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Co-existence of plasmid-mediated *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ in *Escherichia coli* sequence type 167 and ST101 and their discrimination through restriction digestion

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ABSTRACT The concurrent presence of multiple New Delhi metallo-β-lactamase (*bla*$_{NDM}$) variants within an isolate often goes undetected without next-generation sequencing. This study detects and characterizes dual *bla*$_{NDM}$ variants in *Escherichia coli* through Sanger and whole-genome sequencing. Additionally, a rapid identification method utilizing restriction digestion was designed for detecting *bla*$_{NDM}$ variants carrying M154L mutation. Antibiotic susceptibility, minimal inhibitory concentration for meropenem and ertapenem, PCR, and Sanger sequencing of *bla*$_{NDM}$ along with genome sequencing using Illumina and Nanopore technology were conducted. Transmissibility and replicon types of *bla*$_{NDM}$-harboring plasmids were evaluated. Restriction digestion using restriction enzyme, BtsCI was developed to distinguish between *bla*$_{NDM-1}$ and *bla*$_{NDM}$ variants possessing M154L mutation, such as *bla*$_{NDM-5}$, *bla*$_{NDM-7}$ etc. Two isolates belonging to phylogroups A; ST167 and B1; ST101 and resistant to meropenem and ertapenem (≥16 mg/L) were recovered from the blood of a neonate and the rectal swab of a pregnant woman, respectively. *bla*$_{NDM}$ was detected by PCR, and Sanger sequences of *bla*$_{NDM}$ showed two peaks at 262 (G and T) and 460 (A and C) nucleotide positions indicative of more than one *bla*$_{NDM}$ variant. Hybrid assembly confirmed co-existence of *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ in each isolate. *bla*$_{NDM-1}$ was located on IncY (ST167) and IncHI1A/HI1B (ST101), while *bla*$_{NDM-5}$ was on IncFIA/FII (ST167) and IncC (ST101) plasmids in the two isolates. Digestion with BtsC1 could discriminate between *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$. The co-existence of multiple *bla*$_{NDMs}$, *bla*$_{NDM-1}$, and *bla*$_{NDM-5}$ in epidemic clones of *E. coli* is concerning. Restriction digestion method and Sanger sequencing can facilitate quick identification of dual *bla*$_{NDM}$ variants in a single isolate.

IMPORTANCE The global dissemination of antimicrobial resistance genes is a serious concern. One such gene, *bla*$_{NDM}$, has spread globally via plasmids. *bla*$_{NDM}$ confers resistance against all β-lactam antibiotics, except monobactams. Most of the earlier literature reported the presence of single *bla*$_{NDM}$ variant. However, this study reports the prevalence of dual *bla*$_{NDM}$ variants (*bla*$_{NDM-1}$ and *bla*$_{NDM-5}$) located on two separate plasmids identified in two distinct *Escherichia coli* epidemic clones ST167 and ST101 isolated from a septicemic neonate and a pregnant mother, respectively. *bla*$_{NDM-5}$ differs from *bla*$_{NDM-1}$ due to the presence of two point mutations (i.e., V88L and M154L). This study detected dual *bla*$_{NDM}$ variants through Sanger sequences and further validated them through hybrid-genome assembly. Detection of multiple *bla*$_{NDM}$ variants in a single isolate remains difficult until genome sequencing or southern blotting is carried out. Hence, a simple restriction digestion method was devised to rapidly screen dual *bla*$_{NDM}$ variants containing M154L mutation.

KEYWORDS neonatal sepsis, epidemic clone, dual *bla*$_{NDM}$ variants, *bla*$_{NDM-1,-5}$, nanopore, India

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New Delhi metallo-β-lactamase (*bla*$_{NDM}$) is the fastest and most widespread carbapenemase that has triggered an alarming threat worldwide since its identification in 2009 (1, 2). Around 71 variants of *bla*$_{NDM}$ have been reported worldwide, of which *bla*$_{NDM-1}$, *bla*$_{NDM-5}$, and *bla*$_{NDM-7}$ are prevalent (3). NDM confers resistance against all β-lactam antibiotics, including carbapenems, the drug of last resort (4, 5).

Most studies report the presence of a single *bla*$_{NDM}$ variant in a bacterial genome with few exceptions where two copies of *bla*$_{NDM}$-variants (*bla*$_{NDM-1/NDM-5}$) were present either in chromosome or plasmids in a single bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella michiganensis*, and *Acinetobacter johnsonii* (Table S1) (6–12). *E. coli*, being a commensal and an opportunistic pathogen, acts as a reservoir of acquired antimicrobial resistance determinants eventually transferring it to other species (13).

In this study, we identified two *E. coli* isolates from blood (neonate) and rectal swab (adult) possessing two different variants of *bla*$_{NDM}$. To the best of our knowledge, this is the first report where carriage of bi-variant *bla*$_{NDM}$ (*bla*$_{NDM-1}$ and *bla*$_{NDM-5}$) in *E. coli* is being reported along with their characterization and genome analysis.

Two clinical *E. coli* isolates EN5349 and IN-MR210EC, were recovered from the blood of a septicemic neonate and the rectal swab of a hospitalized pregnant mother [as part of a collaborative study, called Burden of antibiotic resistance in neonates from developing societies (BARNARDS)], respectively. Isolates were assessed for antibiotic susceptibility by disk diffusion assay, and minimal inhibitory concentrations (MICs) for meropenem, ertapenem, and colistin (Sigma-Aldrich, Steinheim, Germany) were determined by the broth-micro dilution (14). PCR amplicons of *bla*$_{NDM}$ were sequenced using primer pairs (Table S2) in an Applied Biosystems DNA analyzer (Perkin Elmer, USA) (15). Both short (Illumina NextSeq 500 platform, San Diego, CA) and long read-based (Oxford Nanopore, UK) sequencing technologies were used for genome sequencing. Unicycler was used to generate hybrid assemblies, which were further used for downstream analysis (Supplementary methods) (16). From hybrid assemblies, *bla*$_{NDM}$ harboring plasmids were constructed and searched in the bacterial plasmid database (PLSDB) for similar complete plasmid sequences (17) . Plasmids showing nucleotide identity (≥99%) and same replicon types with similar *bla*$_{NDM}$ variants with reference to study plasmids were compared.

Transfer of *bla*$_{NDM}$ into *E. coli* J53 Az^{r} strain (100 mg/L) was attempted by solid-mating conjugation assay and electro-transformation into *E. coli* DH10B cells (Invitrogen, CA, USA) (Supplementary methods) (14, 15). Plasmid replicon types were determined by PCR-based replicon typing (PBRT, Diatheva, Italy) for wild type isolates (WTs) and transformants (TFs) (18, 19). PCR amplicons of *bla*$_{NDM}$ gene were digested for 4 h using the BtsCI enzyme (New England Biolabs, USA) at 50°C.

Isolates belonged to phylogroups A; ST167 (EN5349) and B1; ST101 (IN-MR210EC), which are epidemic clones conferring resistance to carbapenems and other antibiotics while remaining susceptible to tigecycline and colistin (20). Presence of *bla*$_{NDM}$ in isolates was confirmed by Sanger sequencing. Chromatogram of sequences (forward and reverse) for both isolates depicted the presence of two sharp peaks at 262 (G and T) and 460 (A and C) positions. Two different base calls at a single position suggested amplification of more than one *bla*$_{NDM}$ in a single isolate (Fig. S1). G at position 262 and A at 460 corresponded to sequence of *bla*$_{NDM-1}$, whereas G262→T (V88L) and A460→C (M154L) matched with *bla*$_{NDM-5}$ (21). Meticulous evaluation of chromatogram helped the detection of two distinct *bla*$_{NDM}$ copies.

Genome sequencing confirmed that isolates carried two copies of *bla*$_{NDM}$ (*bla*$_{NDM-1}$ and *bla*$_{NDM-5}$), along with two copies of *bla*$_{TEM-1B}$, which may increase enzyme production and enhance the chance of spread through various plasmids (15, 22). Both isolates EN5349 and IN-MR210EC exhibited a complex resistance profile, harboring multiple resistance determinants. These included genes conferring resistance to β-lactams (*bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{CMY-42}$, *bla*$_{OXA-2}$, *bla*$_{OXA-9}$, *bla*$_{CMY-6}$), aminoglycosides [*rmtB*, *rmtF*, *armA*, *aadA1*, *aadA2*, *aac(6')-Ib*, *aac(6')-Ib-cr*, *aph(6)-Id*, *aph(3'')-Ib*, *aac(3)-IIa*], sulfonamides (*sul1*, *sul2*), trimethoprim (*dfrA12*, *dfrA29*), phenicols (*catA1*), fluoroquinolones (*qnrS1*), and efflux pumps [*qacE*, *msr(E)*, *mph(E)*]

TABLE 1 Genome-based characterization of EN5349 and IN-MR210EC along with characterization of transformants (TFs) in terms of resistance determinants and plasmids[a]

Isolate/Accession number	EN5349/JAPTGK000000000	IN-MR210/JAYKKU000000000
Date of isolation	May, 2017	August, 2017
Source	Blood	Rectal swab
Phylogroup/Sequence types	A/ST167	B1/ST101
Virulence determinants		
Adherence	*ecpABCDER, elfACDG, eaeH, etpA, hcpABC, fimDFG, pilW*	*cfaABCDE, ecpABCDER, elfACDG, eaeH, hcpABC, fimABCDEFGHI, flgC, mrkABD*
Autotransporter	*cah, ehaB*	*ehaAB, upaG/ehaG*
Invasion	*ibeBC, tia*	*ibeBC, tia*
Iron uptake	*sitAD, fyuA, irp1, irp2, ybtAEPQSTUX, hemB*	*fyuA, irp1, irp2, ybtAEPQSTUX*
Toxin	*hlyE/clyA*	*hlyE/clyA*
Serum resistance	*iss, traT*	*iss*
Others	*Wzi, stjC, gale, mntB*	*lpfBCE*
Serotype/C-H type	O101:H9/11–0	O131:H31/41–191
Antibiotic susceptibility	Piperacillin^{R}, Cefoxitin^{R}, Cefotaxime^{R}, Ciprofloxacin^{R}, Trimethoprim-sulfamethoxazole^{R}, Aztreonam^{R}, Amikacin^{R}, Gentamicin^{R}, Meropenem^{R}, Colistin^{S},Tigecycline^{S}	Piperacillin^{R}, Cefoxitin^{R}, Cefotaxime^{R}, Ciprofloxacin^{R}, Trimethoprim-sulfamethoxazole^{R}, Aztreonam^{R}, Amikacin^{R}, Gentamicin^{R}, Meropenem^{R}, Colistin^{S}, Tigecycline^{S}
Minimum inhibitory concentration (MIC)		
Meropenem	128 mg/L	16 mg/L
Ertapenem	64 mg/L	64 mg/L
Size of genome	5253 kb	5417 kb
	Chromosome 5014 kb	Chromosome 4820 kb
	Extra-chromosomal element 315 kb	Extra-chromosomal element 601 kb
GC content (%)	50.80%	50.40%
Core genome sequence type (cgST)	cgST169598	cgST28992
Resistance determinants		
(detected through PCR and WGS)		
Wild type	*bla$_{NDM-1}$, bla$_{NDM-5}$, bla$_{TEM-1B}$, bla$_{TEM-1B}$, bla$_{CTX-M-15}$, bla$_{CMY-42}$, rmtB, rmtF, aadA2, sul1, sul2, dfrA12, ARR-2, qacE, tet(A), mph(A), qnrS1, aac(6')-Ib-cr, aph(6)-Id, aph(3'')-Ib*	*bla$_{NDM-1}$, bla$_{NDM-5}$, bla$_{TEM-1A}$, bla$_{TEM-1A}$, bla$_{TEM-1B}$, bla$_{TEM-1B}$, bla$_{OXA-2}$, bla$_{OXA-9}$, bla$_{CMY-6}$, armA, rmtB, aadA1, aadA2, sul1, sul1, sul1, dfrA12, dfrA29, qacE, qacE, qacE, msr(E), mph(E), catA1, aac(6')-Ib, aac(6')-Ib-cr, aph(6)-Id, aph(3'')-Ib, aac(3)-IIa*
Resistance determinants (detected through PCR)		
TF1 (*bla$_{NDM-1}$$^{+ve}$*)	*bla$_{NDM-1}$,bla$_{TEM}$, bla$_{CTX-M}$,bla$_{CMY}$, qnrS, aac-(6')-Ib-cr*	*bla$_{NDM-1}$,bla$_{TEM}$, aac(6')-Ib*
TF2 (*bla$_{NDM-5}$$^{+ve}$*)	*bla$_{NDM-5}$, bla$_{TEM}$, bla$_{CMY}$, rmtB*	*bla$_{TEM}$,rmtB*
TF3 (*bla$_{NDM-1}$$^{+ve}$*, *bla$_{NDM-5}$$^{+ve}$*)	*bla$_{NDM-1}$,bla$_{NDM-5}$, bla$_{TEM}$, bla$_{CTX-M}$, bla$_{CMY}$,rmtB, qnrS, aac-(6')-Ib-cr*	NF
Plasmid replicon types (PBRT and WGS)		
Wild type	ColRNAI, IncFIA, IncFII, IncIγ, IncY	IncC, IncHI1A, IncHI1B
TF1 (*bla$_{NDM-1}$$^{+ve}$*)	IncY	IncC
TF2 (*bla$_{NDM-5}$ $^{+ve}$*)	IncFII	IncHI1A, IncHI1B

(*Continued on next page*)

TABLE 1 Genome-based characterization of EN5349 and IN-MR210EC along with characterization of transformants (TFs) in terms of resistance determinants and plasmids[a] (*Continued*)

Isolate/Accession number	EN5349/JAPTGK000000000	IN-MR210/JAYKKU000000000
TF3 (bla_{NDM-1}^{+ve}, bla_{NDM-5}^{+ve})	IncFII, IncY	NF
MIC of Meropenem		
TF1 (bla_{NDM-1}^{+ve})	64 mg/L	8 mg/L
TF2 (bla_{NDM-5}^{+ve})	16 mg/L	8 mg/L
TF3 (bla_{NDM-1}^{+ve}, bla_{NDM-5}^{+ve})	32 mg/L	NF
MIC of Ertapenem		
TF1 (bla_{NDM-1}^{+ve})	64 mg/L	32 mg/L
TF2 (bla_{NDM-5}^{+ve})	64 mg/L	32 mg/L
TF3 (bla_{NDM-1}^{+ve}, bla_{NDM-5}^{+ve})	32 mg/L	NF
gyrA-parC mutations	*gyrA:p.S83L, gyrA:p.D87N, parC:p.S80I, parE:p.S458A*	*gyrA:p.S83L, gyrA:p.D87N, parC:p.S80I, parE:p.E460D*
Integrons	In27, In406	In27, In573
Characterization of bla_{NDM-1}-carrying plasmid		
Plasmid Id	P1-EN5349	P1-IN-MR210EC
Size	122 kb	265 kb
Addiction system	relBE	relBE
Replicon type	IncY	IncHI1A, IncHI1B
Carriage of additional resistance determinants	bla_{TEM-1B}, $bla_{CTXM-15}$, *rmtF, sul2, ARR-2, qnrS1, aac-(6′)-Ib-cr, aph(6)-Id, aph(3″)Ib*	bla_{TEM-1A}, bla_{OXA-9}, Δbla_{DHA-1}, *armA, aadA, sul1, qacE, msr(E), mph(E), aac(6')-Ib, aac(6')-Ib-cr*
Genetic Environment	IS*3000*→ΔIS*Aba125*→ bla_{NDM-1}→ ble_{MBL}→ *trpF*→ *dsbD*→ *groES*→*groEL*→IS*3000*	IS*26*→ ΔIS*Aba125*→ bla_{NDM-1}→ ble_{MBL}→ Δbla_{DHA-1} → *ampR*→ *sul1*→ IS*CR1*→ IS*5*
Characterization of bla_{NDM-5}-carrying plasmid		
Plasmid Id	P2-EN5349	P2-IN-MR210EC
Size	125 kb	137 kb
Addiction system	*pemKI*	NF
Replicon type	FIA/FII (F36:A4:B-)	IncC
Carriage of additional resistance determinants	bla_{TEM-1B}, *rmtB, aadA2, sul1, dfrA12, qacE, tetA, mph(A)*	bla_{TEM-1B}, bla_{CMY-6},*rmtB, aadA2, sul1, dfrA12, qacE*
Genetic Environment	IS*26*→ΔIS*Aba125*→bla_{NDM-5} → ble_{MBL}→ *trpF*→ *dsbD*→ IS*CR*→*sul1*→*aadA*→ *DuF1010*→ *dfrA12* → *IntI*→IS*26*	IS*26*→ΔIS*Aba125*→bla_{NDM-5} → ble_{MBL} → *trpF* → *dsbD* → IS*CR1*→*sul1*→*aadA2*→*DuF1010*→*dfrA12*→*IntI1*→Tn*As2*

[a]R, resistant; S, susceptible; TF, transformant; Inc, incompatibility; IS, insertion sequence; Int, integrase; NF, not found.

(Table 1). Additionally, IN-MR210EC carried multiple copies of *bla*$_{TEM-1A}$, *sul1*, and *qacE* (Table 1). Presence of multiple resistance determinants indicated that several antibiotics would be ineffective against these isolates, implying a potential risk of treatment failure with the targeted antibiotics. The isolates exhibited susceptibility to tigecycline and colistin. However, the clinical utility of these two antibiotics is often limited due to potential adverse effects, such as nephrotoxicity with colistin and gastrointestinal disturbances with tigecycline (23, 24).

Hybrid genome assembly revealed that *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ were harbored in two distinct plasmids, *viz*. *bla*$_{NDM-1}$ in IncY [P1-EN5349; 122 kb] and IncHI1A/HI1B [P1-IN-MR210EC; 265 kb], whereas *bla*$_{NDM-5}$ were in IncFIA/FII [P2-EN5349; 125 kb] and IncC [P2-IN-MR210EC; 137 kb] (Fig. 1). Earlier studies have reported the occurrence of double copies of same *bla*$_{NDM}$ variant, either located in the chromosome or in plasmids, among different organisms (Table S1) (6–12). This study reports different *bla*$_{NDM}$ variants in a single *E. coli* isolate.

The genetic environment of *bla*$_{NDM-1}$ featured a truncated IS*Aba125* upstream, along with Tn*3*-like IS*3000* and IS*26*-like family transposase located further upstream of IS*Aba125* in EN5349 and IN-MR210EC, respectively. The downstream regions of *bla*$_{NDM-1}$ varied (Table 1). Moreover, for *bla*$_{NDM-5}$, both isolates possessed an IS*26*-like family transposase and ΔIS*Aba125* upstream, with *ble*$_{MBL}$ downstream, followed by *trpF*, *dsbD*, and IS*91*-like IS*CR1* family transposase (Table 1). The genetic environment of *bla*$_{NDM-5}$ in study plasmids, P2-EN5349 and P2-IN-MR210EC showed >95% similarity (Fig. S2). Genetic environments of the study *bla*$_{NDM}$ variants were individually comparable with the genetic backgrounds of the respective *bla*$_{NDM}$ variants reported worldwide (4, 15, 25).

Conjugation of study plasmids was unsuccessful, and transformants obtained by electroporation carried either *bla*$_{NDM-1}$ or *bla*$_{NDM-5}$. Few TFs of EN5349 (EN5349.TF3) co-harbored *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$ as confirmed by Sanger sequencing. WT and TFs exhibited high MIC values for meropenem and ertapenem (≥16 mg/L) (Table 1). *bla*$_{NDM}$ has been known to be promiscuous in nature, and it is easily transmitted between organisms (4, 26). Study plasmids, such as P2-EN5349, P1-IN-MR210EC, and P2-IN-MR210EC, possessed plasmid transfer/mobilization factors (*tra*-operon system, *mob*I), and P1-EN5349 possessed conjugation/type IV secretion system (T4SS) (*virB*, *virB9*, *virB11*, *virB6*, *virB4*, *Rhs*) (Fig. 1) (27, 28) analyzed through genome sequences. Although conjugation was unsuccessful under laboratory conditions, the presence of such genes in plasmids still suggests the potential for *bla*$_{NDM}$ transmission in natural environments. Such transmission may occur not only in the hospital environment but also in other environments. Multiple *bla*$_{NDM}$ variants in *E. coli* epidemic clones increase the potential for transmission, along with transfer of other resistance genes present in the same plasmid (14, 20).

Study plasmids showed close resemblance with some globally reported plasmids harboring similar *bla*$_{NDM}$ variants and same replicon types in different species of Enterobacterales. P2-EN5349 (pMLST F36:A4:B-) showed similarity with 11 globally reported IncFIA/FII plasmids harboring the *bla*$_{NDM-5}$ gene. These plasmids were identified in diverse *E. coli* isolates obtained from clinical specimens (rectal swabs, urine, faecal swabs, and mastitis milk) across various countries, including Canada, the Czech Republic, Switzerland, Italy, Myanmar, Bangladesh, China, Thailand, and India (Fig. 2; Table S3) (17). In contrast, P2-IN-MR210EC (*bla*$_{NDM-5}$ in IncC plasmid) exhibited similarity only to a plasmid from environmental *E. coli* found in Switzerland. These findings suggest a more widespread dissemination of *bla*$_{NDM-5}$ via IncFIA/FII plasmids compared to IncC. There were very few reports of *bla*$_{NDM-1}$ transmission via IncY and IncHI1A/HI1B plasmids. Notably, P1-IN-MR210EC (*bla*$_{NDM-1}$ in IncHI1A/HI1B) shared similarities with five plasmids found in clinical *K. pneumoniae* isolates from India, Thailand, and New Zealand. (Fig. 2; Table S3) (17).

With the emergence of organisms carrying two different variants of *bla*$_{NDM}$ in different plasmids, spread of such variants and their stability in antibiotic-loaded

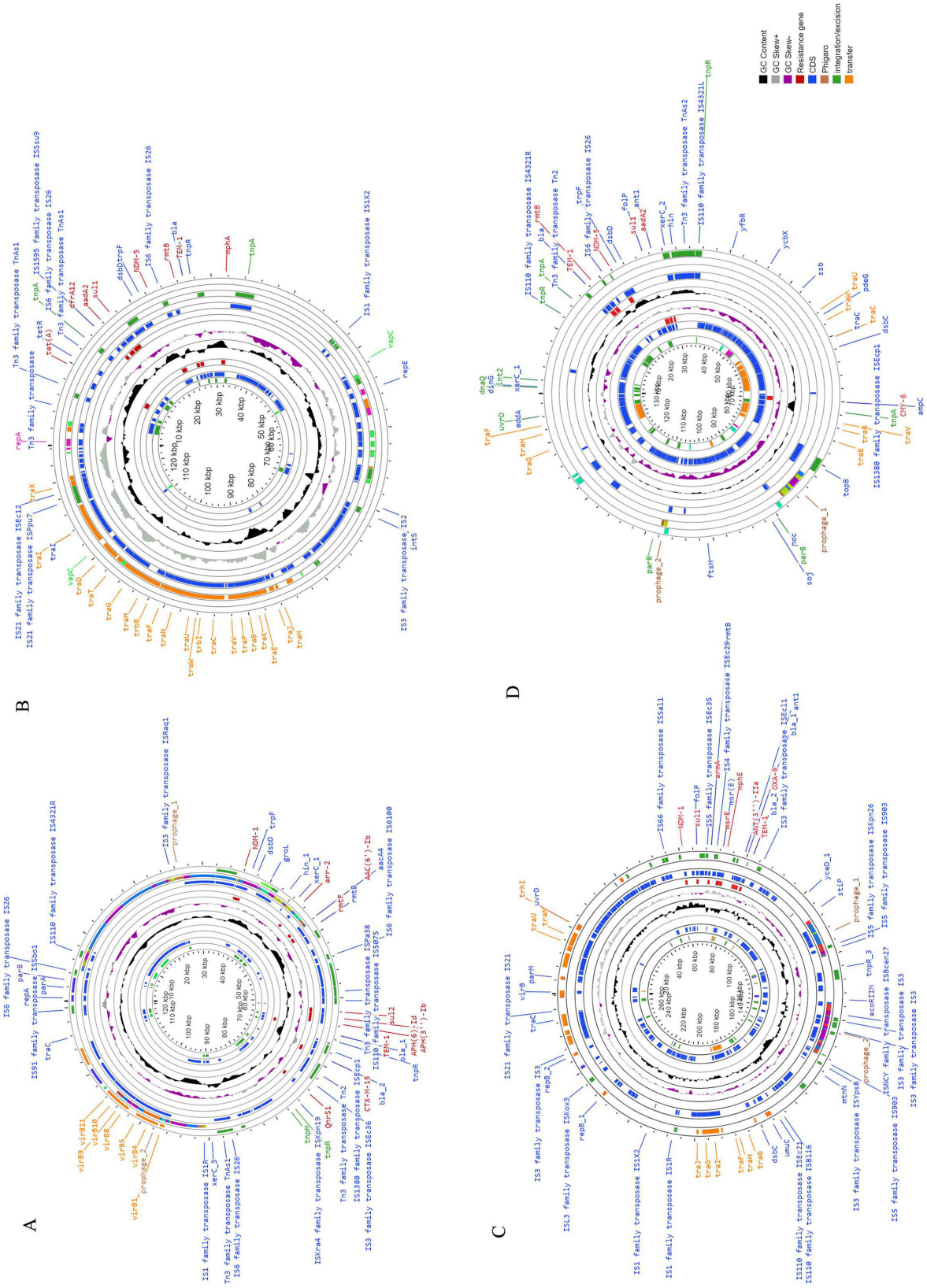

FIG 1 Circular map of the *bla*$_{NDM-1}$ and *bla*$_{NDM-5}$-harbouring plasmids in different *Escherichia coli,* EN5349 (ST167) and IN-MR210EC (ST101). Circular graphs representing the study plasmids, such as IncY [P1-EN5349; 122-kb] with *bla*$_{NDM-1}$ (A), IncFIA/FII [P2-EN5349; 125-kb] with *bla*$_{NDM-5}$ (B), IncHI1A/HI1B [P1-IN-MR210EC; 265-kb] with *bla*$_{NDM-1}$ (C) and IncC [P2-IN-MR210EC; 137-kb] with *bla*$_{NDM-5}$ (D). The inner circle is depicted as GC content in black and the outer circle

(Continued on next page)

Fig 1 (Continued)

represents GC skew in grey and purple. Resistance gene is denoted in red, coding sequences (CDS) in blue, prophage regions (phigaro) in brown, genes involved in transfer is in orange and integration/excision is in green.

environment calls for timely identification of such strains. Dual bla_{NDM} variants in a single isolate may be confirmed by southern blotting and hybrid assembly, which are expensive, time-consuming, and difficult to execute for a diagnostic laboratory. Hence, a novel restriction digestion-based method was introduced to distinguish between bla_{NDM} variants (NDM-4, 5, 7, 8, 12, 13, 15, 16b, 17, 19, 20, 21, 27, 35, 36, 37) with or without M154L mutation. bla_{NDM-1} has two recognition sites for BtsCI (Fig. S3), which generate three DNA fragments (255, 215, and 355 bp). Presence of a mutation (A460→C) corresponding to M154L alters the second recognition site (GGATG→GGCTG), which BtsCI is unable to cleave, resulting in two fragments of 255 and 570 bp (Fig. S3). Hence, most M154L possessing variants (including bla_{NDM-5}) will produce fragments different from the bla_{NDM-1} variant. Since study isolates possessed both bla_{NDM-1} and bla_{NDM-5}, four DNA fragments, that is, 215, 255, 355, and 570 bp (Fig. S3), were generated, which confirmed the presence of bla_{NDM-1} along with a variant possessing M154L mutation (4). Identification of resistance genes in diagnostic laboratories, especially in low- and middle-income countries (LMICs), still remains limited due to the lack of access to PCR-based molecular techniques. Diagnostic laboratories rely on conventional disk diffusion tests or MIC values generated through automated systems, which further guide their treatment decisions. While whole-genome sequencing (WGS) offers a comprehensive approach for identifying resistance determinants, its high cost and lack of expertise in data analysis limit its widespread adoption in LMIC settings.

FIG 2 Worldwide prevalence of plasmids similar to study plasmids. Globally reported plasmids similar (≥99% nucleotide identity) to study plasmids are denoted in four different colours such as yellow (IncY with bla_{NDM-1}), red (IncFIA/FII with bla_{NDM-5}), green (IncHI1A/HI1B with bla_{NDM-1}) and sky blue (IncC with bla_{NDM-5}). The map was obtained from freeworldmaps.net.

Our study adds to the very few studies that reported more than one variant of bla_{NDM} in a single isolate. The presence of multiple bla_{NDM} variants in *E. coli* epidemic clones collected from a septicemic neonate and a pregnant mother (not paired) is worrisome. Emergence and spread of such organisms are of immense public health consequence. Two different variants of bla_{NDM} in different plasmids and their stability in antibiotic-loaded environment calls for timely identification.

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S.B. contributed to the conception of the work, analysis, interpretation of data, and drafting of the manuscript. A.B. performed all experimental work and contributed to the acquisition of laboratory and W.G.S. data with manuscript drafting. P.B. and J.S. performed the W.G.S. data analysis with manuscript editing. S.M. contributed to the analysis of data. S.D. contributed to the review of data. The final manuscript was read and approved by all authors.

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DATA AVAILABILITY

Genome data were submitted to the NCBI database with accession numbers JAPTGK000000000 and JAYKKU000000000 (Table 1).

ETHICAL APPROVAL

This study was conducted on archived strains; hence, ethical approval was not required.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (Spectrum00987-24-s0001.docx). Supplemental methods, Tables S1 to S3, and Figs. S1 to S3.

REFERENCES

1. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, et al. 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10:597–602. https://doi.org/10.1016/S1473-3099(10)70143-2
2. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. 2009. Characterization of a new metallo-β-lactamase gene, *bla*$_{NDM-1}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 53:5046–5054. https://doi.org/10.1128/AAC.00774-09
3. Paul D, Babenko D, Toleman MA. 2020. Human carriage of cefotaxime-resistant *Escherichia coli* in North-East India: an analysis of STs and associated resistance mechanisms. J Antimicrob Chemother 75:72–76. https://doi.org/10.1093/jac/dkz416
4. Khan AU, Maryam L, Zarrilli R. 2017. Structure, genetics and worldwide spread of New Delhi metallo-β-lactamase (NDM): a threat to public health. BMC Microbiol 17:101. https://doi.org/10.1186/s12866-017-1012-8
5. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. 2011. Carbapenems: past, present, and future. Antimicrob Agents Chemother 55:4943–4960. https://doi.org/10.1128/AAC.00296-11
6. Jiang B, Ji X, Lyu Z-Q, Liang B, Li J-H, Zhu L-W, Guo X-J, Liu J, Sun Y, Liu Y-J. 2022. Detection of two copies of a *bla*$_{NDM-1}$-encoding plasmid in *Escherichia coli* isolates from a pediatric patient with diarrhea. Infect Drug Resist 15:223–232. https://doi.org/10.2147/IDR.S346111
7. Shen P, Yi M, Fu Y, Ruan Z, Du X, Yu Y, Xie X. 2017. Detection of an *Escherichia coli* sequence type 167 strain with two tandem copies of *bla*$_{NDM-1}$ in the chromosome. J Clin Microbiol 55:199–205. https://doi.org/10.1128/JCM.01581-16
8. Feng Y, Liu L, McNally A, Zong Z. 2018. Coexistence of two *bla*$_{NDM-5}$ genes on an IncF plasmid as revealed by nanopore sequencing. Antimicrob Agents Chemother 62. https://doi.org/10.1128/AAC.00110-18
9. Jovcić B, Lepsanović Z, Begović J, Rakonjac B, Perovanović J, Topisirović L, Kojić M. 2013. The clinical isolate *Pseudomonas aeruginosa* MMA83 carries two copies of the *bla*$_{NDM-1}$ gene in a novel genetic context. Antimicrob Agents Chemother 57:3405–3407. https://doi.org/10.1128/AAC.02312-12
10. Tang L, Shen W, Zhang Z, Zhang J, Wang G, Xiang L, She J, Hu X, Zou G, Zhu B, Zhou Y. 2020. Whole-genome analysis of two copies of *bla*$_{NDM-1}$ gene carrying *Acinetobacter johnsonii* strain Acsw19 isolated from Sichuan, China. Infect Drug Resist 13:855–865. https://doi.org/10.2147/IDR.S236200
11. Zheng B, Xu H, Yu X, Lv T, Jiang X, Cheng H, Zhang J, Chen Y, Huang C, Xiao Y. 2018. Identification and genomic characterization of a KPC-2-, NDM-1- and NDM-5-producing *Klebsiella michiganensis* isolate. J Antimicrob Chemother 73:536–538. https://doi.org/10.1093/jac/dkx415
12. Yang L, Lin Y, Lu L, Xue M, Ma H, Guo X, Wang K, Li P, Du X, Qi K, Li P, Song H. 2020. Coexistence of two *bla*$_{NDM-5}$ genes carried on IncX3 and IncFII plasmids in an *Escherichia coli* isolate revealed by Illumina and Nanopore sequencing. Front Microbiol 16. https://doi.org/10.3389/fmicb.2020.00195
13. Basu S. 2020. Variants of the New Delhi metallo-β-lactamase: new kids on the block. Future Microbiol 15:465–467. https://doi.org/10.2217/fmb-2020-0035
14. Bhattacharjee A, Sands K, Mitra S, Basu R, Saha B, Clermont O, Dutta S, Basu S. 2023. A decade-long evaluation of neonatal septicaemic *Escherichia coli*: clonal lineages, genomes, and New Delhi metallo-β-lactamase variants. Microbiol Spectr 11:e0521522. https://doi.org/10.1128/spectrum.05215-22
15. Datta S, Mitra S, Chattopadhyay P, Som T, Mukherjee S, Basu S. 2017. Spread and exchange of *bla* $_{NDM-1}$ in hospitalized neonates: role of mobilizable genetic elements. Eur J Clin Microbiol Infect Dis 36:255–265. https://doi.org/10.1007/s10096-016-2794-6
16. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLOS Comput Biol 13:e1005595. https://doi.org/10.1371/journal.pcbi.1005595
17. Schmartz GP, Hartung A, Hirsch P, Kern F, Fehlmann T, Müller R, Keller A. 2022. PLSDB: advancing a comprehensive database of bacterial plasmids. Nucleic Acids Res 50:D273–D278. https://doi.org/10.1093/nar/gkab1111
18. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods 63:219–228. https://doi.org/10.1016/j.mimet.2005.03.018
19. Villa L, García-Fernández A, Fortini D, Carattoli A. 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. J Antimicrob Chemother 65:2518–2529. https://doi.org/10.1093/jac/dkq347
20. Cummins EA, Snaith AE, McNally A, Hall RJ. 2021. The role of potentiating mutations in the evolution of pandemic *Escherichia coli* clones. Eur J Clin Microbiol Infect Dis. https://doi.org/10.1007/s10096-021-04359-3
21. Nakano R, Nakano A, Hikosaka K, Kawakami S, Matsunaga N, Asahara M, Ishigaki S, Furukawa T, Suzuki M, Shibayama K, Ono Y. 2014. First report of metallo-β-lactamase NDM-5-producing *Escherichia coli* in Japan. Antimicrob Agents Chemother 58:7611–7612. https://doi.org/10.1128/AAC.04265-14
22. Maddamsetti R, Yao Y, Wang T, Gao J, Huang VT, Hamrick GS, Son H-I, You L. 2024. Duplicated antibiotic resistance genes reveal ongoing selection and horizontal gene transfer in bacteria. Nat Commun 15:1449. https://doi.org/10.1038/s41467-024-45638-9
23. Kilic I, Ayar Y, Ceylan İ, Kaya PK, Caliskan G. 2023. Nephrotoxicity caused by colistin use in ICU: a single centre experience. BMC Nephrol 24:302. https://doi.org/10.1186/s12882-023-03334-8
24. Greer ND. 2006. Tigecycline (Tygacil): the first in the glycylcycline class of antibiotics. Proc (Bayl Univ Med Cent) 19:155–161. https://doi.org/10.1080/08998280.2006.11928154
25. Mitra S, Mukherjee S, Naha S, Chattopadhyay P, Dutta S, Basu S. 2019. Evaluation of co-transfer of plasmid-mediated fluoroquinolone resistance genes and *bla*$_{NDM}$ gene in *Enterobacteriaceae* causing neonatal septicaemia. Antimicrob Resist Infect Control 8:46. https://doi.org/10.1186/s13756-019-0477-7
26. Wu W, Feng Y, Tang G, Qiao F, McNally A, Zong Z. 2019. NDM metallo-β-lactamases and their bacterial producers in health care settings. Clin Microbiol Rev 32:e00115-18. https://doi.org/10.1128/CMR.00115-18
27. Virolle C, Goldlust K, Djermoun S, Bigot S, Lesterlin C. 2020. Plasmid transfer by conjugation in gram-negative bacteria: from the cellular to the community level. Genes (Basel) 11:1239. https://doi.org/10.3390/genes11111239
28. Christie PJ. 2016. The mosaic type IV secretion systems. EcoSal Plus 7. https://doi.org/10.1128/ecosalplus.ESP-0020-2015