Early Dissemination of NDM-1- and OXA-181-Producing *Enterobacteriaceae* in Indian Hospitals: Report from the SENTRY Antimicrobial Surveillance Program, 2006–2007[▽]

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Among 39 carbapenem-resistant *Enterobacteriaceae* (2.7% overall; *Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* strains) isolated in 2006 and 2007 in India, 15 strains carried *bla*_{NDM-1} and 10 harbored a gene encoding a variant of the carbapenemase OXA-48, named *bla*_{OXA-181}. One *E. cloacae* strain harbored *bla*_{VIM-6}, and one *K. pneumoniae* strain carrying *bla*_{OXA-181} also possessed *bla*_{VIM-5}. Multiple pulsed-field gel electrophoresis patterns and clonal dissemination within and among sites were observed. Isolates producing NDM-1 were disseminated in Indian health care facilities as early as 2006.

NDM-1 (New Delhi metallo-β-lactamase 1), which was initially reported for *Klebsiella pneumoniae* and *Escherichia coli* strains from India, now has been detected in several enterobacterial strains and in different countries (3, 6, 8, 10–13, 15). In this study, we report occurrences and characterizations of carbapenemase-producing *Enterobacteriaceae* collected from Indian hospitals during 2006 and 2007, including isolates producing NDM-1, VIM enzymes, and a variant of the OXA-48 carbapenemase OXA-181.

A total of 1,443 isolates of *Enterobacteriaceae* were collected from 14 hospitals in India as part of the SENTRY Antimicrobial Surveillance Program, 2006–2007. Isolates were susceptibility tested using the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution method (4). Extended MIC ranges were determined using Etest (AB bioMérieux, Marcy l'Étoile, France). Categorical interpretations for all antimicrobials were those found in CLSI document M100-S20-U (5), and quality control results for *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were within specified ranges as published by CLSI (5).

All enterobacterial isolates with reduced susceptibility to imipenem, meropenem, or ertapenem (MIC, ≥2 μg/ml) were screened for production of carbapenemases. The modified Hodge test (MHT) was performed using imipenem and meropenem as substrates. Carbapenem-nonsusceptible isolates were PCR screened using custom primers targeting *bla*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{GES}, *bla*_{KPC}, and *bla*_{OXA-48}. Isolates were also screened for genes encoding plasmid-mediated AmpC enzymes (9) and extended-spectrum β-lactamases (ESBLs) by using custom primers targeting *bla*_{CTX-M}, *bla*_{OXA-2}, *bla*_{OXA-10}, *bla*_{OXA-1/30}, *bla*_{PER}, *bla*_{PSE}, *bla*_{BEL}, and *bla*_{VEB}. PCR amplicons were sequenced for both strands, and sequences were analyzed.

Twenty-six carbapenemase-producing strains (MHT and/or PCR positive) were identified among 39 enterobacterial isolates (2.7%; 1,443 overall) resistant to at least one of the carbapenems tested (imipenem, meropenem, or ertapenem) (3). These 26 isolates were recovered from patients hospitalized in New Delhi (two medical centers), Mumbai, and Pune in 2006 or 2007. Carbapenemase producers were highly resistant to β-lactams, including cefepime (MIC range, 4 to >256 μg/ml) (Tables 1 and 2), ceftazidime (64 to >256 μg/ml), aztreonam (4 to >256 μg/ml), and piperacillin-tazobactam (>64 μg/ml) and were generally resistant to amikacin (MICs, 8 to >32 μg/ml). Only one strain was susceptible to ciprofloxacin (MIC, ≤0.03 μg/ml) (Table 1), and all but two isolates were susceptible to polymyxin B (Tables 1 and 2). Tigecycline was the only antimicrobial agent active against all isolates (MICs, 0.12 to 2 μg/ml).

Fifteen putative carbapenemase-producing strains carried *bla*_{NDM-1} (Table 1), and 10 strains harbored a novel *bla*_{OXA-48}-like gene (Table 2). This new gene displayed 45 nucleotide substitutions leading to four amino acid differences compared to OXA-48 (T104A, N110D, E168Q and S171A) and was named the OXA-181 gene. Additionally, *bla*_{VIM} gene variants (*bla*_{VIM-6} and *bla*_{VIM-5}) were detected for two strains, one also carrying *bla*_{OXA-181} (Table 2). The gene encoding NDM-1 was detected in *E. coli* (six strains) (Table 1), *K. pneumoniae* (six strains), and *Enterobacter cloacae* (three strains), whereas *bla*_{OXA-181} was found in *K. pneumoniae* (10 strains, one also carrying the *bla*_{VIM-5} gene) (Table 2) and *E. cloacae* (1 strain). Another *E. cloacae* strain carried *bla*_{VIM-6} (Table 2).

Pulsed-field gel electrophoresis (PFGE) was used to evaluate clonality among carbapenemase-producing *Enterobacteriaceae* (14). NDM-1 producing *E. coli* isolates were additionally evaluated by multilocus sequence typing (MLST; <http://mlst.ucc.ie/mlst/dbs/Ecoli>). All six carbapenemase-producing *E. coli* strains carried *bla*_{NDM-1} and clustered within two PFGE groups (EC-A and EC-B) (Table 1). The two EC-A strains were recovered from New Delhi, while the four strains belonging to EC-B were from four sites, two in New Delhi and one

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TABLE 1. Demographic information, antimicrobial profile, and molecular results from 15 clinical *Enterobacteriaceae* isolates producing NDM-1 collected in India from 2006 to 2007

Parameter	Characteristic of or result for indicated isolate ^a														
	246-49D	246-34D	246-17D	252-26D	257-25A	258-03D	246-14A	246-05A	258-18A	257-36D	257-42D	246-01A	252-38C	252-41C	258-14C
Species ^a	EC	EC	EC	EC	EC	EC	ECL	ECL	ECL	KPN	KPN	KPN	KPN	KPN	KPN
City	New Delhi	New Delhi	New Delhi	Mumbai	New Delhi	Pune	New Delhi	New Delhi	Pune	New Delhi	New Delhi	New Delhi	Mumbai	Mumbai	Pune
Collection year	2006	2006	2007	2007	2007	2007	2007	2007	2007	2007	2007	2007	2007	2007	2007
Source ^b	SSSI	SSSI	SSSI	SSSI	BSI	SSSI	BSI	BSI	BSI	SSSI	SSSI	BSI	RTI	RTI	RTI
MIC (μ g/ml)															
Imipenem	4	4	8	8	16	64	2	2	8	1*	2	16	8	8	8
Meropenem	8	8	32	32	64	128	2	2	32	2	8	64	16	64	32
Ertapenem	32	32	>32	>32	32	>32	4	4	4	4	32	32	8	32	16
Doripenem	4	2	16	8	>32	>32	1*	2	4	2	4	16	8	16	>32
AMX/CLA ^c	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
PIP/AZ ^d	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Ceftazidime	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
Cefepime	>256	128	>256	>256	>256	>256	16	32	>256	64	64	>256	128	>256	128
Aztreonam	128	64	>256	>256	>256	>256	4	32	>256	>256	>256	>256	64	>256	>256
Amikacin	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Gentamicin	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
Tobramycin	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
Ciprofloxacin	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
Tetracycline	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
Trimethoprim	0.12	0.25	0.25	0.25	0.25	0.25	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Polymyxin B	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	4	4	4	4	4	4	4	4	4
MHT result	Weak pos	Weak pos	Pos	Weak pos	Weak pos	Weak pos	Pos	Pos	Pos	Weak pos	Weak pos	Weak pos	Weak pos	Weak pos	Weak pos
PFGE group	EC-A	EC-A	EC-B	EC-B1	EC-B2	EC-B3	ECL-A	ECL-A	ECL-B	KPN-A	KPN-B	KPN-C	KPN-D	KPN-E	KPN-E
Gene location ^e	P	P	P	C	P	C	P	P	P	P	P	P	P	P	P ^f
Gene															
<i>bla</i> CTX-M-15	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
<i>bla</i> OXA-1-30	Pos	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
<i>bla</i> OXA-2	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
<i>bla</i> CMY	<i>bla</i> CMY-4	<i>bla</i> CMY-4	Pos	Neg	Neg	Neg	<i>bla</i> CMY-6	<i>bla</i> CMY-6	Neg	<i>bla</i> CMY-6	<i>bla</i> CMY-4	Neg	Neg	<i>bla</i> CMY-4	Neg
<i>bla</i> DHA-like	Pos	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Neg

^a EC, *Escherichia coli*; ECL, *Enterobacter cloacae*; KPN, *Klebsiella pneumoniae*.^b SSSI, skin and skin structure infection; BSI, bloodstream infection; RTI, respiratory tract infection.^c AMX/CLA, amoxicillin-clavulanate.^d PIP/AZ, piperacillin-tazobactam.^e P, plasmidic; C, chromosomal.^f Hybridization signals were detected in two plasmid bands.^g *, susceptible by CLSI M100-S20-U criteria; pos, positive; neg, negative.

TABLE 2. Demographic information, antimicrobial profile, and molecular results from clinical *Enterobacteriaceae* isolates producing OXA-181 and/or VIM variants collected in India from 2006 to 2007

Parameter	Characteristic of or result for indicated isolate ^a													
	243-08A	243-09A	243-16C	243-18C	243-21C	257-35A	257-41D	252-36A	252-39A	243-50C	258-04A			
Carbapenemase	OXA-181	OXA-181	OXA-181, VIM-5	OXA-181	OXA-181	OXA-181	OXA-181	OXA-181	OXA-181	OXA-181	VIM-6			
Species ^a	KPN	KPN	KPN	KPN	KPN	KPN	KPN	KPN	KPN	KPN	ECL			
City	Kolkata	Kolkata	Kolkata	Kolkata	Kolkata	New Delhi	New Delhi	Mumbai	Mumbai	Kolkata	Pune			
Collection year	2007	2007	2007	2007	2007	2007	2007	2007	2007	2006	2007			
Source ^b	BSI	BSI	RTI	RTI	RTI	BSI	SSSI	BSI	BSI	RTI	BSI			
MIC (μg/ml)														
Imipenem	64	64	4	2	2	4	64	2	1*	32	2			
Meropenem	64	64	4	2	4	16	64	2	2	64	2			
Ertapenem	>32	>32	8	32	16	>32	>32	4	8	>32	4			
Doripenem	>32	>32	1*	2	2	32	>32	2	1*	>32	2			
AMX/CLA ^c	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16			
PIP/TAZ ^d	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64			
Ceftaxone	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256			
Ceftazidime	128	64	>256	>256	>256	>256	>256	>256	>256	>256	>256			
Cefepime	>256	>256	64	>256	>256	>256	>256	>256	>256	>256	128			
Aztreonam	>256	>256	128	>256	>256	>256	>256	>256	>256	>256	>256			
Amikacin	8	8	>32	16	>32	>256	>32	>32	>32	8	>32			
Gentamicin	≤2	>8	>8	≤2	>8	≤2	>8	>8	>8	>8	>8			
Tobramycin	16	>16	>16	16	>16	16	>16	>16	>16	>16	>16			
Ciprofloxacin	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4			
Tetracycline	>8	>8	8	8	>8	8	>8	≤2	≤2	>8	>8			
Tigecycline	1	1	1	0.5	1	0.5	1	0.25	0.5	2	1			
Polymyxin B	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5			
MHT result	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos			
PFGE group	KPN-F	KPN-F	KPN-G	KPN-H	KPN-H	KPN-E1	KPN-I	KPN-I	KPN-I	ECL-C	ECL-B1			
Gene location ^e	P	P	P	P	P	P	P	P	P	P	P			
Gene														
<i>bla</i> _{CTX-M-15}	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos			
<i>bla</i> _{OXA-1/-30}	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos			
<i>bla</i> _{OXA-10}	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg			
Plasmidic	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg			
AmpC														

^a KPN, *Klebsiella pneumoniae*; ECL, *Enterobacter cloacae*.^b SSSI, skin and skin structure infection; BSI, bloodstream infection; RTI, respiratory tract infection.^c AMX/CLA, amoxicillin-clavulanate.^d PIP/TAZ, piperacillin-tazobactam.^e P, plasmidic.^f *bla*_{OXA-10} was located in the class 1 integron carrying *bla*_{VIM-6}.^g *, susceptible by CLSI M100-S20-U criteria; pos, positive; neg, negative.

FIG. 1. Modified Hodge test (MHT) for NDM-1-producing isolates showing weakly positive results (arrows). Positive and negative controls are included for comparison. MER, meropenem; IMI, imipenem.

each in Mumbai and Pune. MLST analyses showed that EC-A strains belong to ST-167 and that all EC-B subtypes were ST-101. Interestingly, a ST-101 *E. coli* strain carrying *bla*_{NDM-1} was recently described in Australia (11). In the present study, two *E. coli* isolates were collected in 2006, while all remaining NDM-1 strains were recovered in 2007. These are the earliest NDM-1-producing isolates reported to date, indicating that isolates producing this carbapenemase have been present in India earlier than previously appreciated.

Five pulsotypes were observed among the six *K. pneumoniae* strains carrying *bla*_{NDM-1}. Two strains showing identical PFGE profiles were detected in Pune and Mumbai (KPN-E) (Table 1). The remaining nine *K. pneumoniae* strains harbored *bla*_{OXA-181}, were from Kolkata (55.6%), and belonged to five clones. Clonality within medical sites was documented (Table 2). Furthermore, one OXA-181-producing *K. pneumoniae* strain from New Delhi was genetically related to an NDM-1-carrying *K. pneumoniae* type detected in Pune and Mumbai (KPN-E) (Tables 1 and 2). The strain carrying *bla*_{OXA-181} and *bla*_{VIM-5} had a unique genetic background.

A great variety of enzymes was detected for *E. cloacae* strains, and organisms of this bacterial species harbored NDM-1, OXA-181, or VIM-6. Two NDM-1 producers from New Delhi were identical, and isolates from Pune harboring *bla*_{NDM-1} or *bla*_{VIM-6} were also genetically related (Table 2).

A large proportion of carbapenemase-producing isolates also harbored *bla*_{CTX-M-15} (23 of 26 isolates; 88.5%) and/or *bla*_{OXA-30} (20 of 26; 76.9%), and these β -lactamases were detected in strains producing the NDM-1, OXA-181, or VIM enzyme. Three genetically related NDM-1-producing *E. coli* strains from the same medical center were also positive for the *bla*_{OXA-2} gene (Table 1). Furthermore, plasmid-mediated AmpC genes (*bla*_{CMY-4}, *bla*_{CMY-6}, and/or *bla*_{DHA}-like genes) were detected among eight NDM-1-producing isolates (Table 1). None of the OXA-181- or VIM-producing isolates carried plasmid-mediated AmpC genes (Table 2).

Carbapenemase genes were predominantly plasmid located as demonstrated by S1 nuclease and ICEuI experiments fol-

lowed by hybridizations with specific probes. *bla*_{NDM-1} was detected in plasmids ranging in size from 50 to 450 kb in 13 strains, and this metallo- β -lactamase (M β L) gene was embedded in the chromosome of two *E. coli* strains (from Mumbai and Pune). Hybridization signals were noted for two plasmid bands in one *K. pneumoniae* isolate (257-25A) (Table 1). The gene encoding OXA-181 was carried in plasmids of similar molecular sizes (200 to 250 kb) in all strains carrying this gene; however, a few organisms displayed hybridization signals in multiple extrachromosomal bands.

Oligonucleotides targeting *bla*_{NDM-1} and *bla*_{OXA-48} genetic surroundings reported earlier (11, 16) were designed and used against isolates producing these enzymes yielding negative results. Class 1 integron mapping was performed as described previously (1, 2). The gene encoding VIM-6 detected in *E. cloacae* was located in the first position of a 3.9-kb class 1 integron carrying also the *aacA4* and *bla*_{OXA-10} genes. This genetic arrangement was previously described for *P. aeruginosa* isolates from hospitals located in several Asian nations (2), including the medical institution in Pune where this VIM-6-producing *E. cloacae* strain was recovered. The *bla*_{VIM-5} gene was also embedded in a class 1 integron lacking the common structures observed in the 3' conserved sequence (*qacE Δ 1/sulI* or *tmiC* gene) (7); however, genes encoding aminoglycoside resistance (*aacA4* and *aadA1*) were detected downstream of the M β L gene.

Negative or weakly positive MHT results were observed for 11 of 15 NDM-1-producing strains (Table 1 and Fig. 1). All remaining carbapenemase-producing isolates yielded positive MHT results. It is noteworthy that all NDM-1-producing *E. cloacae* strains displayed the lowest carbapenem MIC values (1 to 4 μ g/ml for imipenem and meropenem) among the species tested but were all MHT positive. Disk approximation tests using EDTA or mercaptopropionic acid as ion-chelating agents yielded positive results for all NDM-1-producing strains. These findings are particularly worrisome, because MHT has been broadly recommended to clinical microbiology laboratories for the detection and epidemiological evaluation

of carbapenemase-producing bacteria (5). The newly published CLSI carbapenem susceptibility breakpoints (≤ 1 $\mu\text{g/ml}$) (5) will minimize these false-susceptible errors.

The gene encoding NDM-1 was a prevalent mechanism of carbapenem resistance, being detected in 38.5% of the isolates surveyed and in at least 12 unique strains of three bacterial species. This gene was carried by distinct plasmids. Moreover, *bla*_{NDM-1}-carrying *K. pneumoniae* strain displayed greater clonal diversity than that of the *E. coli* and *E. cloacae* strains harboring the same gene. This suggests greater genetic exchange among strains of this bacterial species.

NDM-1-producing strains appear to be an emerging worldwide problem (3, 6, 8, 10–13, 15), and by our findings, the detection of these strains by the MHT can be problematic (i.e., false-negative results can occur), delaying the implementation of infection control measures and allowing the continued dissemination of the strains. Further studies seem necessary to establish preventive strategies to control the dissemination of strains harboring *bla*_{NDM-1} and to elucidate the mechanism of dissemination of this β -lactamase gene.

Nucleotide sequence accession number. The sequence of the OXA-181 gene was submitted to GenBank under accession number HM992946.

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