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Dissemination of NDM-1-producing Enterobacteriaceae in India

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Amended Abstract

Background: A new metallo-β-lactamase, NDM-1, was identified in a *Klebsiella pneumoniae* from a Swedish patient of Indian origin. We report occurrences and characterization of NDM-1-producing strains collected from Indian sites during the SENTRY Program (2006 – 2007).

Methods: 1443 Enterobacteriaceae from 14 hospitals were susceptibility tested by CLSI methods. Isolates with imipenem or meropenem MICs ≥2 μg/mL were screened by Modified Hodge Test (MHT) and PCR using primers targeting bla_{NDM-1} and surrounding sequences. β-lactamase extracts of NDM-1-positive strains were subjected to spectrophotometric assays. Clonality was assessed by PFGE. Gene location was determined by S1 endonuclease restriction, followed by Southern blot and hybridization.

Results: 15 (1%; 6 *Escherichia coli*, 6 *K. pneumoniae* and 3 *Enterobacter cloacae*) isolates from New Delhi (2 sites), Mumbai and Pune carried bla_{NDM-1}. All isolates were resistant to penicillins, cephalosporins and aminoglycosides, and susceptible only to tigecycline and polymyxin B (two isolates were resistant). Strains showed non-wildtype carbapenem MICs (≥1 μg/mL) and MHT-negative or weak-positive results in 11 (73%) strains, while hydrolytic imipenem activity was not detected. Multiple PFGE patterns were noted in each species, but clonal dissemination within sites was also observed. Distinct plasmids ranging in sizes (ca. 50-450Kb) carried bla_{NDM-1} in 12 isolates, while this gene was embedded in the chromosome of 2 strains. The genetic context was distinct from that previously described for bla_{NDM-1} in all strains.

Conclusions: bla_{NDM-1} was carried by distinct plasmids and one strain showed two gene copies, suggesting high rates for recombination events. Variable carbapenem resistance levels at or below CLSI/EUCAST breakpoints, combined with uncertain results during phenotypic carbapenamase detection tests, will pose a challenge to control bla_{NDM-1} dissemination.

Introduction

The emergence of acquired metallo-β-lactamases (MβLs) among important Gram-negative pathogens, including members of the Enterobacteriaceae family, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. has highlighted this significant clinical problem. MβLs can hydrolyze the vast majority of β-lactam agents available for clinical use and are not inhibited by β-lactamase inhibitors currently marketed or in development.

Several types of MβLs have been reported to date: various IMP- and VIM-types, SPM-1, GIM-1, SIM-1, AIM-1 and KHM-1. Recently, a new MβL was reported from *Klebsiella pneumoniae* and *Escherichia coli* strains from India. This enzyme, called NDM-1 (New Delhi Metallo-β-lactamase) was detected in a Swedish diabetic patient of Indian origin that traveled to New Delhi and acquired a urinary tract infection.

NDM-1 has little similarity with other MβL enzymes, being only 32.4% similar to VIM-1/VIM-2. The gene encoding NDM-1 is located adjacent to a region of *K. pneumoniae* endogenous DNA and a truncated IS*26* element in a 140-kb plasmid. NDM-1 was also detected in various Enterobacteriaceae species (*K. pneumoniae*, *E. coli*, *Enterobacter cloacae* and *Citrobacter freundii*) in the United Kingdom and the United States and the cases were closely linked to receipt of medical care in India or Pakistan.

In this study, we report occurrences and characterization of 15 NDM-1-producing Enterobacteriaceae strains collected from Indian hospitals during the SENTRY Antimicrobial Surveillance Program (2006 – 2007).

Methods

Bacterial isolates: A total of 1,443 Enterobacteriaceae isolates were collected from 14 hospitals in India as part of the SENTRY Program (2006-2007). Only one isolate per patient from documented infections were included in the study. Isolates were collected from bloodstream, respiratory tract and skin and skin-structure infections according to a common protocol. Species identification was confirmed by standard biochemical tests and the Vitek System (bioMerieux, Hazelwood, Missouri, USA), when necessary.

Antimicrobial susceptibility testing: All isolates were susceptibility tested using the broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI, M07-A8). Extended MIC ranges were determined using Etest (AB bioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. Categorical interpretations for all antimicrobials were those found in M100-S20-U (2010) and quality control (QC) was performed using *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. All QC results were within specified ranges as published in CLSI documents (M100-S20-U).

Screening for carbapenemases: All Enterobacteriaceae isolates with reduced susceptibility to imipenem, meropenem or ertapenem (MIC, ≥2 μg/mL) were screened for production of carbapenemases. Modified Hodge test (MHT) was performed using imipenem and meropenem as substrates.

Spectrophotometric detection of carbapenemases: β-lactamase extracts of NDM-1-positive strains were prepared using Bugbuster™ reagent (Novagen/Merck, Darmstadt, Germany). Protein extracts were subjected to spectrophotometric assays at 299 nm to determine hydrolysis of imipenem in 0.1M HEPES buffer.

Genotypic detection of β-lactamase-encoding genes: Carbapenem non-susceptible isolates were PCR screened using primers targeting bla_{NDM-1}. NDM-1-producing isolates were also screened for ESBL production using primers targeting bla_{CTX-M}, bla_{OXA-2}, bla_{OXA-10}, bla_{PER} and bla_{VEB}. PCR amplicons were sequenced on both strands and the nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin, USA). Sequences were compared to others available via internet sources (http://www.ncbi.nlm.nih.gov/blast/).

Gene location analysis: Total cellular DNA embedded in 1% agarose plugs was subjected to partial digestion with S1 nuclease. Plasmids were resolved by electrophoresis performed on the CHEF-DR II (BioRad, Richmond, CA), with the following conditions: 0.5 x TBE, 1% agarose, 13°C, 200V, for 6 hours with switch time ramping from 5 to 25 seconds and 14 hours with the switch time from 30 – 45 seconds. ICeuI digested genomic DNA was also resolved on PFGE as described previously. DNA gels were transferred to nylon membranes by Southern blotting and hybridized with a digoxigenin labeled (Roche Diagnostics GmbH, Mannheim, Germany) bla_{NDM-1}-specific probe.

Results

- Among 38 carbapenem non-susceptible strains, bla_{NDM-1} was detected in 15 (38.5%) Enterobacteriaceae strains: six *E. coli*, six *K. pneumoniae* and three *E. cloacae*. These strains were recovered from patients hospitalized in New Delhi (two hospitals), Mumbai and Pune.
- NDM-1-producing strains showed carbapenem MIC results, ranging from 1 to >8 μg/mL (Table 1) and resistance to penicillins, cephalosporins and aminoglycosides. Only one strain was susceptible to ciprofloxacin (*E. cloacae* 246-14A; MIC, ≤0.03 μg/mL; Table 1).
- All but two isolates were susceptible to polymyxin B (Table 1). Tigecycline was the only antimicrobial agents active against all strains (MIC results, 0.12 to 2 μg/mL).
- Negative or weakly positive MHT results were observed for 11 of the 15 NDM-1-producing strains (Table 1 and Figure 1). Imipenem hydrolysis was not detected when testing protein preparations from NDM-1-producers.
- *E. cloacae* strains displayed the lowest carbapenem MIC values (1 to 4 μg/mL for imipenem and meropenem) among the species tested, but all three were positive for the MHT.
- Among six *E. coli* strains, two were identical and four strains, detected from different hospitals (three cities) were genetically similar by PFGE (Table 1). Two patterns were noted among three *E. cloacae* and five PFGE profiles were observed among six *K. pneumoniae* strains. The two identical *K. pneumoniae* strains were detected in different cities (Mumbai and Pune; Table 1).
- Four NDM-1 positive *E. coli*, two *E. cloacae* and all six *K. pneumoniae* harbored $bla_{CTX-M-15}$. Additionally, three *E. coli* isolates, one from each city, also produced OXA-2.
- Plasmids with sizes ranging from 50 to 450-Kb carried bla_{NDM-1} in 12 isolates (Figure 2). The MβL gene was embedded in the chromosome of two *E. coli* strains (Mumbai and Pune). Hybridization signals were noted for two plasmid bands in one *K. pneumoniae* isolate (257-25A; Table 1 and Figure 2).

Table 1. Demographic information, antimicrobial profile and molecular results from clinical Enterobacteriaceae isolates producing NDM-1 from India evaluated in the present study.

Parameter	Isolate														
	246-49D	246-34D	246-17D	252-26D	257-25A	258-03D	246-14A	246-05A	258-18A	257-36D	257-42D	246-61A	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 (μg/mL)															
Imipenem	4	2	8	8	>8	>8	2	2	4	1*	4	>8	>8	>8	>8
Meropenem	8	8	>8	>8	>8	>8	1*	2	4	1*	2	>8	8	8	8
Ertapenem	>8	8	>8	>8	>8	>8	2	4	>8	4	4	>8	8	>8	>8
Doripenem	4	4	>8	8	>8	>8	2	2	4	1	4	>8	8	8	8
AMX/CLA[c]	>16	>16	>16	>16	>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	>64	>64	>64	>64
Ceftriaxone	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
Ceftazidime	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
Cefepime	>16	>16	>16	>16	>16	>16	8	>16	>16	>16	>16	>16	>16	>16	>16
Aztreonam	>16	>16	>16	>16	>16	>16	8	>16	>16	>16	>16	>16	>16	>16	>16
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	≤0.03	>4	>4	>4	>4	>4	>4	>4	>4
Tetracycline	>8	>8	>8	>8	>8	>8	≤2	≤2	>8	>8	8	>8	>8	4	4
Tigecycline	0.12	0.25	0.25	0.25	0.25	0.5	0.5	0.5	2	1	1	0.5	1	0.5	0.5
Polymyxin B	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	4	4	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
MHT result	weakly positive	weakly positive	positive	weakly positive	weakly positive	weakly positive	positive	positive	positive	weakly positive	weakly positive	weakly positive	weakly positive	weakly positive	weakly positive
PFGE	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]
$bla_{CTX-M-15}$	Neg	pos	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos
bla_{OXA-2}	Neg	neg	neg	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg

*Susceptible by CLSI M100-S20-U criteria.
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/TAZ= piperacillin/tazobactam.
e. P= plasmidic, C=chromosomal.
f. Hybridization signals were detected in two plasmid bands.

Figure 1. Modified Hodge test (MHT) for NDM-1-producing isolates showing weakly positive results (arrows). Positive and negative controls were included for comparison.

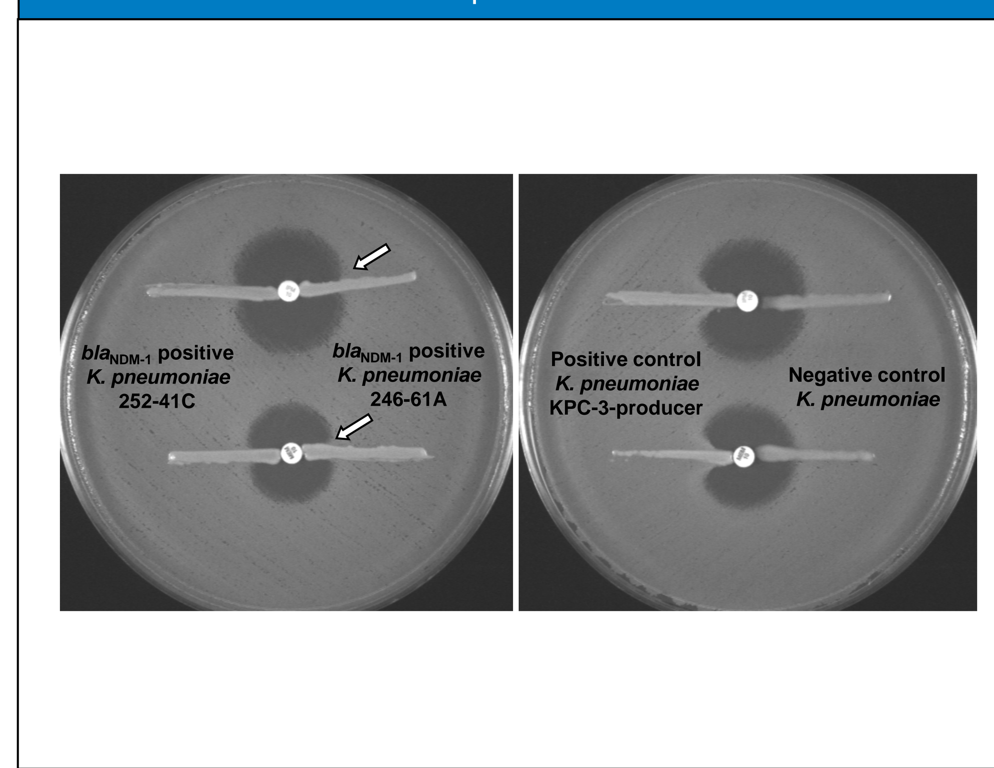
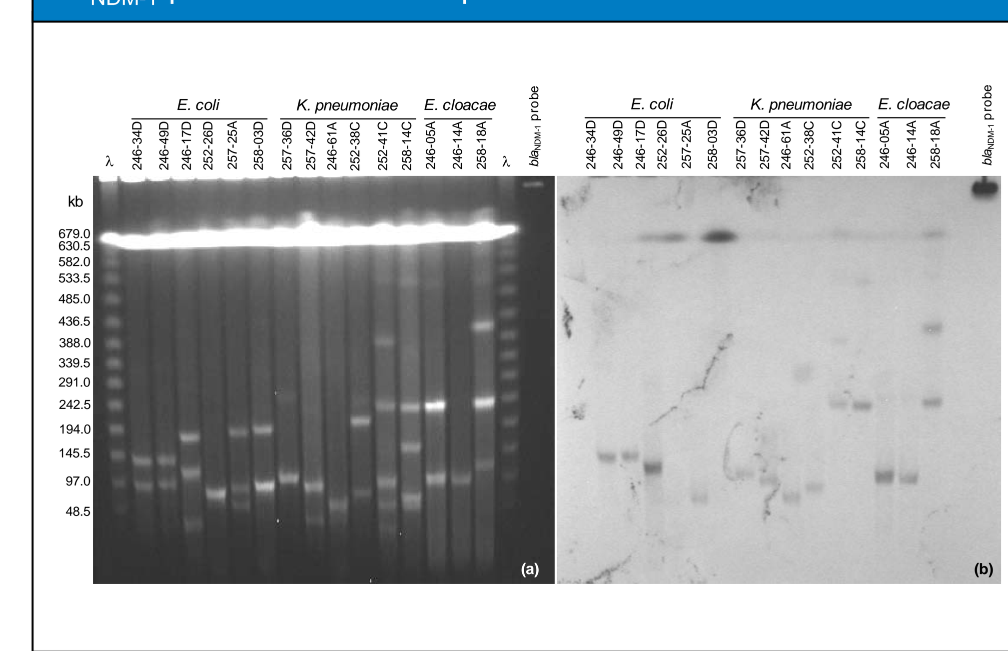


Figure 2. (a) S1 digested DNA profiles in agarose gel electrophoresis and (b) hybridization with digoxigenin labeled bla_{NDM-1}-probe of 15 NDM-1-producing Enterobacteriaceae strains from India (2006-2007). Lambda ladder (λ) was used as molecular weight marker and negative control. Amplicons used to prepare the bla_{NDM-1}-probe were used as positive control.



Conclusions

- We recently demonstrated the dissemination of numerous VIM-variants among *Pseudomonas* spp. strains from India. In the present study, we show that among Enterobacteriaceae displaying elevated MIC values for carbapenems, bla_{NDM-1} is a prevalent mechanism of carbapenem resistance, being detected in 38.5% of the carbapenem-non-susceptible isolates surveyed.
- bla_{NDM-1} was detected in at least 12 unique strains of three bacterial species. Furthermore, this gene was carried by distinct plasmids, with more than one copy found in one isolate or in the chromosome. These results indicate that mobilization of the MβL gene occurred on various occasions and was associated with recombination events that generated multiple copies of this gene within the bacterial cell.
- Eleven of the 15 NDM-1-producing strains from the present study displayed negative results for carbapenemase screening methods recommended by the CLSI. These findings are particularly worrisome, because detection and control of bla_{NDM-1}-carrying strains dissemination can be troublesome. Newly published carbapenem susceptibility breakpoints (≤1 μg/mL) would minimize false-susceptible errors.

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References

1. Castanheira M, Bell JM, Turnidge JD, Mathai D, Jones RN (2009). Carbapenem resistance among *Pseudomonas aeruginosa* strains from India: Evidence for nationwide endemicity of multiple metallo-β-lactamase clones (VIM-2, -5, -6, and -11 and the newly characterized VIM-18). *Antimicrob Agents Chemother* 53: 1225-1227.
2. Clinical and Laboratory Standards Institute (2009). *M07-A8. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: eighth edition.* Wayne, PA: CLSI.
3. Clinical and Laboratory Standards Institute (2010). *M100-S20-U. Performance standards for antimicrobial susceptibility testing: 20th informational supplement (June 2010 Update).* Wayne, PA: CLSI.
4. Detection of *Enterobacteriaceae* isolates carrying metallo-beta-lactamase - United States, 2010. *MMWR Morb Mortal Wkly Rep* 59: 750.
5. Livermore DM, Woodford N (2010). Carbapenemase-producing *Enterobacteriaceae* in the UK: NDM (New Delhi Metallo-β-lactamase: Repeated importation from Indian subcontinent. HPA. Available at http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1248854045473. Accessed April, 2010.
6. 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.