance within a range of 8.2249 to 21.5557. These findings suggest that the acquired CTX-M 15 resistance gene can interfere with antibiotics resistance patterns, particularly for piperacillin and tazobactam, in Klebsiella.

The results of testing Cefotaxime and Ceftazidime against Klebsiella showed that only a small percentage of isolates were sensitive to these antibiotics, with only five isolates out of thirty-two exhibiting sensitivities within a certain range of CTX-M 15 gene expression. Intermediate sensitivity was observed in three isolates within a two-fold change range of the gene expression, but the resistance increased gradually as the fold change of CTX-M 15 expression increased above three. Minimum inhibitory concentrations of cefotaxime also increased rapidly within six Klebsiella isolates, ranging from 128 µg/ml to above 2048 µg/ml as the fold of expression changed from 12.5533 to 21.5557. These findings suggest that Klebsiella resistance to these antibiotics is largely dependent on the level of CTX-M 15 gene expression.

The findings indicate that even a minor change in the fold change of CTX-M 15 expression significantly affects the resistance of Klebsiella isolates towards cefotaxime. Ceftazidime also shows susceptibility towards CTX-M 15 expression, but the impact is less compared to cefotaxime. The evaluation of minimum inhibitory concentrations suggests that for each level of inhibition, there is a gradual increase in the score assigned. The results show that a change in the fold change of CTX-M 15 expression leads to a double change in the minimum inhibitory concentration score for cefotaxime and a single change for ceftazidime.

The statistical analysis conducted revealed a significant correlation between the fold change of CTXM-15 expression and the resistance level of the targeted antibiotics. The findings confirm the extended action of CTXM-15, as evidenced by the statistical results. It was observed that Ampicillin resistance in Klebsiella is not affected by CTXM-15 since the bacterium is intrinsically resistant to Ampicillin. However, the statistical analysis showed a strong positive correlation (0.836) between Piperacillin, particularly in combination with Tazobactam, and the fold change expression of CTXM-15. This correlation indicates that 69.8% of the variance in the minimum inhibitory concentration of Piperacillin can be predicted from the fold change of CTXM-15.

The Analysis of Variance (ANOVA) testing analysis was used to evaluate the relationship between the fold change of CTXM 15 and the minimum inhibitory concentration of piperacillin/tazobactam and ceftazidime. The model was found to be statistically significant at p-value < 0.05, indicating that the fold change of CTXM 15 is a good predictor of the minimum inhibitory concentrations of piperacillin/tazobactam and ceftazidime.For piperacillin/tazobactam, each point change in the fold expression of

blaCTXM 15 is associated with a 1.607-point change in the minimum inhibitory concentration, while for ceftazidime, each one-point change in blaCTXM 15 is linked with a 1.493 point change in the minimum inhibitory concentration. This suggests that a strong or moderate change in the fold expression of CTXM 15 is required to affect the resistance of Klebsiella towards piperacillin/tazobactam and ceftazidime, respectively. These findings suggest that CTXM 15 is a good contributor to the resistance of Klebsiella toward these antibiotics.

The acquisition of the CTXM 15 plasmid in Klebsiella leads to acquired resistance towards cefotaxime. Our analysis of the correlation between cefotaxime minimum inhibitory concentrations and CTXM 15 expression fold change revealed a strong positive correlation of 0.809, with a 65.5% variance in cefotaxime minimum inhibitory concentrations attributed to changes in CTXM 15 expression. The ANOVA testing of this model was statistically significant at p-value < 0.05, indicating that CTXM 15 expression fold change is a good predictor of changes in cefotaxime minimum inhibitory concentrations. Specifically, every one-point change in CTXM 15 expression fold change is accompanied by 1.296 points of change in minimum inhibitory concentrations greater than or equal to 4 µg/ml, indicating a rapid response to changes in CTXM 15 expression fold change.

MIC Testing and Synergy Testing of *K. Pneumonia* MDR Isolates:

In order to determine the antibiotic sensitivity of *Klebsiella*, a total of 101 isolates were tested with sixteen different antibiotics. Out of these, 66 isolates were found to be multi-drug resistant as they exhibited resistance to at least one agent from three different classes of antibiotics. To determine the potential for antibiotic combination therapy, the minimum inhibitory concentration of the targeted antibiotics must be assessed to determine whether the combination leads to synergistic or antagonistic effects. To conduct further analysis, a sample of 22 multi-drug resistant *Klebsiella* isolates was selected using simple random sampling techniques. The results showed that all 22 isolates were resistant to AMP, CTX, NIF, and SXT, with 95% resistance to CAZ and CIP, and 91% resistance to SAM, CZ, FOX, TOB, and LVX. 68% of isolates were found to be resistant to FEP and GEN, while 59% were resistant to TZP. Only 12 (55%) of the isolates exhibited resistance to all of the antibiotics tested. These 12 isolates were selected for further study as they represent the most severe cases of multi-drug resistance, exhibiting complete resistance to all antibiotics used in the study.

The minimum inhibitory concentrations of 22 selected klebsiella isolates were determined for ampicillin/sulbactam, piperacillin/ tazobactam, cefotaxime, gentamicin, and amikacin. The ranges of minimum inhibitory concentrations were found to be 1024-2048 mg/l, 128-512 mg/l, 1024-2048 mg/l, 1024-2048 mg/l, and 32-64 mg/l, respectively. The susceptible ranges for minimum inhibitory concentrations were determined to be ≤ 8/4 µg/ml for ampicillin/sulbactam, ≤ 16/4 µg/ml for piperacillin/tazobactam, ≤ 1 µg/ml for cefotaxime, ≤ 4 µg/ml for gentamicin, and ≤ 16 µg/ml for amikacin.

To overcome the acquired resistance towards cefotaxime, it is crucial to determine which antibiotic combination can be used effectively. The checkerboard synergy testing analysis aims to identify the synergistic or additive effect of antibiotic combinations. The goal of a positive combination is to reduce the minimum inhibitory concentration and reach the susceptible breakpoint range of both antibiotics, according to CLSI 2021. By considering the minimum inhibitory concentration of each antibiotic alone and in combination, the fractional inhibitory concentration index value (FIC) can be determined using the following equation: A/MICA + B/MICB = FICA + FICB = FIC Index. This approach has been used in

previous studies (Orhan *et al.,* 2005; Costa *et al.*, 2019).

The calculation of the FIC Index determines whether a combination of antibiotics has a synergistic or antagonistic effect. An FIC Index value of >1 indicates antagonism, meaning that the combination does not result in a potent effect. Conversely, an FIC Index value of <1 indicates synergism, which produces a potent action when used with another antibiotic. However, the synergism may not be effective enough to reduce the minimum inhibitory concentration to the sensitive breakpoint according to CLSI 2021. Therefore, the ideal combination should have a synergistic effect with an FIC Index value <1 and reduce the minimum inhibitory concentration to the sensitive breakpoint as per CLSI 2021 guidelines.

For instance, the ampicillin/ sulbactam (SAM) minimum inhibitory concentration against the chosen *Klebsiella* isolates ranges from 1024-2048 mg/L. Upon addition of ampicillin/sulbactam to cefotaxime, there was no change in the minimum inhibitory concentration of both antibiotics, and the FIC index value was 2, indicating antagonism. This observation can be attributed to *klebsiella*'s inherent resistance to ampicillin, and the action of sulbactam with cefotaxime is insufficient to overcome the resistance of CTXM 15 towards cefotaxime.

The minimum inhibitory concentration of piperacillin/tazobactam alone ranges from 128-512 mg/l, while for cefotaxime alone, it ranges from 1024-2048 mg/l. When both antibiotics are combined, the FIC index suggests a synergistic effect, but in practical terms, piperacillin/tazobactam does not reach the susceptible breakpoints, and cefotaxime's decline in minimum inhibitory concentration is not sufficient to be beneficial, as it is still above the sensitive breakpoint. However, the addition of tazobactam to the combination has a detectable benefit as it improves the effect of cefotaxime, which is particularly important in cases of neonatal sepsis. The most commonly used combination for neonatal sepsis is ampicillin with gentamicin, but this is not effective against *Klebsiella* due to its intrinsic resistance to ampicillin. Therefore, a better combination for *Klebsiella* would be with cefotaxime to overcome CTXM15 resistance.

The combination of gentamicin with cefotaxime did not show any significant difference in the minimum inhibitory concentration of either antibiotic, as the FIC index was greater than 1. However, the synthetic aminoglycoside, amikacin, may produce different results compared to other aminoglycosides in the same class. In the case of *Klebsiella*, amikacin showed a lower FIC index than gentamicin, indicating synergism El-Demerdash and Bakry (2020). To determine the productivity and usefulness of this combination, minimum inhibitory concentration values were examined. The combination of amikacin with cefotaxime resulted in a decline in the minimum inhibitory concentration of amikacin to a level below the susceptible breakpoint of ≤ 16 µg/ml, and a decline in the minimum inhibitory concentration of cefotaxime to the susceptible breakpoint of ≤ 1 µg/ml. This suggests that the combination of amikacin with cefotaxime could be a productive and beneficial combination, effective in bypassing the resistance of Klebsiella towards cefotaxime, particularly in cases where resistance is acquired through the CTXM 15 plasmid, as previously mentioned by Klastersky *et al.* (1980) and Martino *et al.* (1985).

Cefotaxime (CTX)-EDTA Combined Disc and Double Disc Synergy Test:

Among the tested combinations, the amikacin and cefotaxime combination was found to be the most effective against Klebsiella. This was due to the fact that piperacillin/tazobactam and cefotaxime had a common target of cell wall inhibition, rendering them competitors and producing no additional benefit when used together. The gentamicin and cefotaxime combination was also ineffective due to the natural source of gentamicin, which limited its capability to assist cefotaxime in killing Klebsiella. In contrast, amikacin, as a fully synthetic aminoglycoside, was found to overcome the

resistance towards cefotaxime, as it is less susceptible to bacterial resistance. Amikacin's ability to weaken the cell wall and facilitate the action of cell wall inhibitors like cefotaxime was attributed to its chelation of magnesium ions. A combined disc test and double disk synergy test were conducted with cefotaxime and EDTA to rule out the possibility of Klebsiella producing the Metallo-beta-lactamase enzyme, which is inhibited by amikacin. Twelve MDR *Klebsiella pneumonia* samples were tested, and the absence of a significant increase in the inhibition zone between the cefotaxime disc and the EDTA disc confirmed the absence of the Metallo-beta-lactamase enzyme.

Conclusions

In this study, it was found that neonates with sepsis in some Egyptian hospitals were infected with CTX-M-15-positive K. pneumoniae isolates, which exhibited high-level resistance to a range of antibiotics commonly used for treatment. The high level of antibiotic resistance observed is alarming and highlights the urgent need for the appropriate use of antibiotics to improve treatment outcomes. The expression of CTX-M-15 was found to be closely associated with K. pneumoniae, which is a major contributor to neonatal mortality. Combinations of antibiotics are necessary to overcome the resistance caused by CTX-M-15, and the results suggest that amikacin and cefotaxime may be the best combination for further in-vivo studies in neonates to prevent mortality from MDR Klebsiella infections.

List of Abbreviations

Abbreviation	Definition
NICU	Neonatal intensive care unit
CTXM	Extended-spectrum β-lactamases
EDTA	Ethylenediaminetetraacetic acid
RT-PCR	Reverse transcription polymerase chain reaction
qPCR	Real-time polymerase chain reaction
EGNN	Egyptian Neonatal Network
EOS	Early onset sepsis
LOS	late-onset sepsis
CTX	Cefotaxime
SAM,	Ampicillin/Sulbactam
TZP	Piperacillin/Tazobactam
GEN	Gentamicin
AN	Amikacin
ANOVA	Analysis of Variance
FIC	Fractional inhibitory concentration
MIC	minimum inhibitory concentration
MDR	Multidrug Resistance
MBL	Metallo-beta-lactamase
CDT	Combined Disk Test
DDST	double disc synergy test

Declarations:

Ethical Approval: This research was approved by the Ethical Committee, faculty of medicine, Ain Shams University. Ethical approval number FWA000017585.

Competing interests: No conflict of interest

Authors Contributions: I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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REFERENCES

Abdul-Jabar, H. H., Abd, A. K. H., & Abdulamir, A. S. (2020). Efficacy of combinations of piperacilline/ tazobactam, ceftazidime, amikacin and bacteriophage against enterobacteriaceae sepsis in neonates: In vitro study. *Systematic Reviews in Pharmacy*, 11(10), 165–170. https://doi.org/10.31838/srp.2020.10.28

Awosile, B. B., Agbaje, M., Adebowale, O., Kehinde, O., & Omoshaba, E. (2022). Beta-lactamase resistance genes in Enterobacteriaceae from Nigeria. *African Journal of Laboratory Medicine*, 11(1), 1–10. https://doi.org/10.4102/AJLM.V11I1.1371

Bergès, L., Rodriguez-Villalobos, H., Deplano, A., & Struelens, M. J. (2007). Prospective evaluation of imipenem/EDTA combined disc and Etest for detection of metallo-β-lactamase-producing *Pseudomonas aeruginosa* [10]. *Journal of Antimicrobial Chemotherapy*, 59(4), 812–813. https://doi.org/10.1093/jac/dkm001

Bernardini, A., Cuesta, T., Tomás, A., Bengoechea, J. A., Martínez, J. L., & Sánchez, M. B. (2019). The intrinsic resistome of Klebsiella pneumoniae. *International Journal of Antimicrobial Agents*, 53(1), 29–33. https://doi.org/10.1016/j.ijantimicag.2018.09.012

Bielicki, J. A., Sharland, M., Heath, P. T., Walker, A. S., Agarwal, R., Turner, P., & Cromwell, D. A. (2020). Evaluation of the Coverage of 3 Antibiotic Regimens for Neonatal Sepsis in the Hospital Setting across Asian Countries. *JAMA Network Open*, 3(2), 1–13. https://doi.org/10.1001/jamanetworkopen.2019.21124

Chong, Y., Shimoda, S., Yakushiji, H., Ito, Y., Miyamoto, T., Kamimura, T., Shimono, N., & Akashi, K. (2013). Community spread of extended-spectrum β-lactamase-producing Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis: A long-term study in Japan. *Journal of Medical Microbiology*, 62(PART7), 1038–1043. https://doi.org/10.1099/jmm.0.059279-0

Costa, R. A., Ortega, D. B., Fulgêncio, D. L. A., Costa, F. S., Araújo, T. F., & Barreto, C. C. (2019). Checkerboard testing method indicates synergic effect of pelgipeptins against multidrug resistant Klebsiella pneumoniae. *Biotechnology Research and Innovation*, 3(1), 187–191. https://doi.org/10.1016/j.biori.2018.12.001

Davis, B. D. (n.d.). SESSION I Bactericidal Synergism Between f3-Lactams and Aminoglycosides: Mechanism and Possible Therapeutic Implications. *In Reviews of Infectious Diseases*, (Vol. 4, Issue 2). http://cid.oxfordjournals.org/

Eskandari-Nasab E, MSc, Moghadam pour, M., MSc, & Tahmasebi, A., MSc (2018). Prevalence of blaCTX-M Gene among Extended-Spectrum β-Lactamases Producing Klebsiella pneumoniae Clinical Isolates in Iran: A Meta-Analysis. *Iranian journal of medical sciences*, 43(4), 347–354.

Fey A, Eichler S, Flavier S, Christen R, Höfle MG, and Guzmán CA (2004). Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using Salmonella as a model organism. *Applied and Environmental Microbiology Journal*, 70:3618-3623.

Fursova, N. K., Astashkin, E. I., Ershova, O. N., Aleksandrova, I. A., Savin, I. A., Novikova, T. S., Fedyukina, G. N., Kislichkina, A. A., Fursov, M. V.,

Kuzina, E. S., Biketov, S. F., & Dyatlov, I. A. (2021). Multidrug-resistant klebsiella pneumoniae causing severe infections in the neuro-icu. *In Antibiotics,* (Vol. 10, Issue 8). https://doi.org/10.3390/antibiotics10080979

Galani, I., Karaiskos, I., Angelidis, E., Papoutsaki, V., Galani, L., Souli, M., Antoniadou, A., and Giamarellou, H. (2021). Emergence of ceftazidime-avibactam resistance through distinct genomic adaptations in KPC-2-producing Klebsiella pneumoniae of sequence type 39 during treatment. *European Journal of Clinical Microbiology & Infectious Diseases*, 40, 219–224.

Ghonaim MM, Sahar AM, Nashwa N. Khamis, Fady M. El-Gendy, and Soma E. Ajlan (2021). Detection of virulence genes (magA and rmpA) and resistance gene (CTX-M) in Klebsiella pneumoniae isolated from neonates with septicemia. *Microbes and Infectious Diseases;* 2(4): 767-780.

Giamarellou, H. (1986) 'Aminoglycosides plus beta-lactams against gram-negative organisms: Evaluation of in vitro synergy and chemical interactions', *The American Journal of Medicine,* 80(6), pp. 126–137. Doi :10.1016/0002-9343(86)90490-0.

Hammoud, M. S., Al-Taiar, A., Al-Abdi, S. Y., Bozaid, H., Khan, A., AlMuhairi, L. M., & Rehman, M. U. (2017). Late-onset neonatal sepsis in Arab states in the Gulf region: two-year prospective study. *International Journal of Infectious Diseases*, 55, 125–130. https://doi.org/10.1016/j.ijid.2017.01.006

Harbaoui, S., Ferjani, S., Abbassi, M. S., Saidani, M., Gargueh, T., Ferjani, M., Hammi, Y., & Boutiba-Ben Boubaker, I. (2022). Genetic heterogeneity and predominance of bla CTX- M -15 in cefotaxime-resistantEnterobacteriaceae isolates colonizing hospitalized children in Tunisia. *Letters in Applied Microbiology*. https://doi. org/10.1111/lam.13812

Hasan, B., & Swedberg, G. (2022). Molecular Characterization of Clinically Relevant Extended-Spectrum β-Lactamases blaCTX-M-15-Producing Enterobacteriaceae Isolated from Free-Range Chicken from Households in Bangladesh. *Microbial Drug Resistance*, 28(7), 780–786. https://doi.org/10.1089/mdr.2021.0264

Hassan, H., & Abdalhamid, B. (2014). Molecular characterization of extended-spectrum beta-lactamase producing Enterobacteriaceae in a Saudi Arabian tertiary hospital. *Journal of Infection in Developing Countries*, 8(3), 282–288. https://doi.org/10.3855/jidc.3809

Hassuna, N. A., AbdelAziz, R. A., Zakaria, A., & Abdelhakeem, M. (2020). Extensively- Drug Resistant Klebsiella pneumoniae Recovered From Neonatal Sepsis Cases From a Major NICU in Egypt. *Frontiers in Microbiology*, 11(June). https://doi.org/10.3389/fmicb.2020.01375

Hrbacek, J., Cermak, P., & Zachoval, R. (2021). Current antibiotic resistance patterns of rare uropathogens: survey from Central European Urology Department 2011–2019. *BMC Urology*, 21(1), 1–8. https://doi.org/10.1186/s12894-021-00821-8

Jordan JA, Durso MB (2000). Comparison of 16S rRNA gene PCR and BACTEC 9240 for detection of neonatal bacteremia. *Journal of Clinical Microbiology*,38:2574-8.

Klastersky, J., Gaya, H., Zinner, S. H., Bernard, C., & Ryff, J-C. (1980). Cefotaxime and amikacin: results of in vitro and in vivo studies against Gram-negative bacteria and Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy*, 6(suppl A), 55–61. https://doi.org/

10.1093/jac/6.suppl_a.55

Kaur, J. (2013) 'Modified double disc synergy test to detect ESBL production in urinary isolates of escherichia coli and Klebsiella pneumoniae', *Journal Of Clinical And Diagnostic Research* [Preprint]. doi:10.7860/jcdr/2013/4619.2734.

Leclercq, R., Cantón, R., Brown, D. F. J., Giske, C. G., Heisig, P., MacGowan, A. P., Mouton, J. W., Nordmann, P., Rodloff, A. C., Rossolini, G. M., Soussy, C.-J. ., Steinbakk, M., Winstanley, T. G., & Kahlmeter, G. (2013). EUCAST expert rules in antimicrobial susceptibility testing. *Clinical Microbiology and Infection*, 19(2), 141–160.

Lee, K., Lim, Y. S., Yong, D., Yum, J. H., & Chong, Y. (2003). Evaluation of the Hodge Test and the Imipenem-EDTA Double-Disk Synergy Test for Differentiating Metallo-β-Lactamase-Producing Isolates of *Pseudomonas spp*. and *Acinetobacter spp*. *Journal of Clinical Microbiology*, 41(10), 4623–4629. https://doi.org/10.1128/jcm.41.10.4623-4629.2003

Maillet S, Naas T, Crepin S, Roque-Afonso AM, Lafay F, Efstathiou S, and Labetoulle M (2006). Herpes simplex virus type 1 latently infected neurons differentially express latency-associated and ICP0 transcripts. *Journal of Virology*, 80:9310-9321

Martin, R. M., & Bachman, M. A. (2018). Colonization, infection, and the accessory genome of Klebsiella pneumoniae. *Frontiers in Cellular and Infection Microbiology*, 8(JAN), 1–15.https://doi.org/10.3389/ fcimb. 2018.00004.

Martino, P., Venditti, M., Serra, P., Petti, M. C., & Mandelli, F. (1985). Cefotaxime plus amikacin as empiric therapy in the treatment of febrile episodes in neutropenic patients with hematologic malignancies. *Infection* ,13(3), 125–129. https://doi.org/10.1007/bf01642871

Moghaddam MN, Beidokhti MH, Jamehdar SA, and Ghahraman M (2014). Genetic properties of blaCTX-M and blaPER β-lactamase genes in clinical isolates of Enterobacteriaceae by polymerase chain reaction. *Iranian journal of basic medical sciences*, 17(5): 378–383.

Mohsen, L., Ramy, N., Saied, D., Akmal, D., Salama, N., Abdel Haleim, M. M., & Aly, H. (2017). Emerging antimicrobial resistance in early and late-onset neonatal sepsis. Antimicrobial *Resistance and Infection Control*, 6(1), 1–9. https://doi.org/10.1186/s13756-017-0225-9

Omair, M., Usman, J., Kaleem, F., Hassan, A., Khalid, A., & Fahim, Q. (2012). Evaluation of combined disc method for the detection of metallo-β-lactamase producing Gram negative bacilli. *Malaysian Journal of Microbiology*, May 2014. https://doi.org/10.21161/mjm.33911

Orhan, G., Bayram, A., Zer, Y., & Balci, I. (2005). Synergy Tests by E Test and Checkerboard Methods of Antimicrobial Combinations against Brucella melitensis. *Journal of Clinical Microbiology*, 43(1), 140–143. https://doi.org/10.1128/jcm. 43. 1.140-143.2005

Pot, M. et al. (2021) 'Wide distribution and specific resistance pattern to third-generation cephalosporins of Enterobacter cloacae complex members in humans and in the environment in Guadeloupe (French west indies)', *Frontiers in Microbiology*, 12. doi:10.3389/ fmicb.2021.628058.

Ramdani-Bouguessa, N., Mendonça, N., Leitão, J., Ferreira, E., Tazir, M., & Caniça, M. (2006). CTX-M-3 and CTX-M-15 extended-spectrum β-lactamases in isolates of Escherichia coli from a hospital in Algiers, Algeria. *Journal of Clinical*

Microbiology, 44(12), 4584–4586. https://doi.org/10.1128/JCM.01445-06

Rocha J, Ferreira C, Mil-Homens D. et al. (2022). Third generation cephalosporin-resistant Klebsiella pneumoniae thriving in patients and in wastewater: what do they have in common?. *BMC Genomics*, 23, 72. https://doi.org/10.1186/s12864-021-08279-6

S. El-Demerdash, A., & R. Bakry, N. (2020). Evaluation of the Synergistic Effect of Amikacin with Cefotaxime against Pseudomonas aeruginosa and Its Biofilm Genes Expression. *Gene Expression and Phenotypic Traits.* https://doi.org/10.5772/intechopen.91146

Saleem, A. F., Qamar, F. N., Shahzad, H., Qadir, M., & Zaidi, A. K. M. (2013). Trends in antibiotic susceptibility and incidence of late-onset Klebsiella pneumoniae neonatal sepsis over a six-year period in a neonatal intensive care unit in Karachi, Pakistan. *International Journal of Infectious Diseases*, 17(11), e961–e965. https://doi.org/10.1016/j.ijid.2013.04.007

Sands, K., Carvalho, M. J., Portal, E., Thomson, K., Dyer, C., Akpulu, C., Andrews, R., Ferreira, A., Gillespie, D., Hender, T., Hood, K., Mathias, J., Milton, R., Nieto, M., Taiyari, K., Chan, G. J., Bekele, D., Solomon, S., Basu, S., & Chattopadhyay, P. (2021). Characterization of antimicrobial-resistant Gram-negative bacteria that cause neonatal sepsis in seven low- and middle-income countries. *Nature Microbiology*, 6(4), 512–523. https: //doi. org/ 10. 1038/ s41564-021-00870 -7

Shehab El-Din, E. M. R., El-Sokkary, M. M. A., Bassiouny, M. R., & Hassan, R. (2015). Epidemiology of neonatal sepsis and implicated pathogens: A Study from Egypt. *BioMed Research International,* 2015. https://doi.org/10.1155/2015/509484

Shen P, Berglund B, Chen Y, Zhou Y, Xiao T, Xiao Y and Zhou K (2020). Hypervirulence Markers Among Non-ST11 Strains of Carbapenem- and Multidrug-Resistant Klebsiella pneumoniae Isolated from Patients with Bloodstream Infections. Front. Microbiol., 11, 1199.

Soumya, S., & B. Nagmoti, M. (2017). Comparison of Different Phenotypic Tests for the Detection of Metallo-B-Lactamase (MBL) Producing Pseudomonas aeruginosa. *International Journal of Current Microbiology and Applied Sciences*, 6(10), 3231–3238. https://doi.org/10.20546/ijcmas.2017.610.378

White, R. L., Burgess, D. S., Manduru, M., & Bosso, J. A. (1996). Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrobial Agents and Chemotherapy*, 40(8), 1914–1918. https://doi.org/10.1128/aac.40.8.1914.

Xie, S.; Fu, S.; Li, M.; Guo, Z.; Zhu, X.; Ren, J.; Hu, F. (2020). Microbiological Characteristics of Carbapenem-Resistant Enterobacteriaceae Clinical Isolates Collected from County Hospitals. Infect. Drug Resist., 13, 1163-1169.

Yang, Y., Osawa, K., Kitagawa, K., Hosoya, S., Onishi, R., Ishii, A., Shirakawa, T., Hirai, I., Kuntaman, K., Tanimoto, H., Shigemura, K., & Fujisawa, M. (2021). Differential effects of chromosome and plasmid bla CTX-M-15 genes on antibiotic susceptibilities in extended-spectrum beta-lactamase-producing Escherichia coli isolates from patients with urinary tract infection. *International Journal of Urology*, 28(6), 623–628. https://doi.org/10.1111/iju.14498.