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Molecular Epidemiology of NDM-1-Producing *Enterobacteriaceae* and *Acinetobacter baumannii* Isolates from Pakistan

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The molecular epidemiology of 66 NDM-producing isolates from 2 Pakistani hospitals was investigated, with their genetic relatedness determined using repetitive sequence-based PCR (Rep-PCR). PCR-based replicon typing and screening for antibiotic resistance genes encoding carbapenemases, other β-lactamases, and 16S methylases were also performed. Rep-PCR suggested a clonal spread of *Enterobacter cloacae* and *Escherichia coli*. A number of plasmid replicon types were identified, with the incompatibility A/C group (IncA/C) being the most common (78%). 16S methylase-encoding genes were coharbored in 81% of NDM-producing *Enterobacteriaceae*.

With the worldwide spread of the NDM-1 gene and its variants (NDM-2 to NDM-8) (1, 2), molecular epidemiological studies of global isolates using various genotyping techniques are essential for gaining a better understanding of how this spread is occurring. India, Pakistan, and Bangladesh are clearly major reservoir countries for bla_{NDM}, with numerous factors, such as antibiotic selection pressure, contributing to this current situation (3, 4). This study examines a group of 66 NDM-1-producing isolates from Pakistan for their genetic relatedness, phylotype, plasmid replicon type, and plasmid transferability.

All isolates were acquired from stool samples from 37 distinct patients at two military hospitals in Rawalpindi, Pakistan (5). The samples were collected from inpatients (35%) and outpatients (65%). The isolates were tested for susceptibility to 17 antimicrobials using the Vitek 2 system. The MICs for meropenem, doripenem, fosfomycin, and amdinocillin were determined using a standard agar dilution methodology (5).

The isolates were reconfirmed for the presence of the carbapenem resistance gene bla_{NDM-1} by PCR, as previously described (6). PCR was also performed to detect bla_{OXA-48}, bla_{OXA-23}, bla_{VIM}, bla_{IMP}, bla_{KPC}, $bla_{CTX-M-15}$, bla_{SHV}, bla_{TEM}, bla_{OXA-1} group, AmpC β-lactamases, bla_{CMY-2}, and the 16S rRNA methylase genes *armA*, *rmtB*, *rmtC*, and *rmtF* (6–9). The phylogenetic groups of *Escherichia coli* were determined using a multiplex (PCR)-based method (10).

Repetitive sequenced-based PCR (Rep-PCR)-based typing by the DiversiLab system (bioMérieux, Oakleigh, Australia) was used for assessing clonal relatedness. A cluster of closely related isolates was defined as isolates sharing >95% similarity and indistinguishable isolates of >97% (11, 12). PCR-based replicon typing analysis (PBRT) was performed to determine the plasmid incompatibility (Inc) groups for all *Enterobacteriaceae* isolates (13).

Ten genetically diverse *E. coli* isolates, based on different Rep-PCR profiles and phylogroups, were selected for transformation studies and typing by multilocus sequence typing (MLST). MLST

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TABLE 1 Resistance genes and plasmid replicon types of NDM-1-producing isolates

NDM-producing isolates (no.)	No. (%) of antibiotic resistance genes								
	β-Lactamases[a]				16S rRNA methylases				
	bla_{CMY-2}	$bla_{CTX-M-15}$	bla_{SHV} bla_{TEM}	bla_{OXA-1}	*rmtB*	*rmtC*	*rmtF*	*armA*	Plasmid replicon type(s) (no.)
Escherichia coli (30)	26 (87)	16 (53)	0, 19 (63)	9 (30)	8 (27)	20 (67)	0	15 (50)	IncHI1 (10), IncI1 (2), IncL/M (2), IncN (4), IncFIA (5), IncFIB (6), IncY (2), IncA/C (25), IncFII (9)
Enterobacter cloacae (21)	16 (76)	21 (100)	0, 20 (95)	20 (95)	0	15 (71)	0	14 (67)	IncA/C (19), untypeable (2)
Citrobacter freundii (4)	3 (75)	2 (50)	0, 2 (50)	2 (50)	0	0	0	1 (14)	IncA/C (2), IncFII (1), untypeable (1)
Acinetobacter baumannii (3)	0	0	0	0	0	0	0	0	ND[b]
Klebsiella pneumoniae (3)	1 (33)	3 (100)	3 (100), 3 (100)	3 (100)		1 (33)	0	1 (33)	IncN (1), IncA/C (1), untypeable (1)
Pseudocitrobacter faecalis (2)	0	2 (100)	0, 2 (100)	2 (100)	0	0	0	0	IncN (2)
Providencia rettgeri (2)	2 (100)	0	0, 0	1 (50)	0	1 (50)	0	2 (100)	IncA/C (1), IncN (2), IncY (1)
Citrobacter braakii (1)	1 (100)	0	0, 0	0	0	1 (100)	0	0	IncA/C (1)
Total no. of isolates[c]	49 (78)	44 (70)	3 (5), 46 (73)	37 (59)	8 (13)	38 (60)	0	33 (52)	IncA/C (49)

[a] All isolates were negative for bla_{OXA-48}, bla_{IMP}, bla_{VIM}, and bla_{KPC}.
[b] ND, not determined.
[c] *A. baumannii* excluded from this total.

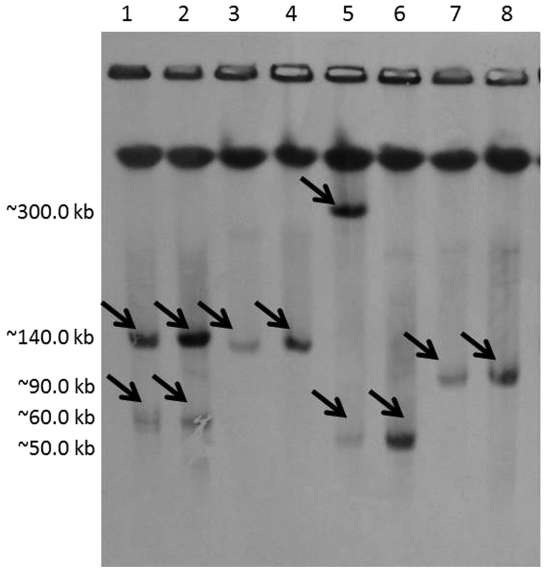

FIG 1 Southern blot hybridization of the PFGE gel with a specific bla_{NDM-1} probe. The black arrows indicate positive signals with the NDM-1 probe in each *E. coli* clinical isolate and its corresponding transformant. Lanes 1 to 8, PN1, PN1 TF1, PN7, PN7 TF1, PN14, PN14 TF1, PN18, and PN18 TF1, respectively.

included seven conserved housekeeping genes and was performed according to the *E. coli* MLST Database (see http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

The transferability of bla_{NDM-1}-carrying plasmids was investigated by electroporation. Plasmid DNA was prepared and electroporated into the recipient TOP10 *E. coli* (Invitrogen, Melbourne, Victoria, Australia), as previously described (6). Successful electrotransformants carrying bla_{NDM-1} were confirmed by PCR. The plasmid replicon type of the transformants acquiring bla_{NDM-1}-carrying plasmids was confirmed by PBRT. Plasmid size was determined by performing S1 endonuclease (Promega; Madison, WI, USA) restriction digestion using pulsed-field gel electrophoresis (PFGE) (14). PCR-amplified DNA probes of bla_{NDM-1} were labeled with digoxigenin nucleic acid (Roche, Mannheim, Germany).

Widespread dissemination of NDM-1 in Pakistan was first described in 2010 (15). In this study, we investigated the molecular epidemiology of a group of NDM-1-producing isolates from Pakistan, and we report here on the clonal relatedness of these isolates, providing an insight into the molecular characterization of bla_{NDM-1}-carrying plasmids.

The majority of the NDM-1-producing *Enterobacteriaceae* isolates coharbored an extended-spectrum β-lactamase (ESBL) gene,

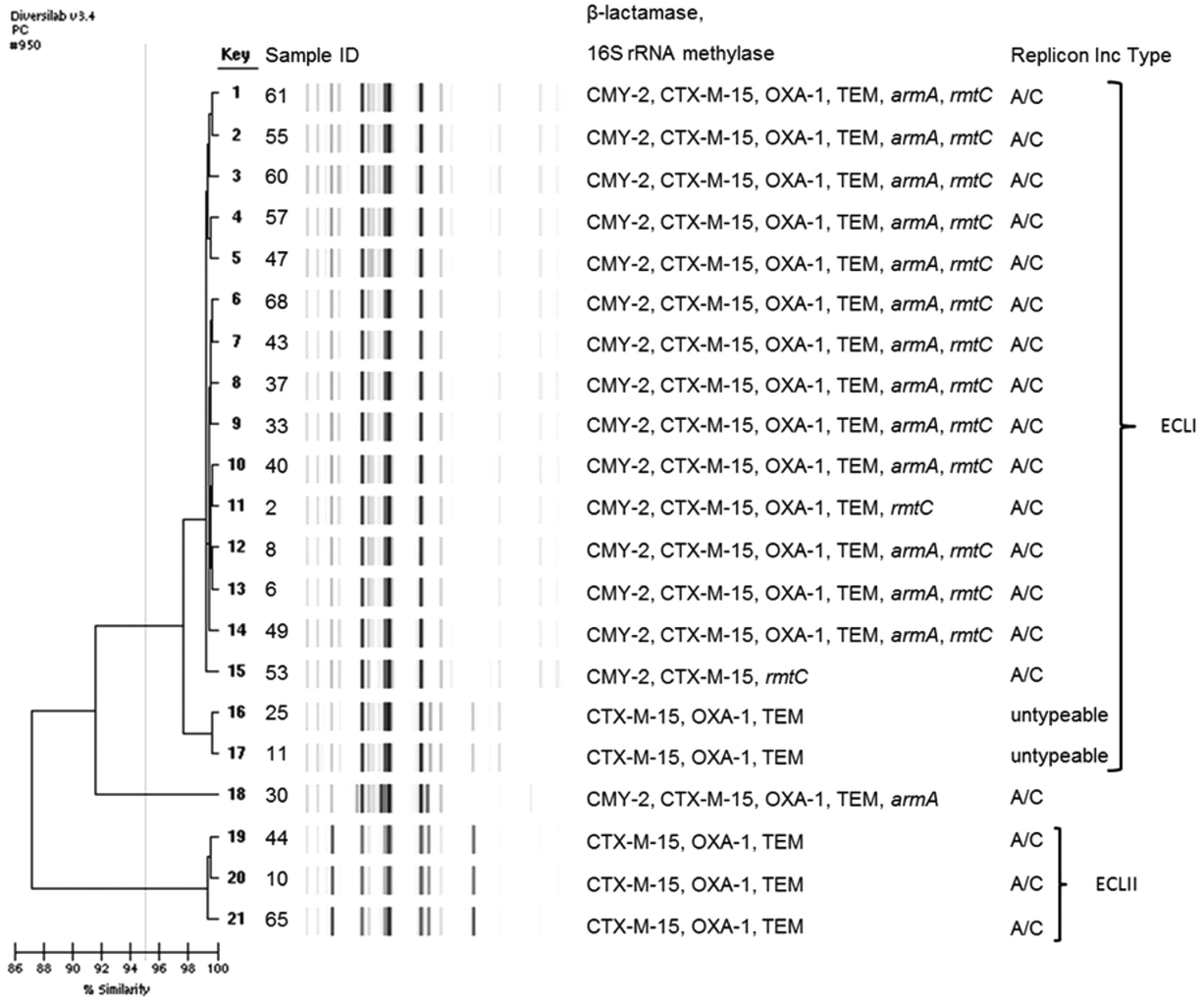

FIG 2 Dendrogram analysis of DiversiLab Rep-PCR fingerprint of NDM-1-producing *E. cloacae* isolates.

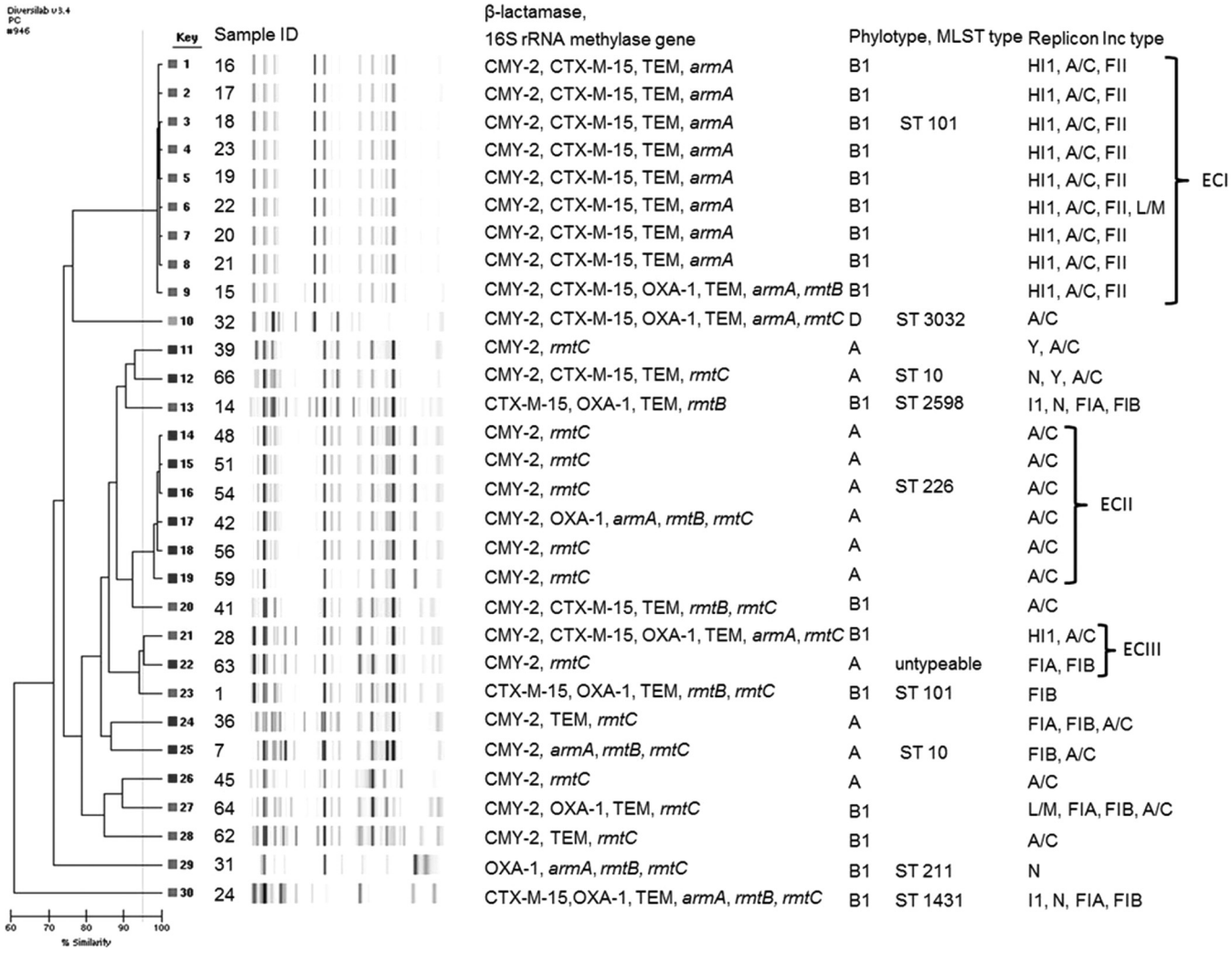

FIG 3 Dendrogram analysis of DiversiLab Rep-PCR fingerprint of NDM-1-producing *E. coli* isolates by phylotype.

$bla_{\text{CTX-M-15}}$ (70%). $bla_{\text{CMY-2}}$ and the cooccurrence of 16S rRNA methylase genes encoding broad-spectrum aminoglycoside resistance, *rmtB*, *rmtC*, or *armA*, was detected in 47 (75%) isolates (Table 1). AmpC β-lactamase production coexisting with an ESBL was also high ($n = 36$ [73%]). The novel 16S rRNA methylase *rmtF* (16) was not detected; however, *rmtB* was found in 8 *E. coli* strains. A strong association between NDM-producing isolates harboring a 16S rRNA methylase-encoding gene has been well documented, particularly with *rmtC*. More recent studies in India, the United Kingdom, South Africa, and Nepal have reported the carriage of *rmtF* among NDM-harboring isolates (9, 17–19). *rmtB* together with bla_{NDM} is a rare association, although a recent outbreak of *E. coli* isolates in Bulgaria, harboring both $bla_{\text{NDM-1}}$ and *rmtB* genes, raises new concerns for the acquisition of resistance determinants (20). Our results suggest that NDM-1-producing *Enterobacteriaceae* from Rawalpindi, Pakistan, have acquired a broad spectrum of singular and distinct resistance genes.

The plasmid incompatibility types HI1, I1, L/M, N, FIA, FIB, Y, A/C, and FII were identified among the NDM-1-producing isolates (Table 1). Plasmid replicon typing revealed the dominance of two incompatibility groups, IncA/C (78%) and IncF (33%), with IncA/C occurring in multiple NDM-carrying species. IncA/C plasmids carrying bla_{NDM} have been reported in Pakistan (21).

Of the 10 *E. coli* isolates subjected to electroporation, the $bla_{\text{NDM-1}}$ plasmids in 4 isolates were successfully electroporated. Plasmid replicon typing of these transformants confirmed that $bla_{\text{NDM-1}}$ resides on IncA/C-, IncN-, IncFIB-, and IncFII-type plasmids. These replicon types have been reported in *Enterobacteriaceae* in many regions of the world (4).

Southern hybridization (Fig. 1) of the *E. coli* donors and their transformants revealed $bla_{\text{NDM-1}}$ plasmid sizes ranging from ~50 kb to ~350 kb. The majority of the $bla_{\text{NDM-1}}$ plasmids were ~140 kb in size. Among the 30 *E. coli* isolates, there was a predominance of the phylogenetic group B1 (57%), followed by phylotypes A (40%) and D (3%). It has been suggested that the distribution of *E. coli* phylotypes may be geographically dependent (22). Mushtaq et al. (23) found a prevalence of phylotype B1 among NDM isolates in Pakistan and no phylotype B2. Our study shows similar results.

Rep-PCR revealed two dominant clones among *Enterobacter cloacae*, one large cluster ($n = 17$), designated ECLI, and one small cluster of three isolates (ECLII) (see Fig. 2). Three clonal types were observed among 17 *E. coli* isolates, and the remaining *E. coli*

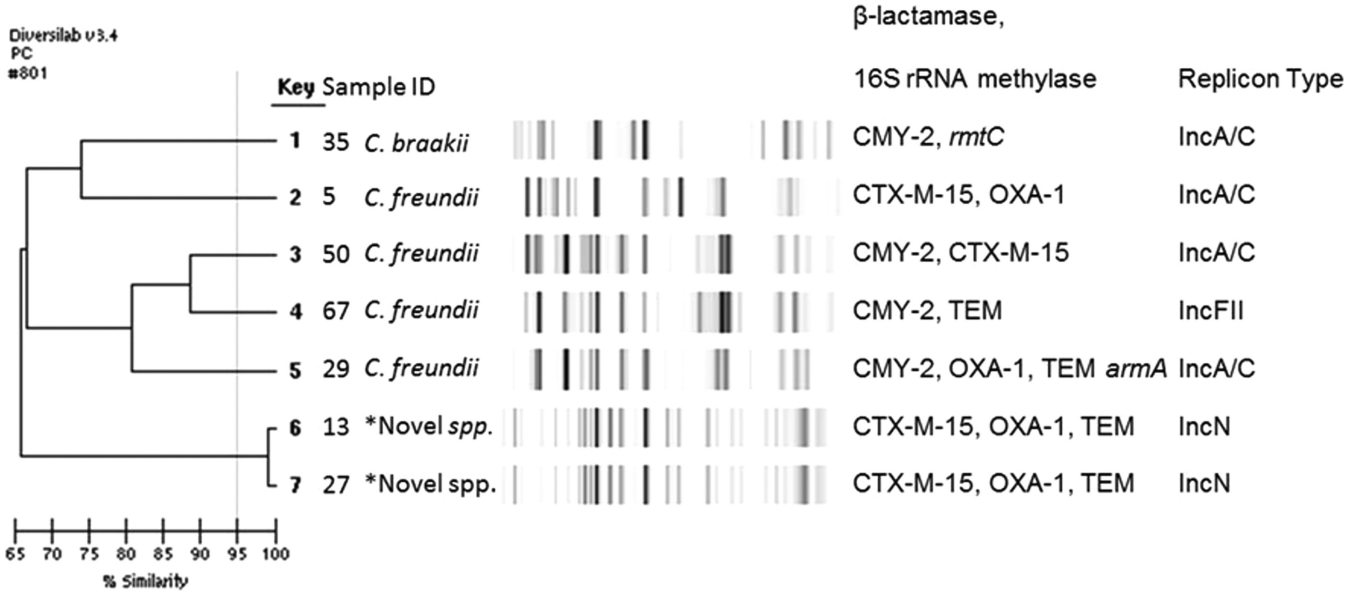

FIG 4 Dendrogram analysis of DiversiLab Rep-PCR fingerprint of NDM-1-producing *Citrobacter* isolates. *, novel species, *Pseudocitrobacter faecalis*.

isolates were diverse (Fig. 3). The three *Klebsiella pneumoniae* isolates were genetically diverse, while the three *Acinetobacter baumannii* isolates were considered identical (>99% similarity) (data not shown). Figure 4 shows the dendrogram for *Citrobacter* spp. and *Pseudocitrobacter faecalis* isolates (>99% similarity).

MLST differentiated the 10 representative *E. coli* strains into seven sequence types and one unknown sequence type (ST) (untypeable). The sequence types included ST10 ($n = 2$), ST101 ($n = 2$), and single isolates representing STs 211, 226, 1431, 2598, and 3032. MLST studies on NDM-1-producing *E. coli* in the literature provide an incomplete and heterogeneous global distribution, suggesting a nonclonal pattern of spread for bla_{NDM-1} (24). In this study, the clinical isolates of *E. coli* representing STs 211, 226, 1431, 2598, and 3032 to our knowledge have not been reported in NDM-1-producing *E. coli*.

There were a number of limitations in our study, including the lack of clinical patient data and using fecal samples from 2 hospitals at a single point in time. It is difficult in this respect to obtain a clear epidemiological picture of NDM-producing isolates more widely in Pakistan.

The spread of bla_{NDM-1} is frequently associated with common and highly promiscuous plasmids resulting in a diverse range of species and clones harboring bla_{NDM-1}. However, the molecular epidemiology of our study may indicate that bla_{NDM-1} additionally disseminates via dominant clones. The potential role of such dominant clones as a factor of bla_{NDM-1} spread may be underrepresented due to the lack of large-scale surveillance and molecular epidemiological studies monitoring bla_{NDM-1} dissemination. In this scenario, we can see a situation in which a single clone of NDM-1 may become epidemic or pandemic.

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