

Neonatal Sepsis: The impact of Hypervirulent *Klebsiella pneumoniae* in a Tertiary Care Hospital

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Abstract

Neonatal sepsis is a blood-stream infection that affects newborns under the age of 28 days. Sepsis is common in NICUs and has a high prevalence of *Klebsiella* species. As a result, the study aims to find the antibiotic resistance profile, virulence factors, and the prognosis of *K. pneumoniae*-infected neonates. A prospective study was conducted which included 140 neonates with clinical sepsis. Characterization of *Klebsiella pneumoniae* isolates was done by conventional methods. Drug resistance and virulence factors were detected by phenotypic methods. Genotypic methods included 16s rRNA amplification and sequencing. Detection of multidrug-resistant genes by PCR was performed. *K. pneumoniae* (26.9%) was the most common pathogen isolated. A high prevalence of ESBL was detected (58.8%). The prevalence of CRKP and M β L was about 29.4%, and 23.5% respectively. Two strains were Strong biofilm producers and nine isolates showed Beta hemolysis. 7 strains were positive for the string test. Four strains were positive for the wcaG gene. 3 positive for magA (K1) and 2 were for gene wzy (K2). Three isolates carried *bla*_{CTX-M}, four isolates harbored *bla*_{VIM}, two for IMP, and one for NDM and KPC gene. *K. pneumoniae* isolates in the NICU increased in frequency and antibiotic resistance. It is a serious hazard to the healthcare system, and it necessitates strict infection control methods in healthcare settings, as well as antibiotic stewardship to prevent the overuse of antibiotics in neonatal sepsis.

Keywords: Neonatal Sepsis, Hypervirulent *Klebsiella pneumoniae*, Drug Resistance, Virulence Factors, Genotypic Methods

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INTRODUCTION

Neonatal sepsis (NS) is a blood-stream infection that affects newborns under the age of 28 days. NS is a primary cause of fatality and morbidity.¹ The impact of newborn death is disproportionately spread worldwide, with developing nations facing the burden of it. India is one of the five countries responsible for half of all newborn fatalities.² The diversity of organisms that cause sepsis differs geographically, and in the same location, it changes with time.³

NS is characterized as early-onset sepsis (EOS) or late-onset sepsis (LOS) depending on the timing of symptoms. The EOS is sepsis that occurs within 72 hours of birth and is primarily caused by organisms acquired before and during delivery. LOS, on the other hand, occurs after the first three days of life and most organisms are acquired after delivery.⁴

Prematurity, low birth weight, and perinatal problems like chorioamnionitis and premature rupture of membranes (PROM) are all linked to early-onset sepsis (EOS). Invasive interventions such as resuscitation and central venous catheterization, are linked to late-onset sepsis (LOS).⁵

Sepsis is common in NICUs, with the rate ranging from 28 to 50%, and has a high prevalence of *Klebsiella* species. *Klebsiella pneumoniae* (KP) infections lead high rate of morbidity and mortality. In neonatal critical care units, it is also one of the most common causes of outbreaks (NICUs).⁶

Multi-drug resistant (MDR) strains are becoming more common, posing a major global challenge in selecting effective antibiotics for treating hospital-acquired infections.⁷ The use of invasive equipment, insufficient diagnostic techniques, immunosuppressed conditions, and overuse of antibiotics have all been linked to the rise of MDR isolates in healthcare institutions.⁸

Unfortunately, with the advent of extensively drug-resistant (XDR) and pan drug-resistant (PDR) *K. pneumoniae* strains in recent years, the situation of antimicrobial resistance has gotten worse.⁹ Carbapenem-resistant *K. pneumoniae* (CRKP) has just emerged and is rapidly spreading over the world. Infection with these “superbugs” frequently results in severe

consequences.¹⁰ As a result, the study aims to find the antibiotic resistance profile, resistance mechanisms, virulence factors, and the prognosis of *K. pneumoniae*-infected neonates.

Study Design, Period and Setting

From January 2020 to December 2021, a hospital-based prospective study was conducted. The sample size (N) was computed with 95% confidence interval and 5% degree of freedom using a single population proportion formula and 13.6 % prevalence from a prior pilot study. As a result, the minimum sample size was calculated to be 140, and participants were recruited as needed until the sample size was reached.

Institutional Ethical Committee clearance was obtained. All neonates suspected of having sepsis were included in the study population. During the data collecting period, all neonates with clinical signs and symptoms of sepsis who had not been given antibiotics were eligible. Neonates having a birth weight of less than 1000 g, as well as those with pneumonia, meningitis or congenital anomalies were excluded.

Maternal data such as maternal chronic illnesses, maternal hypertension, gestational diabetes mellitus, chorioamnionitis, PROM >18 hours, and clinical sequelae were recorded. Clinical symptoms like temperature instability, convulsions, feeding problems, lethargy, and respiratory distress are used to diagnose neonatal sepsis in the study setting, as well as laboratory findings such as C-reactive protein (CRP), complete blood count (CBC), and/or blood cultures.

A standard proforma was specifically constructed to collect patients' demographic data and essential information such as delivery history, risk factors for infection, symptoms, laboratory results, and clinical outcomes.

Laboratory Methods

Phenotypic Identification

Two milliliters (2ml) of venous blood were collected from each neonate. Each sample was then inoculated directly into pediatric brain-heart infusion broth (BHIB) to make a 1:10 dilution and BACTEC PedsPlus™ (Becton Dickinson, Ireland) culture vial. All culture broths were maintained aerobically at 37°C for seven days, with daily observations for the presence or absence of visible

microbial growth. Blood agar, chocolate agar, and MacConkey agar were used for subculturing. The pathogens were identified using standard microbiological procedures.¹¹ Isolates of *K. pneumoniae* were characterized.

Antimicrobial susceptibility pattern was determined. According to the CLSI recommendations, they were assessed for zones of inhibition and classified as sensitive, moderate, or resistant.

Antibiotics like ampicillin, amikacin, cefazolin, cefuroxime, ceftazidime, cefotaxime, cefepime, piperacillin-tazobactam, gentamicin, tobramycin, co-trimoxazole, ciprofloxacin, imipenem, and meropenem were assessed and the results were compared with ATCC standard strain.¹²

Phenotypic Determination of Antibiotic-resistant *K. pneumoniae* Strains

Detection of MDR, XDR, and PDR strains was done based on the disc diffusion method's results.

AmpC Beta-lactamases

- Screened by using ceftiofur (30µg) disc.
- After incubation, if the diameter of a zone to >14mm for ceftiofur - potential AmpC-beta-lactamase producers.¹³

Double Disk Synergy Test

- Bacterial isolates resistant to third-generation cephalosporins were subjected to ESBL producers.
- Cefotaxime 30µg with a distance of 15mm

edge to edge from piperacillin/tazobactam and incubated at 35°C (18-20 hours) and zone of inhibition >5mm increase in diameter - positive for ESBL production.¹⁴

MBL (Metallo-β-lactamase)

- Combined disk test: Two imipenem discs (10µg) and one with 10µl of 0.1 M anhydrous EDTA (Himedia) were used.
- In comparison with the imipenem disc an increase in zone diameter of >5 mm around the EDTA disc is regarded as positive.¹⁵

Determination of Virulence Factors

Hypermucoviscosity (HMV)

- The ability of the colony to stretch as a mucoviscous string.
- It is considered an HMV phenotype, when the string formed stretches in length >10mm.¹⁶
- String test positive for a hypervirulent strain of *Klebsiella pneumoniae* is shown in Figure.

Blood Hemolysis

- The plate hemolysis test was performed by inoculating the isolates on blood agar plates which contain 5% sheep blood.
- Hemolysis was identified after 24 hours of incubation at 37°C.¹⁷

Biofilm Forming Assay

- A microtiter plate method was used to assess biofilm production. The attachment of *Klebsiella* strains to an inert substratum was examined.
- The strains were maintained in BHIB at 37°C

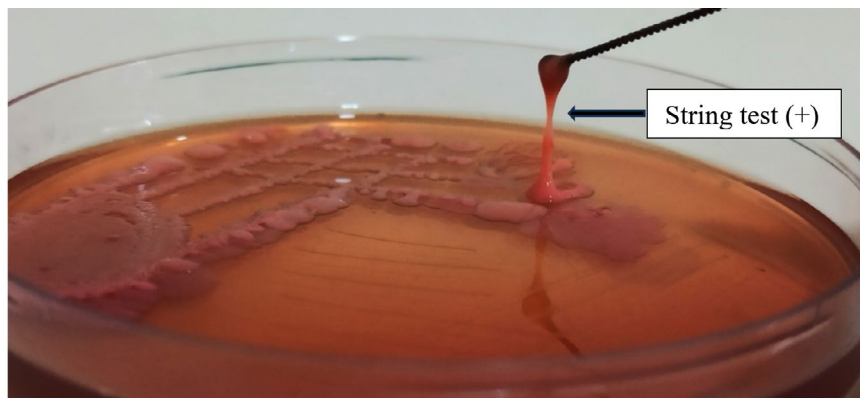


Figure. String test positive for *Klebsiella pneumoniae*

Table 1. Primers used for detecting target genes

PCR	Target gene	Sequences of Primer (5'-3')	Product Size (bp)
Resistant genes			
1.	CTX-M	F- CGCTTTGCGATGTGCAG R- ACCGCGATATCGTTGGT	550
2.	NDM	F-GGGCAGTCGCTTCCAACGGT R-GTAGTGCTCAGTGTCCGCAT	476
2.	IMP	F-TTGACACTCCATTACDG R-GATYGAGAATTAAGCCACYCT	139
2.	VIM	F-GATGGTGTGGTTCGCATA R-CGAATGCGCAGCACCAG	390
2.	KPC	F-CATTCAAGGGCTTTCTTGCTGC R-ACGACGGCATAGTCATTTGC	538
Biofilm gene			
3.	wcaG	F-GGTTGGKTCAGCAATCGTA R-ACTATTCCGCCAACTTTTGC	169
Hyper virulent gene			
4.	magA (K1)	F-GGTGCTCTTACATCATTGC R-GCA ATG GCC ATT TGC GTT AG	1282
5.	Wzy(K2)	F- GACCCGATA TTC ATA CTT GAC AGA G R -CCT GAA GTA AAA TCG TAA ATA GAT GGC	641

for 24 hours. 50µl from culture dilution was transferred into the individual wells of a 96-well, flat-bottomed polystyrene microtiter plate and incubated at 37 °C for 48 hours.

- Plank-tonic bacteria were removed after incubation, wells were gently rinsed thrice with sterile normal saline and methanol fixation was done for 20 min.
- Then each well was stained with crystal violet and washed. Biofilm-associated crystal violet was decolorized in 1 mL of ethanol. Finally, the optical density of the solution was read at 620nm.¹⁸

Blood Parameters

CRP levels were measured using CRP-turbidatex method. It is a quantitative turbidimetric test for the measurement of CRP.¹⁹

Genomic DNA Extraction and Amplification of 16S rRNA Gene

The DNA Extraction kit (Qiagen blood mini Kit -HIMEDIA) was used to extract DNA from samples. The content and purity of DNA were determined by measuring absorbance at wavelengths of 280 nm. Gel electrophoresis was

used to check the integrity of the genomic DNA.²⁰

The 16SrRNA gene amplification was performed using Qiagen HotStarTaq VR Master Mix and universal primer pair forward primer F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer R 5'-ACGGTTACCTTGTTACGACTT-3'. The final volume of 25 ml PCR reaction mixture contains a master mix of 12.5 ml, forward and reverse primer 2 ml, DH2O 5.5 ml, and Template DNA 3 ml.

The PCR setting was 94°C for 15 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, 52°C for 1 min at annealing, extension at 72 °C for 1 min 30 s, and final polymerization extension of 72°C for 5 min (The Applied Biosystems Veriti 96- Well Thermal Cycler). The amplified PCR sample was taken to agarose gel electrophoresis (1%) by 1X TAE (Tris-Acetate-EDTA) buffer with ethidium bromide. The amplified bands were visualized on UV transilluminator and the image was taken under UV illumination using the gel documentation (Bio-Rad) method.²¹

The PCR amplified products have been purified with a QIAquick PCR purification kit. The Purified PCR gene amplicons by sequence,

Table 2. PCR – Amplification conditions

PCR	Initial Denaturation	Denaturation	Annealing	Elongation	Final Elongation	Cycle
1	95°C 7 min	95°C 1 min	58°C 1 min 30 sec	72°C 2 min	72°C 5 min	40cycles
2	95°C 5min	95°C 2 min	55°C 2 min	72°C 1 min	72°C 7 min	35cycles
3	95°C 10 min	95°C 5 min	56°C 1 min 50 sec	72°C 1 min	72°C 10 min	45 cycles
4&5	95°C 7 min	95°C 2 min	59°C 1 min 35 sec	72°C 2 min	72°C 7 min	40 cycles

Table 3. Demographic and Clinical presentation of Neonatal sepsis

Variable	Other bacteria	<i>K. Pneumoniae</i>
Sex(male : Female)	61:39 (%)	59.6:40.4 (%)
Hospitalization duration (day)(mean ± SD)	8 ± 2	12 ± 4
Weight(g)(mean ± SD)	2,850 ± 500	2,700 ± 800
No. Of preterm neonates	3	1
No. of cases with PROM	8	5
White blood cell count	10,110 ± 253	9,250 ± 560
ESR(mean ± SE)	17 ± 2.1	15 ± 5.1
The onset of Septicemia(day)(mean ± SD)	2 ± 2	1 ± 2
Fever (mean ± SD)	37.1 ± 0.7	37.3 ± 0.8
No. of cases with meconium-stained liquor	9	7
CRP(positives: negatives)	40:6 (86.9:13.1%)	15:2(88.2:11.8%)
Present condition at sepsis onset		
Hypothermia	19 (41.3%)	4 (23.5%)
Respiratory difficulty	21 (45.6%)	7 (41.1%)
Apnea	8 (17.3%)	1 (5.8%)
Jaundice	4 (8.6%)	1 (5.8%)
Prematurity	12 (26%)	4 (23.5%)
Convulsion and neurological alterations	1 (2.1%)	-
Poor feeding	14 (30.4%)	2 (11.7%)

(ESR- Erythrocyte sedimentation rate; SD-standard deviation; CRP-C-reactive protein).

the reaction was performed in the PCR thermal cycler using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The purified air-dried samples were sequenced in ABI 3730XI 96-capillary DNA Analyzers. The sequence quality was validated using Finch TV version 1. 5. 0.²²

Sequencing

The characterization and recognition of *Klebsiella* strains were done by 16S rRNA sequences. The individual sequences were subjected to similarity search and comparison in the NCBI database using the similarity tool BLAST. The identification of strains was based on the maximum similarity hits obtained from BLAST. The 16S rRNA sequence strains were submitted

to the GenBank nucleotide database of NCBI, the sequence was accepted and the accession number for each sequence was obtained separately.²³

Multidrug Resistance Genes Detection

The following genes were identified using Polymerase chain reaction (PCR) and the specific primers are given in Table 1 and their amplification conditions are given in Table 2.

- ESBL-encoding genes (*bla_{CTX-M}*)
- Carbapenemases genes (*bla_{VIM}*, *bla_{KPC}*, *bla_{NDM}*, IMP)
- Biofilm gene (WCaG)
- Hyper virulent Kp genes -magA(K1),wzy(K2)

Amplification reactions were carried out and Electrophoresis and Visualization of DNA fragments using a Gel imaging system were performed.²⁴

Table 4. Antibiotic-resistant pattern of *Klebsiella pneumoniae* isolates

Antibiotics	<i>K. Pneumoniae</i> Total no.: 17		Other bacteria Total no.: 46		p-value
	No.	%	No.	%	
Amikacin	2	11.8	4	8.7	0.657
Ciprofloxacin	12	70.6	7	15.2	<0.001***
Cotrimoxazole	15	88.2	9	19.6	<0.001***
Cefepime	15	88.2	5	10.9	<0.001***
Ampicillin	17	100	9	19.6	<0.0001***
Ceftriaxone	15	88.2	5	10.9	<0.001***
Cefazolin	10	58.8	3	6.5	<0.001***
Cefuroxime	14	82.4	5	10.9	<0.001***
Cefotaxime	13	76.5	3	6.5	<0.001***
Piperacillin-Tazobactam	10	58.8	3	6.5	<0.001***
Tobramycin	8	47.1	6	13.04	0.007**
Gentamicin	11	64.7	8	17.4	0.001***
Imipenem	4	23.5	2	4.3	0.041*
Meropenem	4	23.5	2	4.3	0.041*

Note: Chi-square test was used and Fisher's exact test was used when at least one of the expected cell counts was less than 5. If the p-value is less than 0.05 it is assigned with one star (*). If the p-value is less than 0.01 it is assigned with two stars (**). If the p-value is less than 0.001 it is assigned with three stars (***).

Statistical Analysis

The statistical analysis was done with the SPSS software version 24.0. A p-value < 0.05 was considered to be statistically significant.

RESULTS

This study included 63 newborns with culture-proven sepsis caused by 11 different types of pathogens. The rate of neonatal sepsis cases in the NICU was 39.2 %. The higher proportion of the new-born with septicemia was male. 44 (70.3%) of newborns were belonging to Early onset sepsis (EOS) cases. Newborns with EOS had lower gestational age, decreased birth weight, and infection age at a younger age than infants with LOS (P < 0.05). EOS infants stayed in the hospital for substantially longer than those in the LOS group.

The majority of the isolates, 35(74.05%) were Gram-negative bacteria. *K. pneumoniae* n=17(26.9%) was the most common pathogen both in the EOS and LOS groups. Demographic and clinical presentations of neonatal sepsis are given in Table 3. The Antibigram pattern of *Klebsiella pneumoniae* is shown in Table 4.

Infections with MDR-KP were found in both the Early and late-onset sepsis groups. The MDR-KP infection group had a lower birth weight, lower gestational age, and longer time of antibiotic exposure (P < 0.05). There were no changes in maternal variables, neonate gender, or delivery pattern (P > 0.05). In addition, in the MDR group, the period of hospitalization was greater.

A high prevalence of ESBL was detected in the *K. pneumoniae* isolates reaching 58.8%. Among the *Klebsiella pneumoniae* isolates, the prevalence of CRKP and MBL producers were 29.4% and 23.5% respectively. Sepsis risk factors including PROM and neutropenia were significantly associated with CRKP.

Two strains were Strong biofilm producers, three were moderate, five were weak and seven were non-biofilm producing *Klebsiella pneumoniae* and four strains were positive for biofilm-producing wcaG gene. Nine isolates showed Beta hemolysis.

In *Klebsiella pneumoniae* isolates, a string test was done for phenotypic confirmation of hvKp and 7(41.1%) strains were positive. In these 7 hvKp strains, 3 positive and 4 were negative for magA(

Table 5. BLAST analysis, similarity, and NCBI Gene bank accession numbers of *Klebsiella pneumoniae*

No.	Sample	Bacterial strain	Similarity	Blast Result	Accession No.
1.	Blood	<i>K.pneumoniaehv</i> kp1	100 %	<i>K.pneumoniae</i>	OM442431
2.	Blood	<i>K.pneumoniaehv</i> kp2	100 %	<i>K.pneumoniae</i>	OK635329
3.	Blood	<i>K.pneumoniaehv</i> kp3	100 %	<i>K.pneumoniae</i>	OM442431
4.	Blood	<i>K.pneumoniaehv</i> kp4	100 %	<i>K.pneumoniae</i>	OM066754
5.	Blood	<i>K.pneumoniaehv</i> kp5	100 %	<i>K.pneumoniae</i>	OM066754

K1). 2 were positive and 5 were negative for gene *wzy* (K2).

In terms of ESBL genes, three isolates in our investigation carried *bla*_{CTX-M} and regarding the carbapenemases, four isolates harbored *bla*_{VIM}, two for IMP, one for NDM and KPC gene.

16s rRNA Sequence Analysis

In the current study, *Klebsiella pneumoniae* strains are exposed to 16S rRNA sequences. The different lengths of the nucleotide bases created by this study have been exposed to BLAST analysis. BLAST results in the 16S rRNA gene sequence showed almost all isolates had greater than 99.9% similarity and database reference sequences are given in Table 5.

Treatment

According to the sensitive data, all of the infants were given antibiotics. When necessary, other treatment procedures were used, such as entire parental nourishment and intensive care.

DISCUSSION

Neonatal sepsis has different etiological agents in developed and developing nations. Gram-negative organisms were the most common pathogens in this study, as well as earlier studies from India and Nigeria.²⁵ Coagulase-negative staphylococcus (CONS) is frequently isolated from developed nations.²⁶

Klebsiella pneumoniae is typically encountered in the neonatal critical care unit. It can be found in the hands of healthcare professionals as colonizers. In addition, outbreaks of neonatal septicemia caused by *Klebsiella pneumoniae* have been reported often in nurseries and NICUs.²⁷

Klebsiella pneumoniae was found to be the common cause of early-onset sepsis in this investigation, although no Group B Streptococcus

was found in any of the newborns. Although Group B Streptococcus was once thought to be a major cause of early-onset sepsis, new research reveals that the incidence of this pathogen is decreasing.²⁸

Escherichia coli, *Staphylococcus aureus*, *Coagulase-negative staphylococcus* (CONS), and *Klebsiella* species are the common pathogens in LOS, which are mainly acquired in hospital settings. *Klebsiella pneumoniae* was the prevalent etiological agent of late-onset sepsis in this investigation.²⁹

In our study, the majority of the newborns were male. It has been also observed in previous studies. This could be attributable to a gender bias in health practices. Furthermore, males have been found to have a higher prevalence of ESBL-producing *K. pneumoniae*.³⁰

Infants' immune systems are immature, making them vulnerable to nosocomial infections. An upsurge in ESBL-producing *K. pneumoniae* sepsis has been observed over the world, with the NICU accounting for the majority of cases.³¹ Antimicrobial resistance in *Klebsiella* is mostly linked with the formation of ESBL. In 2017, the WHO included ESBL-producing *Klebsiella* as most dangerous superbugs along with *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.³²

Multidrug resistance was seen in our *K. pneumoniae* isolates, with carbapenem resistance increasing. There are a few antibiotics that can be used to treat CRKP: imipenem or meropenem. A combination of antibiotics can also be used along with the removal of invasive devices which can be beneficial. Polymyxin B can be used to treat multidrug-resistant CRKP.³³

The majority of *Klebsiella pneumoniae* isolates evaluated in this study were resistant to many antibiotics except amikacin, meropenem, and imipenem. In our study *Klebsiella* were resistant to second and third-generation cephalosporins. This shows the importance of testing gram-negative

bacterial isolates for cephalosporin resistance and ESBL development regularly. Due to the increased prevalence of ESBL, increased resistance to aminoglycosides, which are widely used for empirical therapy, was also seen in our study.³⁴

ESBL-producing *K. pneumoniae* isolates have been found in high numbers in several studies. Most of the isolates found in this study originated in neonatal and critical care units. Infections caused by ESBL-producing organisms in infants are usually acquired in hospitals and are associated with invasive procedures. These units frequently utilize ampicillin + gentamicin as a first-line antibiotic and cefotaxime as a second-line antibiotic.³⁵

Carbapenemases are thought to be the primary mechanism that causes CRKP to exist, based on the genes. According to PCR analysis, four isolates harbored *bla*_{VIM}. The majority of them possessed the *bla*_{VIM} gene. Our genotypic profile contrasts with that of another study by Ghaith et al. who found a higher prevalence of *bla*_{OXA-48} and no blaVIM in any of their isolates.³⁶

In terms of ESBL genes, three isolates in our investigation carried *bla*_{CTX-M}. It is consistent with research by Amer et al. who found similar rates in isolates.³⁷

Additional carbapenemases, such as IMP and VIM, were discovered in the isolates investigated. In the meantime, several studies have found VIM and KPC generating *K. pneumoniae*. In our study, four isolates were positive for blaVIM, two for IMP, and one for NDM and KPC gene.^{32,25}

CONCLUSION

In conclusion, *K. pneumoniae* isolates in the NICU increased in frequency and antibiotic resistance. *K. pneumoniae* is a serious hazard to the healthcare system, and it necessitates strict infection control methods in healthcare settings, as well as antibiotic stewardship to prevent the overuse of antibiotics in neonatal sepsis. As the signs and symptoms are so varied, diagnosing and treating them can be difficult. Furthermore, the extended turnaround time of laboratory diagnosis adds to the difficulties, necessitating the continuation of empirical antibiotic therapy until the suspected sepsis is eradicated. To present, the growing appearance of multidrug-

resistant organisms has resulted in only a few therapeutic options being available, delaying effective therapy. In hospital settings, developing preventative and management strategies should be implemented. Infection control activities such as antibiotic stewardship programs with continuous surveillance are required to track new CRKP infections as soon as possible, especially in high-risk units like NICUs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The study was conducted according to the guidelines of the Institutional Human Ethical Committee (No.010 /IHEC/Jan 2021) - Chettinad Academy of Research and Education, Kelambakkam, India

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