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OXA-181-Like Carbapenemases in *Klebsiella pneumoniae* ST14, ST15, ST23, ST48, and ST231 from Septicemic Neonates: Coexistence with NDM-5, Resistome, Transmissibility, and Genome Diversity

Sharmi Naha,[a] Kirsty Sands,[b] Subhankar Mukherjee,[a]* Bijan Saha,[c] Shanta Dutta,[a] (ID) Sulagna Basu[a]

[a]Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata, West Bengal, India
[b]Department of Medical Microbiology and Infectious Disease, Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom
[c]Department of Neonatology, Institute of Post-Graduate Medical Education & Research and SSKM Hospital, Kolkata, West Bengal, India

ABSTRACT Studies on the epidemiology and genomes of isolates harboring OXA-48-like genes in septicemic neonates are rare. Here, isolates producing these carbapenemases which emerged and persisted in an Indian neonatal unit were characterized in terms of their resistome, transmissibility, and genome diversity. Antibiotic susceptibility and whole-genome sequencing were carried out. The sequence types, resistome, virulome, mobile genetic elements, and transmissibility of carbapenem-resistant plasmids were evaluated. Core genome analysis of isolates was shown in a global context with other OXA-48-like carbapenemase-harboring genomes, including those from neonatal studies. Eleven OXA-48-like carbapenemase-producing *Klebsiella pneumoniae* ($bla_{OXA-181}$, $n = 7$ and $bla_{OXA-232}$, $n = 4$) isolates belonging to diverse sequence types (ST14, ST15, ST23, ST48, and ST231) were identified. $bla_{OXA-181/OXA-232}$ and bla_{NDM-5} were found in a high-risk clone, ST14 ($n = 4$). $bla_{OXA-181/OXA-232}$ were in small, nonconjugative ColKP3 plasmids located on truncated Tn*2013*, whereas bla_{NDM-5} was in self-transmissible, conjugative IncFII plasmids, within truncated Tn*125*. Conjugal transfer of $bla_{OXA-181/OXA-232}$ was observed in the presence of bla_{NDM-5}. The study strains were diverse among themselves and showed various levels of relatedness with non-neonatal strains from different parts of the world and similarity with neonatal strains from Tanzania and Ghana when compared with a representative collection of carbapenemase-positive *K. pneumoniae* strains. We found that $bla_{OXA-181/OXA-232}$-harboring isolates from a single neonatal unit had remarkably diverse genomes, ruling out clonal spread and emphasizing the extent of plasmid spreading across different STs. This study is probably the first to report the coexistence of $bla_{OXA-181/232}$ and bla_{NDM-5} in neonatal isolates.

IMPORTANCE Neonatal sepsis is a leading cause of neonatal mortality in low- and middle-income countries (LMICs). Treatment of sepsis in this vulnerable population is dependent on antimicrobials, and resistance to these life-saving antimicrobials is worrisome. Carbapenemases, enzymes produced by bacteria, can make these antimicrobials useless. Our study describes how OXA-48-like carbapenemases in neonatal septicemic *Klebsiella pneumoniae* shows remarkable diversity in the genomes of the strains and relatedness with strains from other parts of world and also to some neonatal outbreak strains. It is also the first to describe such resistance due to coproduction of dual carbapenemases, (OXA)-48 and New Delhi metallo-β-lactamase-5, in *Klebsiella pneumoniae* from neonatal settings. Carbapenemase genes situated on plasmids within high-risk international clones, as seen here, increase the ease and transfer of resistant genetic material. With the WHO treatment protocols not adequately poised to handle such infections, prompt attention to neonatal health care is required.

KEYWORDS OXA-181/232, NDM-5, neonates, sepsis, dual carbapenemases, ColKP3, WGS, core genome, India

Address correspondence to Sulagna Basu, basus.niced@gov.in.

* Present address: Subhankar Mukherjee, Department of Zoology, Government General Degree College, Singur, Hooghly, West Bengal, India.

Neonatal sepsis is one of the primary causes of neonatal deaths (23%) in middle- and low-middle-income countries (1). Multidrug-resistant bacteria complicate the treatment of sepsis in this vulnerable population (2). *Klebsiella pneumoniae*, belonging to the *Enterobacteriaceae* family, is one such species that has high rate of acquisition of resistance compared to other bacteria of this family (3). In addition, *K. pneumoniae* is also the leading cause of neonatal sepsis in developing countries (4). With escalating resistance to all available β-lactam antibiotics for neonates (penicillins, monobactam, cephalosporins, etc.), use of carbapenems has gradually increased, ultimately leading to a global upsurge of carbapenem-resistant *K. pneumoniae* (CR-*Kp*) in the last 2 decades (1, 3). According to the Centre for Disease Dynamics, Economics & Policy (CDDEP), there has been an increase in CR-*Kp* from 24% (2008) to 59% (2017) in India (1), a country that bears the burden of one-fourth of all neonatal deaths that occur globally each year (5).

K. pneumoniae is known to produce different carbapenemases, including Ambler class A carbapenemases (e.g., KPC), Ambler class B metallo-β-lactamases (e.g., NDM, IMP, VIM, etc.) and Ambler class D carbapenemases (e.g., OXA-48) (3, 6, 7). The New Delhi metallo-β-lactamase (NDM) is the most prevalent and worrisome, as it confers resistance not only to carbapenems but to almost all hydrolyzable β-lactams and has rapidly spread worldwide (8). To date, 29 variants of NDM have been reported (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/ndm). NDM-1 is the most disseminated variant, followed by NDM-5, which was first detected in *Escherichia coli* from the United Kingdom (9, 10). bla_{NDM-5} differs from NDM-1 at two amino acid positions, V88L and M154L, and exhibits enhanced resistance to carbapenems and extended-spectrum cephalosporins (9, 10).

Though NDM has gained prominence, oxacillinase (OXA)-48-like carbapenemases (OXA-48), first reported from Turkey in *K. pneumoniae* (2001) (6), has now spread to different genera of *Enterobacteriaceae*. Outbreaks and case reports throughout Europe, North Africa, the Middle East, and South Asian countries are increasingly documented (11–13). Reports of emergence or outbreak in neonatal units from Middle Eastern countries have also surfaced (14). Detection of OXA-48-producing microorganisms is not limited to clinical settings and is often detected in environmental surface samples, companion animals, livestock, production animals, and wild animals (11, 15, 16).

To date, 39 variants of OXA-48 have been reported (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/oxa-48). Currently, OXA-181 and OXA-232 constitutes the 2nd and 3rd most common global OXA-48-like derivatives after OXA-48 (14). OXA-181 was first reported from India (17) and differed from OXA-48 by four amino acid substitutions (T104A, N110D, E168Q, S171A) but did not evolve from it. On the other hand, OXA-232 first reported from France is a derivative of OXA-181 with a single amino acid substitution at R214S (14). OXA-48-like enzyme hydrolyzes penicillins and narrow-spectrum cephalosporins efficiently but does not hydrolyze extended-spectrum cephalosporins and exhibits poor activity toward meropenem while also showing the highest known catalytic efficiency for imipenem (6). Therefore, OXA-48 producers often remain undetected during surveillance because they are categorized as susceptible to carbapenems according to CLSI and EUCAST (6, 14). Like other carbapenemases, OXA-48-like carbapenemases are not inhibited by conventional β-lactamase inhibitors, but nowadays, use of avibactam (a non-β-lactam β-lactamase inhibitor) has been put forward. However, increasing reports of resistance toward avibactam have been documented (18). Hence, specific phenotypic detection of class D carbapenemases is still confusing. High-level resistance to temocillin (MIC, >64 mg/liter) has been suggested as a criterion to screen OXA-48-like carbapenemase; however, due to a similar resistance profile toward KPC and other metallo-β-lactamases (19), this is not suitable. This further emphasizes the difficulty in the identification of OXA-48, which inevitably leads to poor tracking of emergence and spread, and infection control measures. Carriage of such resistance markers on plasmids is often associated with international clones such

as sequence type 11 (ST11), ST14, ST15, ST63, ST147, ST231, etc., which aided in their rapid dissemination across boundaries (10, 14, 20).

Studies focusing on the epidemiology and genomic characterization of isolates harboring OXA-48-like genes particularly in neonatal septicemic cases are rare, with few reports of outbreaks or sporadic infections (13, 14). This study, however, monitors the presence of these genes in a neonatal unit over a period of 4 years (2013 to 2016) and evaluates the isolates in terms of their STs, production of multiple carbapenemases, their transmissibility, and associated mobile genetic elements. We performed core genome analysis incorporating isolates in this study in a global context with other OXA-48-like carbapenemase-harboring genomes, including those from other neonatal studies, to explore the genomic epidemiology and variability of carbapenemase lineages, focusing on the context of neonatal sepsis.

RESULTS

Bacterial isolates, their susceptibility, and genotypic profiles. During 2013 to 2016, 195 nonduplicate *Enterobacteriaceae*, including *Escherichia coli* ($n = 35$, 18%), *Klebsiella pneumoniae* ($n = 146$, 75%), *Enterobacter aerogenes* ($n = 3$, 1.5%), *Enterobacter cloacae* complex ($n = 11$, 5.6%) were identified which were resistant to piperacillin (89%), cefotaxime (80%), aztreonam (78%), and ciprofloxacin (70%). Resistance to meropenem was 47%, whereas few were resistant to tigecycline (2%) or colistin (5%).

Out of 195 strains identified, 11 strains (6%) were found to harbor $bla_{\text{OXA-48-like}}$ genes by conventional PCRs. Other carbapenemases detected were bla_{NDM} ($n = 73$, 38%) and bla_{KPC} ($n = 4$, 2%). In 2013, OXA-48-like carbapenemase was observed for the first time in this neonatal unit, prompting a thorough investigation of these isolates.

Detailed characterization of OXA-48-like carbapenemase-producing strains. All the OXA-48-like producers were *Klebsiella pneumoniae* (*Kp1* to *Kp11*). Some of the neonates from whom the *K. pneumoniae* was isolated did not survive, and most were "outborns" referred from some other hospitals (data not shown).

Kp1 to *Kp11* were resistant to most of the antimicrobials tested, *viz.*, piperacillin and its inhibitor (tazobactam), amikacin or gentamicin, cefotaxime, cefoxitin, ciprofloxacin, imipenem, ertapenem, meropenem, and aztreonam, and were fully susceptible to tigecycline (Table 1), although few strains were susceptible to meropenem and cefoxitin.

Two types of OXA-48-like carbapenemases namely, $bla_{\text{OXA-181}}$ and $bla_{\text{OXA-232}}$, were found among the study strains, henceforth called $bla_{\text{OXA-181-like}}$. $bla_{\text{NDM-5}}$ was the only class B carbapenemase detected and was found in four of the $bla_{\text{OXA-181-like}}$ positive strains. All 11 $bla_{\text{OXA-181-like}}$ strains harbor $bla_{\text{CTX-M-15}}$ along with different β-lactamases and aminoglycoside resistance and quinolone resistance genes in various combinations (Table 2).

Molecular typing of OXA-48-like carbapenemase-producing strains. Pulsed-field gel electrophoresis (PFGE) revealed 7 pulsotypes among the 11 $bla_{\text{OXA-181-like}}$ *K. pneumoniae* isolates. Of them, *Kp3* and *Kp9* to *Kp11* were found to be clonal (Fig. 1).

Multilocus sequence typing (MLST) revealed the presence of 5 diverse STs, *viz.*, ST14 (*Kp3*, *Kp9* to *Kp11*), ST15 (*Kp4*, *Kp5*), ST23 (*Kp6*, *Kp7*), ST48 (*Kp1*, *Kp2*), and ST231 (*Kp8*) (Table 2). Though *Kp3* and *Kp9* to *Kp11* belonged to same pulsotype and were ST14, their isolation was temporally distant, i.e., *Kp3* in 2014 but *Kp9* to *Kp11* in 2016. They also harbor two different variants of OXA-48-like carbapenemases, *viz.*, $bla_{\text{OXA-232}}$ (*Kp3*) and $bla_{\text{OXA-181}}$ (*Kp9* to *Kp11*).

The 5 STs collate within 4 clonal complexes (CCs), CC15 (ST14 and ST15), CC23 (ST23), CC48 (ST48), and CC231 (ST231) by goeBURST (Table 2). ST15, ST23, ST48, and ST231 of this study are the founder STs of their respective CCs, harboring the largest number of single-locus variants (SLVs) in their group. ST15, being a single-locus variant of ST14, contains more SLVs than ST14 and has been assigned as the founder of CC15. Hence, ST14 is categorized under CC15 as a subgroup founder. In our study, the presence of $bla_{\text{OXA-181}}$ was found in ST14, ST15, and ST48, while $bla_{\text{OXA-232}}$ was found in ST14, ST23, and ST231 (Table 2). On the other hand, $bla_{\text{NDM-5}}$ was found in ST14 only.

TABLE 1 Susceptibility profiles of *K. pneumoniae* strains and their transconjugants (TCs)/transformants (TFs) along with transmissibility of $bla_{OXA-181-like}$ and genotypic characterization of TCs/TFs established with PCR-based techniques

Strain ID	MIC (mg/liter):[a]													Resistance genes present/transferred[b]	Insertion sequence (IS) element	Plasmid type
---	AN	CN	AT	CT	FX	CI	IP	ETP	MP	CO	TGC	PP	PTZ			
EN5153 (Kp1)	8	96	>256	>256	48	4	>32	>32	12	1	0.75	>256	96	$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{SHV-1}, bla_{OXA-1}, $bla_{OXA-181}$, oqxA, oqxB, aac(6')-Ib-cr	Truncated ISEcp1 (167 bp)	IncFIIK, IncFIB(K), IncFIB (pQil), ColKP3
Kp1 TF2	3	0.5	0.094	0.38	12	<0.002	8	1	0.38	0.25	0.125	128	48	**$bla_{OXA-181}$**	ND	ColKP3
EN5172 (Kp2)	>256	>1,024	48	>256	6	32	16	>32	2	1	0.38	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{OXA-1}, $bla_{OXA-181}$, aac(6')-Ib, qnrB, oqxA, oqxB, aac(6')-Ib-cr	ISEcp1 absent but 303 bp of its RTE Ext present	IncFIIK, IncFII, ColKP3
Kp2 TF2	4	0.5	2	2	6	<0.002	4	12	1	0.5	0.125	128	96	**$bla_{OXA-181}$**	ND	ColKP3
Kp2 TF3	2	0.38	0.19	0.5	4	<0.002	4	6	1	0.125	0.125	128	96	bla_{TEM-1B}, **$bla_{OXA-181}$**	ND	ColKP3, IncKP3
EN5199 (Kp3)	>256	>1,024	>256	>256	>256	>32	16	>32	>32	1	1	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{SHV-28}, bla_{OXA-1}, bla_{OXA-9}, bla_{NDM-5}, $bla_{OXA-232}$, rmtB, aac(6')-Ib, oqxA, oqxB, aac(6')-Ib-cr	ISEcp1 absent but 335 bp of its RTE Ext present	IncR, IncFIIK, IncFII, IncFIB (K), IncFIA (HI1), ColKP3
Kp3 TC1	>256	>256	16	96	48	>32	>32	32	24	0.5	0.19	>256	>256	**bla_{NDM-5}**, rmtB	ND	IncFII
Kp3 TC4	>256	>1,024	16	96	48	>32	>32	32	24	0.5	0.75	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1B}, **bla_{NDM-5}**, **$bla_{OXA-232}$**, rmtB	ND	ColKP3, IncR, IncFII
EN5213 (Kp4)	>256	>1,024	>256	>256	12	32	16	>32	3	1	1	>256	>256	$bla_{CTX-M-15}$, bla_{SHV-28}, $bla_{OXA-181}$, aac(6')-Ib, qnrB, oqxA, oqxB, aac(6')-Ib-cr	ISEcp1 absent but 339 bp of its RTE Ext present	IncFIIK, ColKP3
Kp4 TF1	2	0.25	0.19	0.75	3	<0.002	16	32	3	0.125	0.19	>256	>256	**$bla_{OXA-181}$**	ND	ColKP3
EN5218 (Kp5)	32	2	32	256	4	>32	8	>32	1	1	1	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1A}, bla_{SHV-28}, bla_{OXA-1}, bla_{OXA-9}, **$bla_{OXA-181}$**, aac(6')-Ib-cr, aac(6')-Ib, qnrB1, oqxA, oqxB, aac(6')-Ib-cr	NF	IncFIIK, IncFII
Kp5 TC2	32	3	32	32	1	>32	4	0.5	0.032	0.5	1	>256	>256	$bla_{CTX-M-15}$, bla_{SHV-28}, **$bla_{OXA-181}$**, aac(6')-Ib-cr, qnrB, oqxA, aac(6')-Ib-cr	ND	IncFIIK
EN5275 (Kp6)	>256	>1,024	>256	>256	>256	>32	32	>32	>32	0.25	0.5	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1B}, $bla_{SHV-190}$, bla_{CMY-4}, $bla_{OXA-232}$, armA, rmtF, aac(6')-Ib-Hangzhou, qnrB1, oqxA, oqxB, aac(6')-Ib-cr	ISEcp1 absent but ~128 bp of its RTE Ext present	Col44011, Col44011, ColKP3, IncA/C2, IncFIIK, IncX3, IncFIB (pQil)
Kp6 TF1	2	1.5	0.5	0.5	32	0.006	8	8	1	0.5	0.38	>256	>256	**$bla_{OXA-232}$**	ND	ColKP3
EN5280 (Kp7)	>256	>1,024	>256	>256	>256	>32	32	>32	>32	0.5	1	>256	>256	$bla_{CTX-M-15}$, bla_{SHV-11}, bla_{CMY-4}, $bla_{OXA-232}$, armA, aac(6')-Ib, qnrB1, oqxA, oqxB, aac(6')-Ib-cr	ISEcp1 absent but 335 bp of its RTE Ext present	IncA/C, IncFIIK, IncX3, ColKP3
Kp7 TF1	2	0.25	0.125	1	24	<0.002	8	6	1.5	0.125	0.19	>256	>256	**$bla_{OXA-232}$**	ND	ColKP3
EN5338 (Kp8)	>256	>1,024	>256	>256	>256	>32	>32	>32	>32	0.5	1	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{SHV-28}, bla_{OXA-1}, bla_{OXA-9}, $bla_{OXA-232}$, rmtF, aac(6')-Ib-Hangzhou, oqxA, oqxB, qnrS1	ISEcp1 absent but ~320 bp of its RTE Ext present	ColKP3, IncFIA, IncHI1B, IncFIB (Mar), IncFIB (pQil), IncFIIK, IncFII (pAMA1167-NDM-5)
Kp8 TF1	3	0.25	0.5	0.75	16	0.16	8	16	1.5	<0.25	0.38	>256	>256	**$bla_{OXA-232}$**	ND	ColKP3
EN5339 (Kp9)	>256	>1,024	>256	>256	>256	>32	>32	>32	>32	64	1	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1A}, bla_{OXA-1}, bla_{OXA-9}, bla_{NDM-5}, rmtB, aac(6')-Ib, oqxA, oqxB, aac(6')-Ib-cr	ISEcp1 absent but 335 bp of its RTE Ext present	ColKP3, IncFIA (HI1), IncFIB (K), IncFIB (pKPHS1), IncR, IncFIIK, IncFII
Kp9 TC2	>256	>1,024	32	>32	64	>32	>32	>32	6	0.5	1	>256	>256	**bla_{NDM-5}**, rmtB, oqxA, oqxB	ND	IncFII
Kp9 TC3	>256	>1,024	32	>32	96	>32	>32	>32	12	0.5	1	>256	>256	**bla_{NDM-5}**, **$bla_{OXA-181}$**, rmtB, oqxA, oqxB	ND	IncR, IncFII, ColKP3
EN5340 (Kp10)	>256	>1,024	>256	>256	>256	>32	>32	>32	>32	64	0.75	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{OXA-1}, bla_{OXA-9}, bla_{NDM-5}, rmtB, aac(6')-Ib, oqxA, oqxB, aac	ISEcp1 absent but 335 bp of its RTE Ext present	ColKP3, IncFIA (HI1), IncFIB, IncR, IncFIIK, IncFII
Kp10 TC1	>256	>1,024	48	>32	96	>32	>32	24	32	0.25	1	>256	>256	**bla_{NDM-5}**, **$bla_{OXA-181}$**, oqxA, oqxB	ND	ColKP3, IncFII, IncR
EN5343 (Kp11)	>256	>1,024	>256	>256	>256	>32	>32	>32	>32	64	1	>256	>256	$bla_{CTX-M-15}$, bla_{TEM-1A}, bla_{OXA-1}, bla_{OXA-9}, bla_{NDM-5}, rmtB, aac(6')-Ib, oqxA, oqxB, aac	ISEcp1 absent but 335 bp of its RTE Ext present	ColKP3, IncFIA (HI1), IncFIB, IncR, IncFIIK, IncFII
Kp11 TC1	>256	>1,024	32	>32	32	>32	>32	24	>32	0.5	0.25	>256	>256	**bla_{NDM-5}**, rmtB, oqxA, oqxB	ND	IncFII
Kp11 TC2	>256	>1,024	48	>32	48	>32	>32	12	24	0.5	1	>256	>256	**bla_{NDM-5}**, **$bla_{OXA-181}$**, rmtB, oqxA, oqxB	ND	ColKP3, IncR, IncFII

*a*Abbreviations: TC, transconjugant; TF, transformant; AN, amikacin; CN, gentamicin; AT, aztreonam; CT, cefotaxime; FX, cefoxitin; CI, ciprofloxacin; IP, imipenem; ETP, ertapenem; MP, meropenem; CO, colistin; TGC, tigecycline; PP, piperacillin; PTZ, piperacillin-tazobactam; ND, not done; NF, not found; RTE Ext, right-end extremity of ISEcp1.
*b*Transferred carbapenem-resistant genes have been boldfaced.

TABLE 2 Characterization and comparative analysis of the strains by two different methods, viz., PCR and whole-genome sequencing (WGS)[a]

	Strain characteristics			PCR-based findings				
Strain ID	Year of isolation	ST/CC[b]	Aminoglycoside resistance genes	Beta-lactamases and carbapenemases (bla)	Quinolone resistance genes	Virulence determinants	PBRT and primer walking	Integron/integrase/GC array
Kp1	2013	ST48/CC48	Not found	CTX-M-15, TEM-1B, SHV-1, OXA-1, OXA-181	oqxAB, aac(6')-Ib-cr	wabG, uge, fimH	IncFIIK, Col	intI1
Kp2	2014	ST48/CC48	aac(6')-Ib	CTX-M-15, TEM-1B, OXA-1, OXA-181	qnrB, oqxAB, aac(6')-Ib-cr	wabG, uge, fimH	IncFII, IncFIIK, Col	In27, intI1
Kp3	2014	ST14/CC15	rmtB, aac(6')-Ib	NDM-5, CTX-M-15, TEM-1B, SHV-28, OXA-1, OXA-232	oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD	IncFII, IncFIIK, IncR, Col	intI1
Kp4	2015	ST15/CC15	aac(6')-Ib	CTX-M-15, SHV-28, OXA-1, OXA-181	qnrB, oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD	IncFIIK, Col	intI1
Kp5	2015	ST15/CC15	aac(6')-Ib	CTX-M-15, TEM-1A, SHV-28, OXA-1, OXA-181	qnrB, oqxAB, aac(6')-Ib-cr	uge, fimH, mrkD	IncFII, IncFIIK	intI1
Kp6	2016	ST23/CC23	armA, aac(6')-Ib	CTX-M-15, TEM-1B, SHV-190, OXA-232, CMY-4	qnrB, oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD, kfuBC, wcaJ, rmpA, magA	IncA/C, IncFIIK, IncX3, Col	intI1
Kp7	2016	ST23/CC23	armA, aac(6')-Ib	CTX-M-15, TEM-1B, SHV-11, OXA-232, CMY-4	qnrB, oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD, kfuBC, wcaJ	IncA/C, IncFIIK, IncX3, Col	intI1/aadA2, dfrA12, orfF
Kp8	2016	ST231/CC231	aac(6')-Ib	CTX-M-15, TEM-1B, SHV-28, OXA-232	qnrS, oqxAB	wabG, uge, fimH, mrkD, kfuBC	IncFIIK, IncFIA, IncFIB-M, IncHIB-M, Col	In27/intI1
Kp9	2016	ST14/CC15	rmtB, aac(6')-Ib	NDM-5, CTX-M-15, TEM-1A, SHV-28, OXA-1, OXA-181	oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD, kfuBC	IncFII, IncFIIK, IncR, Col	intI1
Kp10	2016	ST14/CC15	rmtB, aac(6')-Ib	NDM-5, CTX-M-15, TEM-1A, SHV-28, OXA-1, OXA-181	oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD, kfuBC	IncFII, IncFIIK, IncR, Col	intI1
Kp11	2016	ST14/CC15	rmtB, aac(6')-Ib	NDM-5, CTX-M-15, TEM-1A, SHV-28, OXA-1, OXA-181	oqxAB, aac(6')-Ib-cr	wabG, uge, fimH, mrkD, kfuBC	IncFII, IncFIIK, IncR, Col	intI1

TABLE 2 Continued

Strain characteristics			WGS-based findings								
Strain ID	Year of isolation	ST/CC[b]	Aminoglycoside resistance genes	Beta-lactamases (bla)	Carbapenemases (bla)	Quinolone resistance genes	Other resistance genes (family)	Virulence determinants; CPS cluster genes; capsular type; virulence sequence type; integrative conjugative element	Plasmid type	Integron/ GC array	GenBank accession no.
Kp1	2013	ST48/CC48	aac(3)-IIa, aph(6)-Id, aph(3")-Ib, aadA2	TEM-1B, SHV-1, OXA-1, CTX-M-15	OXA-181	oqxAB, aac(6')-Ib-cr	fosA (fosfomycin); mph(A) (macrolide); catA1, catB3 (phenicol); sul1, sul2 (sulphonamide); dfrA12 (trimethoprim); arsABCDR (arsenic); pcoABCDERS (copper); silABCEFGPRS (silver)	mrkABCDFHIJ; fimABCDEFGHIK; iutA; entABCDEFS, fepABCDG, fes; iroEN; fyuA, irp1, irp2; ybtAEPQSTUX; rcsA, rcsB; T6SS-I/II/III; LPS rfb locus, wzi62, wzc62; K62, O1/O2v1; ybt14; ICEKp5	IncFIIK, IncFIB (K), IncFIA (pQil), ColKP3	In27	VSLB000000000
Kp2	2014	ST48/CC48	WGS ND	WGS ND	WGS ND, NDM-5,	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND, In27,	WGS ND
Kp3	2014	ST14/CC15	rmtB, aac(6')-Ib, aadA1, aadA2, aph(6)-Id, aph(3")-Ib, aac(3")-IId	TEM-1B, SHV-28, OXA-1,9, CTX-M-15	OXA-232	oqxAB, aac(6')-Ib-cr	fosA (fosfomycin); mph(A), ere(A), erm(B) (macrolide); catB3, cmlA1, catA1 (phenicol); sul1, sul2 (sulphonamide); dfrA1, dfrA12 (trimethoprim); arsABCDR (arsenic); pcoABCDER (copper); silACEFGPRS (silver); merACDEPRT (mercury)	mrkABCDEFGHIK; pilW; iutA; entABCDEFS, fepABCDG, fes; iroEN; fyuA, irp1, irp2; ybtAEPQSTUX; rcsA, rcsB; T6SS-I/II/III; LPS rfb locus, wzi2, wzc2; K2, O1/O2v1; ybt14; ICEKp5	IncR, IncFIIK, IncFIB, IncFII, IncFIB (K), IncFIA (HI1), ColKP3	In578	VSLC000000000
Kp4	2015	ST15/CC15	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND
Kp5	2015	ST15/CC15	aac(6')-Ib, aadA1, aph(3")-Ib, aph(6)-Id, aac(6')-Ib3	TEM-1A, SHV-28, OXA-1,9, CTX-M-15	OXA-181	qnrB1, oqxAB, aac(6')-Ib-cr	fosA (fosfomycin); mph(A) (macrolide); catB3 (phenicol); sul2 (sulphonamide); tet(A) (tetracycline); dfrA14 (trimethoprim); arsABCR (arsenic); pcoABCDERS (copper); silABCEFGPRS (silver)	mrkCDH; fimCDHK; iutA; entCEFS, fepABCDG, fes; iroE; fyuA, irp1, irp2; ybtAEPQSTUX; rcsA; T6SS-I/II/III; LPS rfb locus, wzi151; K48, O1v1; ybt1; ICEKp4	IncFIIK, IncFII	In191	WMCH00000000
Kp6	2016	ST23/CC23	armA, rmtF, aac(6')-Ib, aph(3")-Ib, aph(6')-Id	TEM-1B, SHV-190, CTX-M-15, CMY-4	OXA-232	qnrB1, oqxAB, aac(6')-Ib-cr	fosA (fosfomycin); msr(E), mph(E) (macrolide); catA1 (phenicol); arr-2 (rifampin); sul1, sul2 (sulphonamide); dfrA14 (trimethoprim); pcoABCDER (copper); silCERS (silver); terABDEWZ (tellurite); pbrAR (lead)	mrkABCDFIJ; fimABCDEFGHIK; iutA, iucABCD; entABCDEFS, fepABCDG, fes; iroBCDEN; fyuA, irp1, irp2; ybtAEPQSTUX; allABCDRS; rmpA, rmpA2; magA; rcsA, rcsB; T6SS-I/II/III; LPS rfb locus, wzi1, wzc1; K1, O1v2; ybt9; ICEKp3	Col4401I, Col440II, ColKP3, IncA/C2, IncFIIK, IncX3, IncFIB (pQil)	aacA4, arr-2, dfrA14b	VINI0000000
Kp7	2016	ST23/CC23	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND, In27,	WGS ND
Kp8	2016	ST231/ CC231	rmtF, aac(6')-Ib, aac(6')-Ib-Hangzhou, aadA2	TEM-1B, SHV-28, CTX-M-15	OXA-232	qnrS1, oqxAB	fosA (fosfomycin); erm(B) (macrolide); catA1 (phenicol); arr-2 (rifampin); sul1 (sulphonamide); dfrA12 (trimethoprim); terABCDEXZ (tellurite)	mrkABCDFHIJ; fimABCDEFGHIK; pilW; iucABCD, iutA; entABCDEFS, fepABCDG, fes; iroEN; fyuA, irp1, irp2; ybtAEPQSTUX; sitC, sitABCD; rcsA, rcsB; T6SS-I/II/III; LPS rfb locus; stbABCDE; wzi104; KL51, O1v2; ybt14; ICEKp5	ColKP3, IncFIA, IncHI1B, IncFIB (Mar), IncFIB (pQil), IncFIIK, IncFII (pAMA1167-NDM-5)	In406	JAAGUA000000000
Kp9	2016	ST14/CC15	rmtB, aac(6')-Ib, aadA1, aadA2, aph(6)-Id, aph(3")-Ib	TEM-1A, SHV-28, OXA-1,9, CTX-M-15	NDM-5, OXA-181	oqxAB, aac(6')-Ib-cr	fosA (fosfomycin); mph(A), ere(A), erm(B) (macrolide); cmlA1, catA1 (phenicol); sul1, sul2 (sulphonamide); dfrA1, dfrA12 (trimethoprim); arsABCDR (arsenic); pcoABCDER (copper); silACEFGPRS (silver); merACDEPRT (mercury)	mrkABCDFHIJ; fimABCDEFGHIK; pilW; iutA; entABCDEFS; fepABCDG, fes, iroEN, fyuA, irp2, ybtAEPQSTUX; rcsA, rcsB; T6SS-I/II/III; LPS rfb locus, wzi2, wzc2; K2, O1v1; ybt 14; ICEKp5	ColKP3, IncFIA (HI1), IncFIB (K), IncFIB (pKPHS1), IncR, IncFIIK, IncFII	In27, In1329	VSJI000000000
Kp10	2016	ST14/CC15	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND
Kp11	2016	ST14/CC15	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND	WGS ND

[a]Terms used: Inc, incompatibility group; intI1, integrase 1; GC, gene cassette; mrk, type-3 fimbriae operon; fim, type-1 fimbriae operon; pil, type IV pili; juc and iut, aerobactin; ent, fep, and fes, enterobactin; iro, salmochelin; all, allantoin utilization/nutritional factor; sitC, ferrous iron transporter; sit, iron/manganese transporter; rcs, RcsAB operon; T6SS, type-6-secretion system; LPS rfb locus, for serum resistance; fyuA, irp, and ybt, yersiniabactin; stb, fimbrae adherence determinants; YbST, yersiniabactin sequence type; ICE, integrative conjugative element; WGS ND, whole-genome sequencing not done.
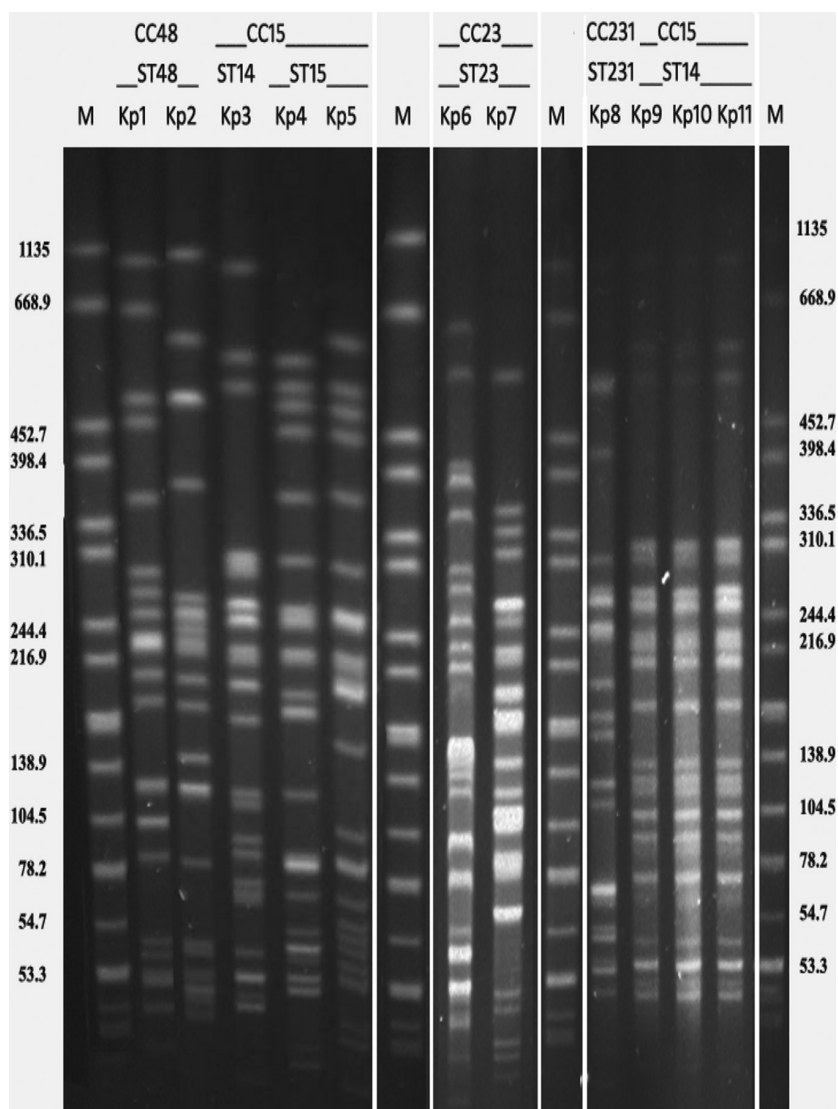[b]ST, sequence type; CC, clonal complex.

FIG 1 Pulsed-field gel electrophoresis with XbaI macrodigestion of $bla_{OXA-181-like}$-harboring *K. pneumoniae* isolated from blood of septicemic neonates. Lanes 1, 7, 10, and 15: M (marker) *Salmonella* serotype Braenderup H9812 as reference standard; lane 2: *Kp1*; lane 3: *Kp2*; lane 4: *Kp3*; lane 5: *Kp4*; lane 6: *Kp5*; lane 8: *Kp6*; lane 9: *Kp7*; lane 11: *Kp8*; lane 12: *Kp9*; lane 13: *Kp10*; lane 14: *Kp11*. Sequence types found in the strains are listed above each strain. ST, sequence type; CC, clonal complexes.

Resistome and virulome analysis of OXA-181-like carbapenemase-producing strains. One strain from each different ST (ST15, ST23, ST48, and ST231) along with the 2 strains of ST14 (*Kp3* and *Kp9*) possessing different $bla_{OXA-181-like}$ genes were subjected to whole-genome sequencing (WGS). Other strains (*Kp2*, *Kp4*, *Kp7*, *Kp10*, and *Kp11*) not processed for WGS were screened by PCR followed by Sanger sequencing of the relevant resistance genes. Resistome analysis (≥98% identity and coverage) showed the presence of $bla_{CTX-M-15}$ in all the strains together with several other β-lactamases, aminoglycoside, and fluoroquinolones (Table 2). Apart from these, the presence of several heavy metal and other antibiotic resistance genes was also noted, as listed in Table 2. Out of 11, 7 were found to carry $bla_{OXA-181}$ (*Kp1*, *Kp2*, *Kp4*, *Kp5*, and *Kp9* to *Kp11*), and the remaining 4 (*Kp3*, *Kp6* to *Kp8*) harbored $bla_{OXA-232}$.

Strains were found to possess virulence genes (Table 2) such as *iut*, *ent*, *fep*, *fes*, *ybt*, *irp*, *iro*, etc. (iron-chelators). The occurrence of serum resistance and antiphagocytosis
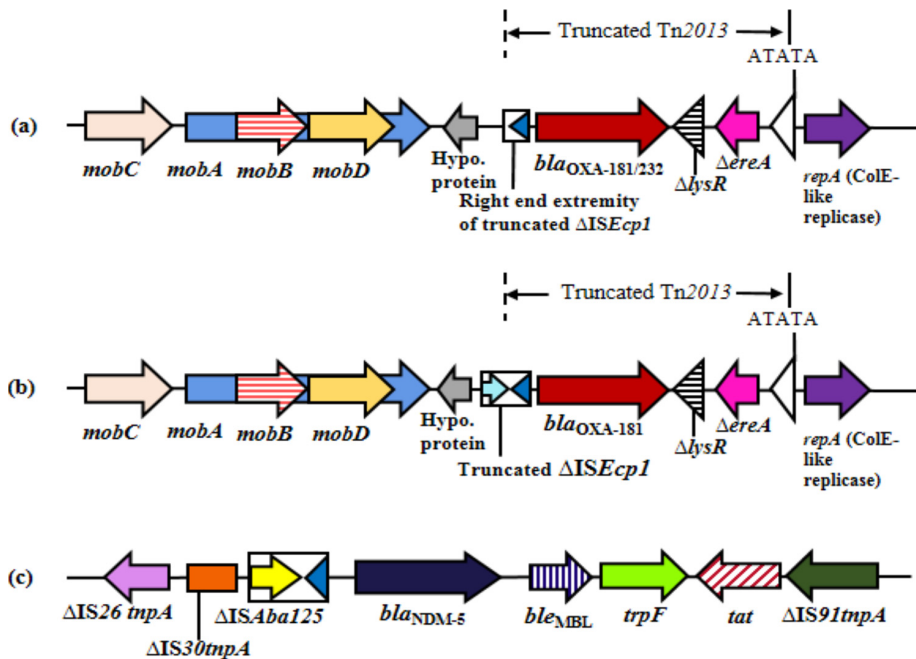
FIG 2 Schematic presentation of MGEs associated with $bla_{OXA-181/232}$ and bla_{NDM-5} in the *K. pneumoniae* strains isolated from neonates. Heterogeneity of the genetic environment found in the studied carbapenemases: (a) $bla_{OXA-181/232}$ (*Kp2-Kp4*, *Kp6-Kp11*), (b) $bla_{OXA-181}$ in *Kp1*, and (c) genetic environment of transposon 125 (Tn*125*) harboring bla_{NDM-5} (*Kp3*, *Kp9-Kp11*). Genes and their corresponding transcription orientations are indicated by horizontal arrows. Target site duplications (ATATA) generated by the insertion of Tn*2013* are indicated by white triangles. *mobA*, *mobB*, *mobC*, and *mobD*, mobilization relaxosome proteins; Δ*lysR*, truncated LysR-type transcriptional regulator; Δ*ereA*, truncated erythromycin esterase; *repA*, replicase; *tnpA*, transposase; IS, insertion sequence; ble_{MBL}; bleomycin resistance gene; *trpF*, N-(5′-phosphoribosyl) anthranilate isomerase; *tat*, twin-arginine translocation pathway signal sequence protein; Hypo. protein, hypothetical protein; Δ, denotes deletion or truncation.

capsular factors along with different K- and O-loci were found in the strains. Strains also possessed various integrative conjugative elements. The presence of *rmpA*, *rmpA2*, and *magA* responsible for hypermucoidy and hypervirulence was found in *Kp6*, which has already been reported in a separate study (16).

Transmissibility of $bla_{OXA-181}$, $bla_{OXA-232}$, and bla_{NDM-5}. Conjugal transfer of an OXA-48-like-bearing plasmid was successful for 5 strains (*Kp3*, *Kp5*, and *Kp9* to *Kp11*); for others, transformants were obtained. The presence of resistance genes was assessed in the transconjugants (TCs)/transformants (TFs) (Table 1). bla_{NDM-5} was borne on large conjugative plasmids (ranging between ~100 and 200 kb), while $bla_{OXA-181/OXA-232}$ were present on small (~6 to 8 kb) nonconjugative plasmids. Interestingly, conjugal transfer of $bla_{OXA-181/OXA-232}$ was successful when coexisting with bla_{NDM-5}, though on separate plasmids.

Most of the TCs/TFs with only $bla_{OXA-181-like}$ showed the presence of similar plasmid scaffolds, i.e., ColKP3, except for one (*Kp5*) with IncFIIK (Table 1). WGS data also specified the association of ColKP3 with the $bla_{OXA-181-like}$ (Table 2). On the other hand, bla_{NDM-5} was present on IncFII (Table 1).

The MIC of the TCs/TFs for different antimicrobials were assessed (Table 1). TCs/TFs with only $bla_{OXA-181-like}$ exhibited high MICs for imipenem followed by ertapenem compared to meropenem. However, TCs where coexistence of bla_{NDM-5} and $bla_{OXA-181-like}$ were observed showed higher MIC for meropenem.

Analysis of mobile genetic elements (MGEs). The genetic environment of $bla_{OXA-181-like}$ revealed the presence of a mobilization relaxosome (*mobA*, *mobB*, *mobC*, and *mobD*) upstream, and Δ*lysR* (transcription regulator), Δ*ereA* (erythromycin esterase), and Col replicase (*repA*) downstream, respectively (Fig. 2a and b).
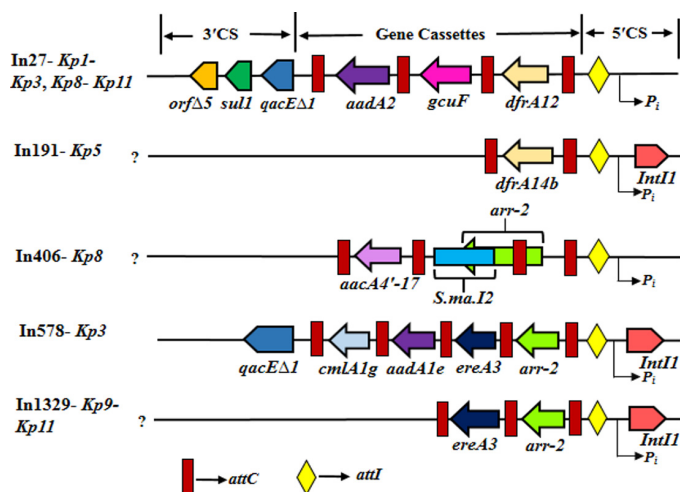
FIG 3 Diagrammatic representation of class 1 integron found in the strains under study. *arr-2*, ADP-ribosyl transferase; *qacEΔ1*, quaternary ammonium compound resistance protein; *sul1*, sulfonamide resistant dihydropteroate synthase; orf*Δ5*, an open reading frame of unknown function; *aadA1e* and *aadA2*, aminoglycoside adenyltransferase; *gcuF*, DUF1010 domain-containing protein; *dfrA12* and *dfrA14b*, dihydrofolate reductases type-A; *S.ma.I2*, group IIc intron; *aacA4'-17*, aminoglycoside 6'-*N*-acetyltransferase; *ereA3*, erythromycin esterase; *cmlA1g*, chloramphenicol resistance gene; *attI*, site of recombination; *intI1*, integrase gene; *attC*, site of attenuation; P_i, promoter of integrase; CS, conserved sequence.

Deletion of IS*Ecp1* was found with varying stretches of its right-end extremity except for *Kp1* and *Kp5* (Table 1). All study strains were found in truncated Tn*2013*.

On the other hand, *bla*$_{NDM-5}$ was bracketed between truncated IS*Aba125* and bleomycin resistance genes (*ble*$_{MBL}$) found upstream and downstream, respectively. IS*Aba125* is preceded by a truncated transposase of the IS*30* family and truncated IS*26*, while *ble*$_{MBL}$ is succeeded by N-(5'-phosphoribosyl) anthranilate isomerase (*trpF*), twin-arginine translocation pathway signal sequence protein (*tat*), and the truncated transposase of IS*91* (Fig. 2c). *Kp3* and *Kp9-Kp11* have similar genetic environments with truncated Tn*125*.

Five different integrons, In27, In191, In406, In578, and In1329, were detected (Table 2 and Fig. 3). In27 was found to be the most prevalent integron (*Kp1* to *Kp3*, *Kp8* to *Kp11*) (Table 2), but *bla*$_{OXA-181/OXA-232}$ or *bla*$_{NDM-5}$ was not found to be allied to any of the integrons obtained.

A phylogenetic global comparison of OXA-48-like genomes and *K. pneumoniae* isolated from neonates. The maximum likelihood core genome phylogenetic tree was constructed with 197 *K. pneumoniae* from (i) a global collection of OXA-48-like and NDM carbapenemase-carrying isolates and (ii) published genomic data of septicemic neonatal *K. pneumoniae* (Fig. 4). As few neonatal studies with published sequence data (either GenBank NCBI or ENA-EMBL) were available, all possible sequences were incorporated, irrespective of carbapenem resistance.

bla$_{OXA-48-like}$ *K. pneumoniae* detected from 21 countries and 20 sample sources, including human, animal, and environmental samples, were remarkably diverse, with 40 different STs identified.

The diversity at the core genome level of the strains within this study was vast, spanning multiple lineages, showing both diversity among themselves as causative agents of neonatal sepsis and varying levels of relatedness compared to strains from different parts of the world. EN5153 (*Kp1*) showed similarities with strains from Tanzania and Ghana; EN5218 (*Kp5*), with strains from China, Spain, and Norway; EN5275 (*Kp6*), with distantly related strains from Romania; EN5338 (*Kp8*), with strains from Thailand, Pakistan, the United States, and Switzerland; and EN5199 (*Kp3*) and EN5339 (*Kp9*), with strains from the United Kingdom, the United States, South Korea, Pakistan, Thailand, and Tanzania. Also, EN5199, EN5338, and EN5339 showed
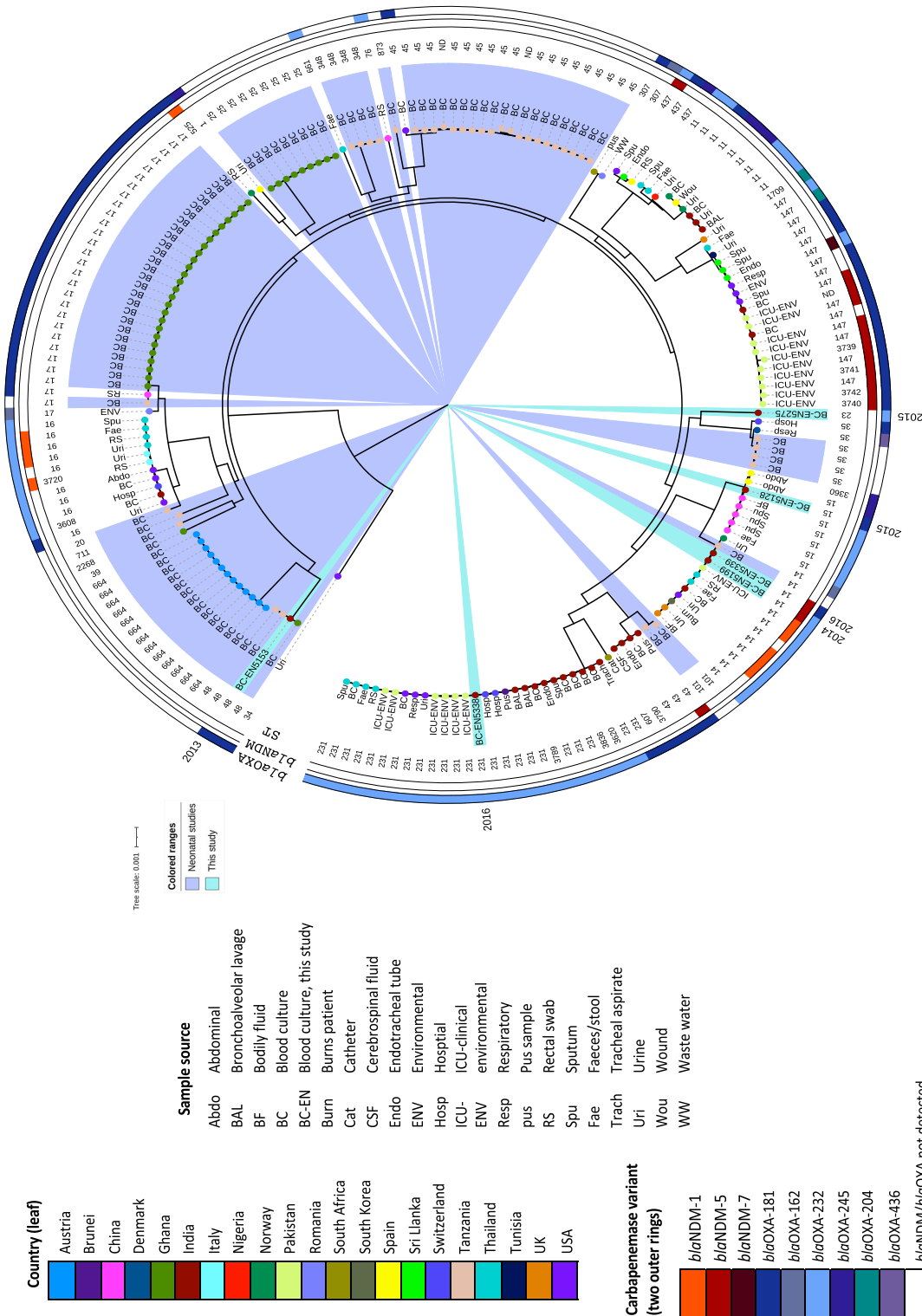
FIG 4 Core genome phylogeny of 197 *Klebsiella pneumoniae* isolates using Roary (v3.12.0) and FastTree (v2.1.11). Isolates are colored at the endpoint according to country, and the outer ring abbreviation is labeled according to the sample source. The additional two outer rings denote the presence of *bla*NDM and *bla*OXA-48-like antibiotic resistance genes. Clades containing isolates from this study are highlighted in teal, and light blue clade highlights indicate *K. pneumoniae* neonatal sepsis isolates from other studies. The year of sample collection for isolates in this study has been added external to the tree phylogeny.

Tree scale 100

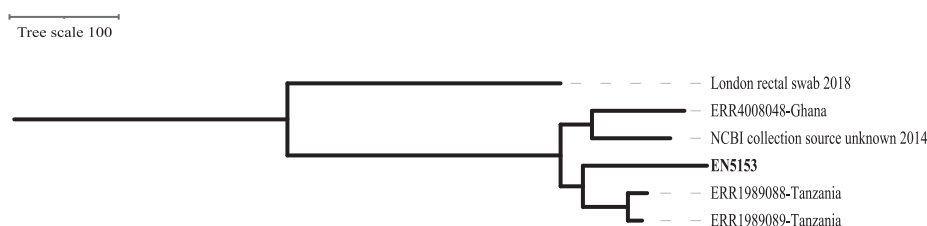**FIG 5** Core genome SNP phylogeny of EN5153 (*Kp1*) with other ST48 neonatal isolates. An outgroup rooted tree was built using the most distant isolate from the Mash genome estimation analysis (an isolate from London, submitted to the NCBI database in 2018). Isolates beginning with ERR are from other ST48 neonatal isolates and another isolate submitted to NCBI on 2014.

similarities with strains reported from various parts of India. When genomes of bacteria causing neonatal infections are compared, EN5153, EN5199, and EN5339 showed similarities with neonatal strains from Tanzania and Ghana. Interestingly, core genome single nucleotide polymorphism (SNP) phylogeny of EN5153 suggests that all ST48 neonatal isolates sit within the same cluster, and the additional ST48 with the greatest similarity from the NCBI database (an isolate from a rectal swab in London from 2018) sits on a single branch (Fig. 5).

Six variants of $bla_{OXA-48-like}$ were identified in the collective core genome phylogeny, of which only $bla_{OXA-181}$ or $bla_{OXA-232}$ were detected from neonatal *K. pneumoniae* in both Ghana and this study. Apart from these, none of the neonatal strains harbor carbapenem-resistant genes.

DISCUSSION

In this study, we characterized $bla_{OXA-181-like}$-producing *K. pneumoniae* in a neonatal setting over 4 years, showing the diversity of the genomes. We identified 11 $bla_{OXA-181/232}$ carbapenemases-producing *K. pneumoniae*. bla_{NDM-5} was found in some of the strains. OXA-48-like carbapenemases have been found to be the most common carbapenemases among *Enterobacteriaceae* family pathogens in certain parts of the world, such as Europe, the Middle East, North America, etc., while NDM carbapenemases are endemic to India and Southeast Asia (10, 14, 20). The presence of $bla_{OXA-181/OXA-232}$ along with bla_{NDM-5} has been reported in patients from South Korea, the United States, Chad, and Nepal, having travel history from India or the Indian subcontinent (8, 21–23). The existence of dual carbapenemases ($bla_{OXA-181/232}$ and bla_{NDM-5}) among the strains reduced their susceptibility to all carbapenems (imipenem, ertapenem, and meropenem), thereby making them extremely drug resistant. Infection with these organisms is dreadful, especially in neonates with limited therapeutic options. Following an extensive PubMed search for reports of $bla_{OXA-181/OXA-232}$ along with bla_{NDM-5} in neonates, we found no matches; however, $bla_{OXA-232}$ has been reported in neonatal infections from China (20). Hence, to the best of our knowledge, this is the first study to report the coexistence of $bla_{OXA-181/OXA-232}$ with bla_{NDM-5} in septicemic neonates.

Strains were found to be diverse and belonged to 5 different STs, some of which are well-known international clones (ST14). OXA-48-like carbapenemases are well known for triggering outbreaks involving specific sequence types, such as ST11, ST14, ST15, ST101, ST147, and ST307 recorded from various parts of Europe, Mediterranean regions, China, North America, and South Africa (12, 14). Carriage of $bla_{OXA-181}$ with STs such as ST11, ST14, ST16, ST25, ST43, ST61, ST147, ST231, ST307, and ST709 and $bla_{OXA-232}$ with ST11, ST14, ST15, ST16, ST17, ST101, ST147, ST231, ST307, ST395, ST570, and ST2040 have been previously reported (11, 12, 14, 24). Major hospital outbreaks were noted with ST14 and ST15, harboring $bla_{OXA-181}$ and $bla_{OXA-232}$, respectively, in Canada and China, the latter involving a neonatal unit (14). Reports of $bla_{OXA-181-like}$ with ST11, ST14, ST43, ST101, ST147, ST231, and ST2040 were documented from India (11, 24). However, in this study, the occurrence of $bla_{OXA-181}$ in

ST14, ST15, and ST48 and $bla_{OXA-232}$ in ST14, ST23, and ST231 was noted (Table 2). *K. pneumoniae* isolates with bla_{NDM-5} are mostly reported among ST15, ST45, ST147, ST182, ST395, and ST476 (21, 25–28). But the present study, like a few other studies (25, 29), reported bla_{NDM-5} in ST14 *K. pneumoniae*. The presence of $bla_{OXA-181/OXA-232}$ with bla_{NDM-5} in high-risk international clone ST14 further highlights the spread of resistance across continental boundaries.

A plethora of resistance and virulence genes were identified among the strains, which supports the survival of the pathogen in antibiotic-laden environments of health care settings as well as their successful colonization in the host. The occurrence of resistance genes on plasmids and virulence genes on integrative conjugative elements instigates the spread of these genes in the community. Hence, the presence of drug-resistant virulent strains of *K. pneumoniae* in neonates can cause severe infection leading to critical consequences.

In the current study, two specific plasmid scaffolds were seen to be associated with the studied carbapenemases genes. $bla_{OXA-181-like}$ were found on a nonconjugative ColKP3 plasmid on a truncated Tn*2013*, as reported previously (14, 30, 31). $bla_{OXA-232}$ has always been reported in Tn*2013*, but $bla_{OXA-181}$ has been found in Tn*2013* and in other transposons, such as Tn*6360* (14). Deletion of IS*Ecp1* from the upstream of $bla_{OXA-181/232}$ was noted among the strains, which must have restricted its transposase activity, resulting in stabilization of $bla_{OXA-181/OXA-232}$ on pKP3/pOXA232-like plasmids (30, 31). bla_{NDM-5} was found in a conjugative IncFII plasmid within truncated Tn*125* with a comparable plasmid background reported from a nontraveler in Spain (32), although the association of bla_{NDM-5} is predominantly reported in IncX3, but they have also been found in IncFII (32). This study also indicated the presence of $bla_{OXA-181/OXA-232}$ and bla_{NDM-5} on separate plasmids, suggesting two independent events of gene acquisition by the organism. The majority of previous reports have proposed that the spread of $bla_{OXA-181-like}$ is through clonal dissemination, but this study corroborated the results from few earlier reports (14, 30, 31), describing the involvement of a helper plasmid (bla_{NDM-5}) that facilitated conjugal transfer of $bla_{OXA-181-like}$, reinforcing the role of helper plasmids in their transmission. Such a phenomenon underlines the threat these carbapenemases pose when present with bla_{NDM}, not only in terms of increased resistance and further treatment limitations, but also in the ease of transfer.

WGS analysis of neonatal strains is largely limited to outbreak cases, and studies of isolates collected over longer periods are rare. This study is probably the first to incorporate a global collection of *K. pneumoniae* harboring OXA-48-like and NDM carbapenemases with special reference to septicemic neonatal strains. Strains of this study belonged to diverse sequence types, which ruled out clonal spread of $bla_{OXA-181-like}$-carbapenemases and were similar to outbreak strains from neonates in Tanzania, Ghana, and Austria (33–35). Genomes were diverse, but the plasmid scaffold (ColKP3) harboring $bla_{OXA-181-like}$ was similar across the study strains as also reported by other studies (14, 30, 31). Diversity among the isolates studied here could be, in part, due to many neonatal referrals from other hospitals within this study, and therefore neonates were exposed to both different health care and environmental factors.

Although there are limitations of short read sequencing with respect to plasmid assembly, holistic understanding of the genomes and their spread across the globe and in specific populations or patients is possible. The presence of carbapenem-resistant *K. pneumoniae* in low-middle-income countries (LMIC) such as India, where neonatal deaths amount to nearly 0.75 million per year (5), is a serious concern which requires rapid investigation. With increasing WGS facilities and decreasing cost of sequencing, short read sequencing is an extremely useful tool to aid routine antimicrobial resistance (AMR) surveillance. This study thus gives an insight about such strains not only in a particular setting but also in a wider global context.

MATERIALS AND METHODS

Ethical approval. The study protocol was approved by the Institutional Ethics Committee of the ICMR-National Institute of Cholera and Enteric Diseases (no. A-1-2/2018/IEC). Patient information was anonymized and deidentified prior to analysis.

Identification and susceptibility testing. During 2013 to 2016, bacteria were isolated from blood of septicemic neonates from the neonatal intensive care unit of a tertiary care hospital of Kolkata, West Bengal, India. Isolates were identified with in-house biochemical tests and the Vitek 2 compact system (bioMérieux, Marcy-l'Étoile, France). MICs were determined with Etest (bioMérieux) for all antimicrobials tested, except for colistin. Broth microdilution was carried out for colistin as described previously (36). Results were analyzed according to CLSI and EUCAST guidelines (37, 38).

Genotypic characterization of β-lactamases, carbapenemases, fluoroquinolones, and 16S rRNA methylases. PCR was carried out for the following resistance genes: β-lactamase genes ($bla_{CTX-M,TEM,SHV,OXA-1}$), AmpC genes ($bla_{MOX,CMY,DHA,ACC,MIR/ACT,FOX}$), aminoglycoside resistance genes [$aac(6')$-Ib, $rmtA$, $rmtB$, $rmtC$, $rmtD$, and $armA$], carbapenemase genes ($bla_{VIM,IMP,SPM-1,GIM-1,SIM-1,NDM-1}$, bla_{OXA-48}, $bla_{KPC,SME,IMI,GES,NMC}$), and flouroquinolone resistance genes [qnr-A,B,S, $qepA$, $aac(6')$-Ib-cr, $oqxA$, $oqxB$], depending upon the susceptibility profile (39, 40).

Multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE). For sequence typing (ST), seven housekeeping genes were amplified, sequenced, and submitted to the MLST database (https://bigsdb.web.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef). The goeBURST algorithm (http://www.phyloviz.net/goeburst/) was used for assigning clonal complexes to the STs (41).

Strains producing $bla_{OXA-181-like}$ were subjected to PFGE using XbaI and were visually interpreted according to Tenover criteria (42).

Transmissibility of carbapenem-resistant genes. Transfer of carbapenemase genes was performed by conjugation with the *E. coli* J53 Az^r strain as the recipient by the solid-mating conjugation technique. Electroporation was carried out with purified plasmid DNA (43) into *E. coli* DH10B (Invitrogen, California, USA) using a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA, USA) for every failed conjugation. Transconjugants (TCs) were selected on Luria Bertani (LB) agar plates supplemented with (i) sodium azide (100 mg/liter) and ertapenem (0.25 mg/liter) and (ii) sodium azide and cefoxitin (8 mg/liter) (Sigma-Aldrich, St. Louis, MO, USA) for $bla_{OXA-181-like}$-producing strains possessing bla_{NDM}. Transformants (TFs) were selected on LB agar with ertapenem (0.25 mg/liter). The TCs/TFs retrieved were subjected to confirmation of carbapenem-resistant genes and other β-lactamase genes by PCR followed by susceptibility testing.

Plasmid analysis was performed with wild-type strains and their TCs/TFs according to Kado and Liu (43), followed by plasmid typing using PCR-based replicon typing (PBRT) (44). To map the entire integron structure and determine their types and possible association with carbapenem-resistant genes, PCRs were performed as described previously (45, 46), followed by Sanger sequencing, and submitted to the INTEGRALL site.

Whole-genome sequencing (WGS). Total genomic DNA was isolated and DNA libraries were prepared for paired-end sequencing using Nextera XT and NEBNext Ultra II DNA library prep kits according to the manufacturer's instruction. Sequencing was performed using the Illumina platform (San Diego, CA). Quality and adaptor trimming were completed using Trim Galore (v0.4.3). *De novo* assembly was accomplished using different assemblers, such as SPAdes (v.3.9.0), Velvet (v.1.2.10), and Shovill (v.0.9.0), and Pilon (v1.22) was used on the resulting contigs to correct any mapping errors. Evaluation of assembly metrics and annotation were carried out using Quast (v2.1) and Prokka (v1.12), respectively, and were viewed in Artemis (Sanger, UK) and the SnapGene viewer.

With the contig files, the following online servers were used for analysis: (i) ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and pathogenwatch (https://pathogen.watch/) for resistance genes, (ii) PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) for plasmid types, (iii) the MLST database for sequence typing, (iv) the BIGSdb-Kp database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) and the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) for virulence genes and the Kaptive database (https://kaptive-web.erc.monash.edu/) for K- and O-antigen capsular typing, (v) the Integrall site for nomenclature of the integron sequences, (vi) TETyper for identification of transposon type, and (vii) ISfinder for IS elements (https://isfinder.biotoul.fr/).

A core genome phylogeny tree was built using Roary (v3.12.0) and FastTree (v2.1.11) with isolates from this study along with *K. pneumoniae* possessing different OXA-48-like and NDM variants submitted to National Center for Biotechnology Information (NCBI) database. Initially 8,663 *K. pneumoniae* genomes were downloaded from NCBI on 27 March 2020. Abricate (v0.9.7) was used to screen the genomes for the presence of OXA-48-like and NDM antibiotic resistance genes. Similarly, *in silico* MLST (v2.17.6) was performed to assign STs. Based on the presence/absence of carbapenemase variants, and ST, a selection of strains was chosen for comparative analysis. From the BioSample database within NCBI, data of the country and source of the isolate were collected, where applicable. Additionally, and following a literature search for studies with neonatal sepsis *K. pneumoniae* WGS data available, raw sequencing reads were downloaded from the ENA repository. All FASTQ reads were subject to the same quality control (QC) parameters as previously described, assembled using Shovill (v0.9.0), and annotated using Prokka (v1.12). Based on relatedness to other neonatal sepsis isolates in the core genome phylogeny, isolates within the same clade were further analyzed to create a core SNP phylogeny using Snippy, Gubbins (47), and RAxML (48) (GTRCAT model) within the Snpiphy (v0.5.0) pipeline with the default 85% coverage cutoff.

To complement this analysis, a genome estimation of all NCBI genomes ($n = 8663$) compared to the study strains was performed using Mash (v2.0), and isolates with a similarity of >950/1,000 shared hashes were additionally incorporated into this analysis.

Data availability. All genome sequences were submitted to the NCBI database with accession numbers VSLB00000000, VSLC00000000, WMCH00000000, VINI00000000, JAAGUA000000000, VSJI00000000 (Table 2).

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