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Molecular Characterization of Colistin Resistant *Klebsiella Pneumoniae* ST11 Responsible for Nosocomial Outbreak in Neonatal Intensive Care Unit, India

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ABSTRACT

The dramatic increase of resistance in Klebsiella pneumoniae (K. pneumoniae) due to the misuse of antibiotics poses one of the greatest health threats and is a global health concern. Colistin, a last resort antibiotic is used extensively to treat carbapenem-resistant Klebsiella pneumoniae infections. Present research carried out to analyze the molecular mechanism, clonal types, and outcomes of the infections caused by colistin-resistant K. pneumoniae in neonates during an outbreak in neonate intensive care unit. Twenty-eight cases of colistin-resistant, carbapenem-resistant K. pneumoniae were identified between March and April 2016. Isolates were genotyped using multi-locus sequence typing and the molecular mechanism of colistin resistance was ascertained. All the colistin-resistant K. pneumoniae isolated from neonates during outbreak have insertional inactivation by ISL3 family transposes in the mgrB gene and were clonally related belong to ST11. PCR screening confirmed the presence of the bla_{OXA-48} *and* bla_{SHV-34} *genes. The observed mortality was 35.7% in two-month periods. The present baseline report of colistin-resistant K. pneumoniae ST11 outbreak suggested the emergence of clones with this phenotype that required paramount importance for future health monitoring and assessment.*

Keywords: Antibiotics, β-lactamase, Colistin-resistance, *Klebsiella pneumoniae*, plasmid

INTRODUCTION

Klebsiella pneumoniae has emerged as a superbug and poses as one of the world's greatest health threats [1]. It is the causative agent of a variety of diseases, commonly including urinary tract, soft tissue infections, bacteremia, and pneumonia; however, community-acquired invasive infections have also emerged as of late in the form of a pyogenic liver abscess, brain abscess, meningitis, and ankylosing spondylitis [2,3]. Eradication of this superbug is extremely difficult as it has acquired resistance to almost all available antibiotics and is consequently associated with high morbidity and mortality, thus it has been included among the six multidrug-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) [4]. Neonates are more vulnerable to infection due to their immature immune systems. Low birth weight, prematurity, febrile illness in the mother, rupture of membranes, unhygienic conditions, delays in recognition and prolong hospitalization are some recognized risk factors. In India, neonatal septicemia is ported incidence rate of 30 cases per 1000 live births, with *K. pneumoniae* as the most frequently isolated pathogen in both intramural births (32.5%) and extramural neonates (27%) [5]. The use of cephalosporin and carbapenem leaped forward the fight against infections caused by *K. pneumoniae*. However, co-production of multiple β-lactamases, especially ESBLs (e.g. bla_{SHV}, $bla_{TEM,}$ and/or bla_{CTX}) and carbapenemase (bla_{KPC} and/or bla_{OXA}), in *K. pneumoniae* has been reported from many countries, rendering β-lactam antibiotics completely futile [6-8]. The situation is further complicated as Extended-Spectrum β-Lactamase (ESBL)-producing *K. pneumoniae* isolates exhibited very high (up to 56%) resistance to quinolones [9].

Due to the unavailability of therapeutic alternatives for the treatment of Carbapenem-Resistant *K. pneumoniae* (CRKP), colistin has been extensively used instead. Colistin Sulfate (CS) is used for oral and topical therapy, whereas Colistin Methanesulfonate Sodium (CMS) is used for parenteral and aerosol therapy [10,11]. Colistin being positively

charged, alters the permeability of the bacterial cell membrane by displaying divalent cations, $Ca^{2+}$and Mg^{2+}, from the phosphate groups of lipid A, thus causing an outflow of cell contents which ultimately results in bacterial death [12]. However, with excessive use, colistin resistance in *K. pneumoniae* began to increase, which highlights an emerging threat in the treatment of healthcare-related infections [13,14]. PhoP/PhoQ and PmrA/PmrB are the main systems responsible for the modification of the Lipopolysaccharide (LPS) layer. Changes in these regulatory systems cause colistin resistance. Insertional inactivation/mutation of the *mgr*B gene, which encodes the key regulatory protein of the PhoP/PhoQ system, or the presence of a plasmid-mediated *mcr-1* gene are the primary mechanisms driving colistin resistance [15-17].

Between March-July 2016, an outbreak of colistin-resistant *K. pneumoniae* bacteremia was reported in the neonatal intensive care unit, Jamshedpur, Jharkhand [18]. Keeping the progressive resistance mechanism of *K. pneumoniae* in mind which is a global health concern, the present research investigation has been carried out to analyze the molecular mechanism of colistin resistance, clonal types, clinical futures, and outcomes of the infections caused by colistin-resistant *K. pneumoniae* isolated from neonates during this outbreak. Our findings will set the stage for future research on this emerging health crisis.

METHODS

Bacterial Isolates

Thirty (28 from neonates and 2 from hospital environment) non-duplicate Colistin-Resistant Carbapenem-Resistant *K. pneumoniae* (COL^R-CRKP) isolates from the neonatal intensive care unit of a tertiary care hospital (Jharkhand, India) during an outbreak were included in this study. One ml of blood was drawn from the neonates suspected to have sepsis and inoculated in the BacT/ALERT (bioMerieux, USA) microbial detection system. After the prediction of an outbreak, surveillance swabs from the nursery (air, warmer, suction, IV/PIC line, and oxygen nozzle), gynecology OT (table, light, and instruments), and labor room were taken for culture. Random hand swabs of staff, doctors, and mothers were also taken for culture. Identification and antibiotic susceptibility of isolates were determined by aVITEK® 2 compact system (bioMerieux, USA) using the ID-GNB and AST-N280 cards following the manufacturer's instructions. The antimicrobial susceptibility testing card included the following antibiotics: Ampicillin (AMP), Cefuroxime (CXM), Ceftriaxone (CRO), Cefepime (FEP), Imipenem (IPM), Meropenem (MEM), Cefoperazone/Sulbactam (CSS), Piperacillin-Tazobactam (TZP), Amoxicillin-Clavulanic Acid (AMC), Ciprofloxacin (CIP), Trimethoprim-Sulfamethoxazole (SXT), Amikacin (AMK), Gentamicin (GEN), Nitrofurantoin (NIT), Ertapenem (ERT), Co-Trimoxazole (COT) and Colistin (COL). Ertapenem resistance was defined as a MIC of $\geq$ 8 mg/l and colistin resistance was defined as a MIC of $\geq$ 4 mg/l [19]. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains for each batch of MIC tests.

Patients and Clinical Epidemiology

Demographic data such as gender, age, clinical diagnosis, location, clinical outcome, and the time of admission, the time of discharge, and the length of hospital stay were extracted from the patient administration system.

Detection of β-lactamase Genes

Phenotypic detection of ESBLs, MBLs, and carbapenemase was carried out using a double disk synergic test, and imipenem EDTA double-disc synergy test, and Modified Hodge Test (MHT) respectively, following the protocol as described by CLSI 2014 manual [20]. The phenotypic detection of *Amp*C was performed using a ceftazidime-boronic acid combined disc diffusion test as described by Yilmaz, et al. [21]. PCR amplification was carried out in a thermal cycler (Eppendorf, Germany) to detect the genes encoding for bla_{TEM}, bla_{SHV}, bla_{IMP}, bla_{VIM}, bla_{NDM-1}, bla_{OXA-48}, $bla_{KPC,}$ and *Amp*C according to their respective product size. PCR products were separated in a 1% agarose-TAE gel containing ethidium bromide and visualized in a Chemidoc imager (Bio-Red, USA).

PCR Amplification of *mgr*B and *mcr-1* Genes

PCR amplification of the *mgr*B gene and *mcr-1* gene were carried out to determine the colistin resistance mechanism. The presence of plasmid DNA of all the *K. pneumoniae* isolates were screened using QIAGEN plasmid mini kit (QIAGEN GmbH, Germany). Amplified PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN) and sequenced with an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced

protein sequences were analyzed using the NCBI website. The Insertion Sequence (IS) was analyzed using the IS finder website.

Molecular Typing

Strain typing of *K. pneumoniae* isolates was performed by ERIC-PCR using primer setERIC-2/ERIC-1026 according to Munoz, et al. [22]. Multilocus sequence typing of isolates was performed as described by Diancourt, et al. and analyzed using the MLST website for *K. pneumoniae* [23]. All the seven house-keeping gene sequences were submitted to get the gene bank accession number.

RESULTS

A total of 28 colistin-resistant *K. pneumoniae* were isolated between March and April 2016 from the blood culture of neonates admitted to the neonatal intensive care unit (Table 1). Out of 28 neonates, 57% (n=16) were males and 43% (n=12) were females. Besides two more colistin-resistant *K. pneumoniae* strains (EKpCR1 and EKpCR2) were isolated from the gynecological oxygen nozzle and suction of the Operation Table (OT) of the same hospital. However, all the *K. pneumoniae* isolates showed uniform antibiogram, pattern, and resistance to the first and second line of cephalosporins, β-lactam/β-lactamase inhibitor combination drugs, carbapenems, aminoglycosides, quinolones, and colistin. The only drugs which were sensitive in all of the isolates were tigecycline and co-trimoxazole, which revealed that all of the isolates belonged to a single clone.

The isolates were phenotypically negative for ESBL, MBL, and *Amp*C production, whereas determined to be positive for the production of carbapenemases by a modified Hodge test. PCR screening confirmed the presence of the $bla_{\text{OXA-48}}$ and $bla_{\text{SHV-34}}$ genes.

Table 1 Isolates ID and detail of the patients during an outbreak

Isolates ID	Date of isolation of *K. pneumoniae*	MIC (mg/l)				Gender of patient	A hospital stay of the patient (days)	Outcome of patient
		Col	ERT	MER	TGC			
CKpCR1	2-Mar-16	>16	>8	>16	2	F	33	Alive
CKpCR2	26-Mar-16	>16	>8	>16	2	M	7	Alive
CKpCR3	28-Mar-16	>16	>8	>16	2	F	5	Dead
CKpCR4	10-Apr-16	>16	>8	>16	2	M	7	Dead
CKpCR5	10-Apr-16	>16	>8	>16	2	M	23	Alive
CKpCR6	1-Mar-16	>16	>8	>16	2	F	4	Alive
CKpCR7	16-Apr-16	>16	>8	>16	2	F	26	Dead
CKpCR8	5-Mar-16	>16	>8	>16	2	M	5	Dead
CKpCR9	21-Apr-16	>16	>8	>16	2	F	2	Alive
CKpCR10	16-Apr-16	>16	>8	>16	2	F	29	Alive
CKpCR11	14-Mar-16	>16	>8	>16	2	M	10	Alive
CKpCR12	26-Mar-16	>16	>8	>16	2	F	7	Dead
CKpCR13	29-Mar-16	>16	>8	>16	2	M	11	Dead
CKpCR14	16-Apr-16	>16	>8	>16	2	F	8	Alive
CKpCR15	22-Mar-16	>16	>8	>16	2	M	9	Alive
CKpCR16	26-Mar-16	>16	>8	>16	2	M	7	Dead
CKpCR17	6-Apr-16	>16	>8	>16	2	M	15	Alive

CKpCR18	30-Mar-16	>16	>8	>16	2	M	7	Alive
CKpCR19	16-Apr-16	>16	>8	>16	2	M	18	Alive
CKpCR20	13-Mar-16	>16	>8	>16	2	F	13	Alive
CKpCR21	13-Mar-16	>16	>8	>16	2	F	21	Alive
CKpCR22	19-Apr-16	>16	>8	>16	2	M	6	Dead
CKpCR23	12-Apr-16	>16	>8	>16	2	F	16	Alive
CKpCR24	10-Apr-16	>16	>8	>16	2	M	4	Alive
CKpCR25	10-Apr-16	>16	>8	>16	2	M	10	Alive
CKpCR26	26-Mar-16	>16	>8	>16	2	F	15	Alive
CKpCR27	12-Apr-16	>16	>8	>16	2	M	10	Dead
CKpCR28	28-Mar-16	>16	>8	>16	2	M	7	Dead
#EKpER1	4-Apr-16	>16	>8	>16	2	-	-	-
#EKpER2	4-Apr-16	>16	>8	>16	2	-	-	-
MIC: Minimum Inhibitory Concentration; Col: Colistin; ERT: Ertapenem; MER: Meropenem, TGI: Tigecycline								
#Colistin resistant and carbapenem-resistant *K. pneumoniae* from the hospital environment								

All isolates in the present study contain plasmids. However, PCR amplification of *mcr-1* gene was negative for all isolates. PCR amplification targeting *mgr*B gene was carried out to determine the presence of colistin resistance. Targeted 250 bp PCR amplification gave an amplicon of 510 bp (Figure 1), indicating the presence of an insertion sequence. Sequence analysis of the amplicon revealed that the *ISL3* family transposase (*ISKpn25* ORF4) of 363 bp targeted in the reverse orientation, downstream of 133 bp of *mgr*B gene led to insertional inactivation (Figure 2). The NCBI accession number of the seven house-keeping gene sequences used for MLST and the *mgr*B gene sequence of colistin-resistant carbapenem-resistant *K. pneumoniae* CKpCR1 were MG367191-MG367198.

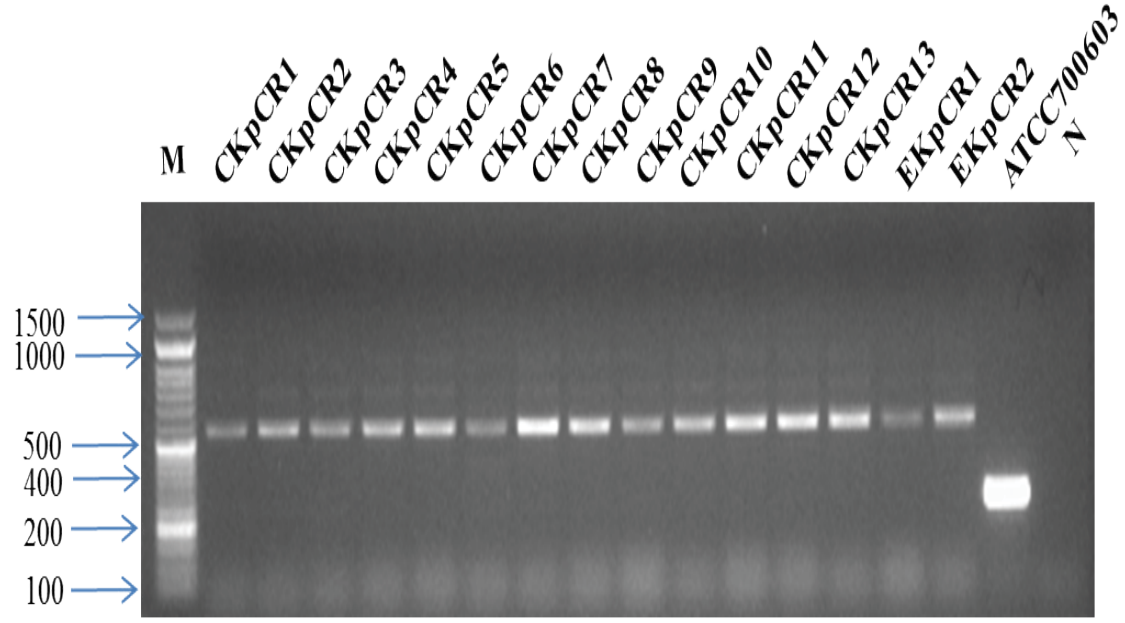

Figure 1 PCR amplification of *mgr*B gene; Lanes 1-13 were colistin-resistant *K. pneumoniae* isolated from neonates and Lane 14-15 were colistin-resistant *K. pneumoniae* isolated from hospital environment; M: DNA ladder (NEB, size as indicated) and N: Nontemplate control

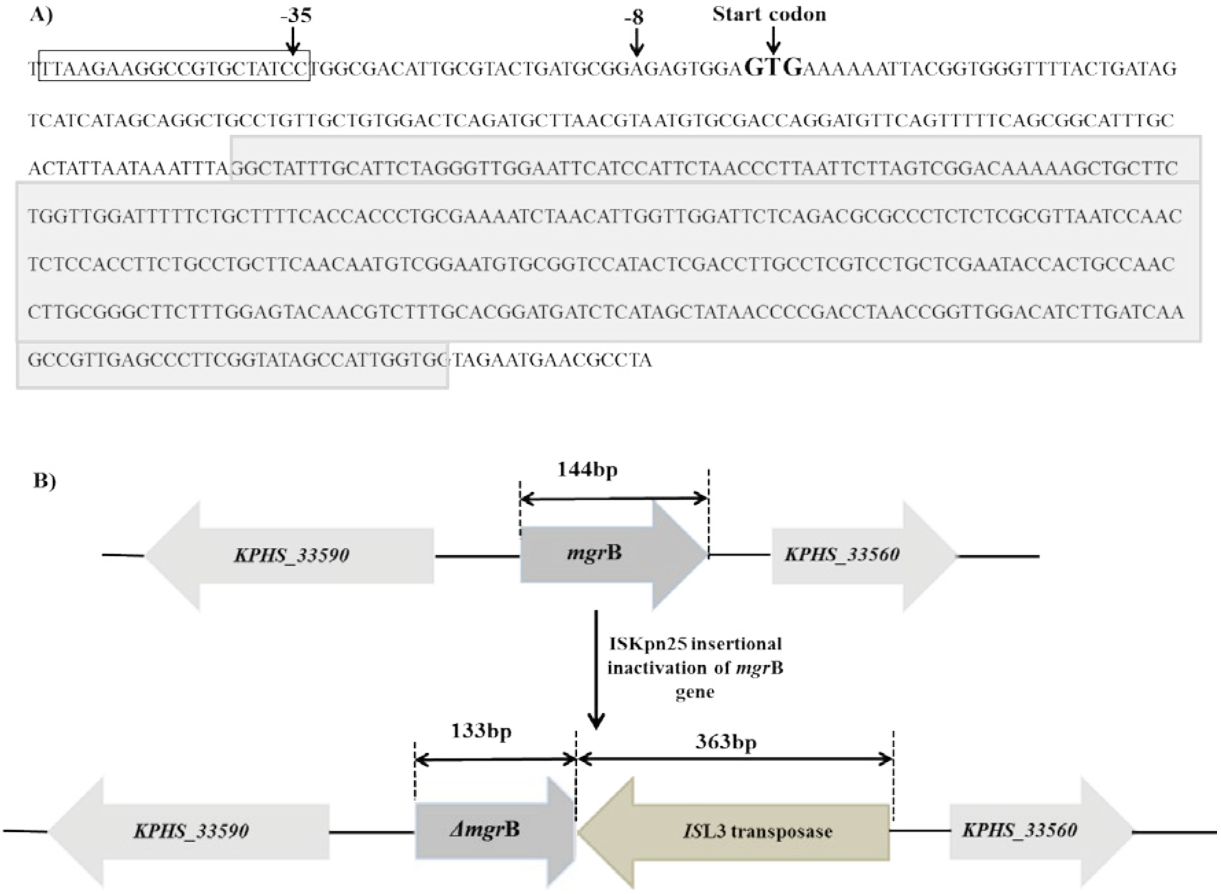

Figure 2 Insertional inactivation of mgrB gene; A) Sequence of Δ*mgr*B gene and ISKpn25-like insertional sequence; the *mgr*B start codon is in bold, grey shaded area indicates ISKpn25 sequence; (B) Schematic representation of insertional inactivation of *mgr*B gene by ISKpn25 insertion sequence

Based on ERIC-PCR band similarity (Figure 3), *K. pneumoniae* strains were deemed to be representative of a clonal. Multi-locus sequence typing showed that isolates belonged to ST11 with allelic variation 3, 3, 1, 1, 1, 1, 4.

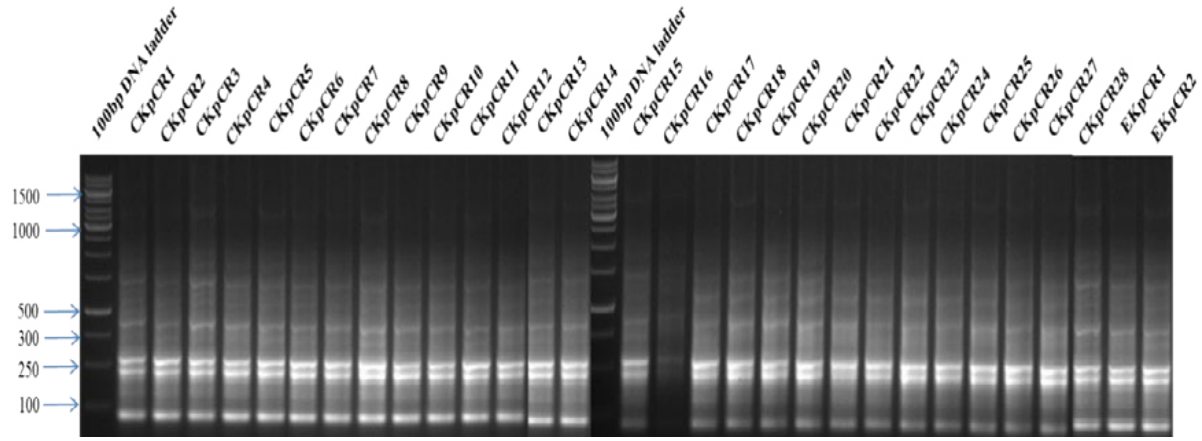

Figure 3 ERIC-PCR of colistin resistant and carbapenem-resistant *Klebsiella pneumoniae* isolated from neonates and hospital environment during outbreak; *Klebsiella pneumoniae* isolated from neonates were designated as CKpCR and hospital environment isolates were designated as EKpCR

DISCUSSION

In this study, we described and characterized a clonal outbreak of *K. pneumoniae* bacteremia in the NICU of a tertiary care hospital in the state of Jharkhand, India. All of the neonates in the study exhibited respiratory distress, low platelets associated with diminished spontaneous activity and feeding intolerance, positive blood culture for bacteria with similar cultural characteristics, and antibiotic resistance pattern. The observed mortality was 35.7% over two months. Majhi, et al. have reported detailed clinical parameters during this outbreak, including the severity of illness, risk factors such prematurity, birth asphyxia, babies who had surgery, double volume exchange transfusion, and prior antibiotic usages [18]. This outbreak was caused by a colistin-resistant $bla_{\text{OXA-48}}$ and $bla_{\text{SHV-34}}$ producing *K. pneumoniae* ST11 that was susceptible only to tigecycline and co-trimoxazole. Proper surveillance and actions like removal of gynecological OT oxygen nozzle and suction, lead to overcome the outbreak.

At present, reserved antibiotic colistin and newly developed glycylcycline antibiotic tigecycline are the few available antibiotics for treating carbapenem-resistant bacteria. Following excessive use, bacteria have progressively developed resistance toward colistin. In 2004, the first clinical outbreak of colistin-resistant *K. pneumoniae* was reported in Greece [24]. Since then, the emergence of colistin-resistant *K. pneumoniae* was reported from various parts of the world [25]. Several recent studies highlight the loss□of□function mutations of the *mgr*B gene responsible for the emergence of colistin resistance in *K. pneumoniae*. However, other mechanisms, such as the role of efflux pump and plasmid-mediated colistin resistance protein *mcr-1* were reported to be associated with colistin resistance in *K. pneumoniae* [26,27]. In the present study, we found insertional inactivation of the *mgr*B gene by transposase of *ISL3* family that might be responsible for colistin resistance in all the *K. pneumoniae* isolates. Similar *ISL3*, a transposable restriction-modification system of 8154 nucleotides inserted in *mgr*B nucleotide position 133 was reported in colistin-resistant *K. pneumoniae* ST258 isolated from two Italian and eight Greek patients [28]. They reported that *ISL3* was located on pKpQIL-like plasmids and may transpose into the chromosome. Single locus variant of ST11, colistin-resistant *K. pneumoniae* ST258 with the insertional inactivation of *mgr*B gene by an IS5-like element was reported [15].

In clinical isolates of *Klebsiella pneumoniae*, colistin resistance has been reported to be mainly associated with the sequence types ST 258, ST 512, and ST147 [29,30]. However, in our study, colistin-resistant *K. pneumoniae* has been observed in sequence type ST11. In a SMART surveillance program during 2008 and 2009, K. pneumoniae ST11 producing $bla_{\text{OXA-48}}$-like and NDMs were reported from India and many parts of the world [31]. In India, colistin-resistant *K. pneumoniae* ST11 were reported from a tertiary care hospital carried β-lactam, fluoroquinolones, aminoglycoside, and fosfomycin resistant genes on the genomic DNA [32]. We found that all the isolated *K. pneumoniae* have ESBL ($bla_{\text{SHV-34}}$) and carbapenemase ($bla_{\text{OXA-48}}$) genes though the fluoroquinolones and aminoglycoside resistance study were not carried out in the present study.

CONCLUSION

In conclusion, this study showed the emergence of bacterial resistance to the last-resort antibiotic, especially in the intensive care unit of the hospital is of great concern and required judicious use of antibiotics with proper susceptibility analysis as well as continuous surveillance to minimize the emergence of new clones.

DECLARATIONS

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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