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Original Article

Molecular detection of *bla*_{NDM-1} (New Delhi metalloβ-lactamase-1) in nosocomial *Enterobacteriaceae* isolates by nested, multiplex polymerase chain reaction

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ARTICLE INFO

Article history:

Received 9 February 2016

Accepted 17 February 2017

Available online 22 March 2017

Keywords:

Carbapenemases

New Delhi Metallobetalactamase (NDM)

Multiplex PCR (*bla*_{NDM-1}*bla*_{IMP}*bla*_{VIM} and *bla*_{KPC})

ICU

ABSTRACT

Background: Carbapenems are considered “drugs of last resort” in many life-threatening infections. Advent of carbapenemases like KPC, OXA-48, VIM, IMP, and NDM have greatly affected the efficacy of these drugs, posing serious threat to global health and infection control. NDM bears special significance to the India subcontinent, labeled as place of origin and reservoir. NDM tends to escape detection by routine phenotypic methods, requiring molecular confirmation. This study utilizes nested, multiplex polymerase chain reaction (PCR) for reliable detection of *bla*_{NDM-1} in nosocomial *Enterobacteriaceae* isolates.

Methods: This study was conducted to detect prevalence of *bla*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{KPC} genes by multiplex PCR among multidrug/carbapenem-resistant nosocomial *Enterobacteriaceae* isolates. From March 2013 to April 2014, 100 consecutive non-repeat isolates of *Enterobacteriaceae* from various inpatient clinical samples were analyzed. Imipenem-resistant isolates identified by Kirby Bauer disk diffusion method with Clinical and Laboratory Standards Institute guidelines were further subjected to nested, multiplex PCR to simultaneously detect *bla*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{KPC} genes.

Results: Out of 100 isolates, 17 (17%) were found to be imipenem-resistant. *bla*_{NDM-1} was detected in all 17 isolates by nested, multiplex PCR. *bla*_{VIM} was co-carried in 4 isolates while one isolate co-harbored *bla*_{IMP} with *bla*_{NDM-1}. Imipenem resistance and NDM-1 carriage was predominant amongst *Klebsiella* isolates. Maximum NDM-1 producers were isolated from the intensive care unit (70.6%).

Conclusion: NDM-1 prevalence in nosocomial *Enterobacteriaceae* isolates in our hospital was found to be 17%. A nested, multiplex PCR was used for rapid detection of various carbape-

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<http://dx.doi.org/10.1016/j.mjafi.2017.02.009>

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nemase genes with high sensitivity and specificity which is essential not only for favorable patient outcome but also for timely implementation of appropriate infection control practices to prevent further spread of such organisms.

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Introduction

β -Lactam resistance in *Enterobacteriaceae* has shown an alarming increase in frequency and spread, posing an ominous threat to public health. Among the most important issues in the antimicrobial resistance today, resistance to carbapenems, colloquially considered the 'drugs of last resort' for life-threatening infections in *Enterobacteriaceae* is mediated primarily by carbapenemases, functionally divided into serine-dependent (e.g., KPC, OXA-48) and metallo- β -lactamase (e.g., VIM, IMP, and NDM). New Delhi Metallobetalactamase-1 (NDM-1) bears special significance to India, labeled as the country of origin of this deadly enzyme, first identified in a *Klebsiella pneumoniae* isolate from a Swedish patient of Indian origin hospitalised in New Delhi in 2007.¹ Since then, studies worldwide have reported the emergence of NDM-1, most linking it to a possible reservoir in the Indian subcontinent.²

Earlier restricted largely to the *Enterobacteriaceae*, the plasmid-mediated *bla*_{NDM-1} gene³ has since spread rapidly to non-clonally related isolates. The same plasmid often carries other drug-resistance genes as well, giving rise to multidrug resistant organisms (MDROs). Non-plasmid-mediated resistances may confer complete resistance to all antibiotics, including tigecycline and colistin³ severely limiting treatment options and increasing risk of nosocomial spread.

These often escape detection by routine laboratory methods, giving a false susceptibility report which in turn leads to ineffective and unnecessary antibiotic use, further aggravating the problem of resistance. Hence, rapid detection of these organisms is of utmost importance in limiting their emergence as well as spread, improving patient outcome and enhancing infection control. This has gained great interest amongst researchers in these past few years. Absence of phenotypic manifestation of gene, susceptible albeit elevated carbapenem minimum inhibitory concentrations (MICs) and inability of automated systems in distinguishing from other resistance mechanisms (e.g., extended spectrum beta-lactamases (ESBLs) and/or porin loss)³ necessitate molecular detection of the gene as the gold standard in diagnosis.^{12–16,19–22}

This prospective study was undertaken at a tertiary care hospital in New Delhi over a period of one year (March 2013 to April 2014) to detect prevalence of *bla*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{KPC} genes by multiplex polymerase chain reaction (PCR) among MDR and carbapenem-resistant nosocomial isolates of the *Enterobacteriaceae* family.

Material and methods

100 consecutive non-repeat isolates of *Enterobacteriaceae* isolated from various clinical specimens such as urine, pus,

blood, sputum and body fluids from various medical and surgical wards and the intensive care unit (ICU) were included in the study.

Identification of *Enterobacteriaceae*: The isolates were identified till species level based upon standard bio-chemical methods.⁴

Antibiotic susceptibility testing: The antimicrobial susceptibility testing was done by the Kirby Bauer disk diffusion method on Mueller Hinton agar and commercially available antimicrobial discs (HiMedia Laboratories Pvt. Ltd., 23, Vadhani Ind. Est., LBS Marg, Mumbai, India) were used. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S20-U Jun 2010). For isolates with imipenem zone size <13 mm, MICs of carbapenems were determined by Vitek 2 Compact automated system (bioMérieux, Marcy-l'Etoile, France) in accordance with the CLSI recommendations and manufacturers' instructions. MIC values of colistin and tigecycline were not carried out. All *Enterobacteriaceae* isolates resistant to imipenem were directly subjected to molecular detection of *bla*_{NDM-1}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{KPC} genes by multiplex PCR. No phenotypic methods of carbapenemase detection were undertaken.

Molecular methods

All carbapenem resistant isolates were subjected to an automated multiplex PCR technique provided by Aus Diagnostics Pty Ltd for rapid detection of resistance genes *bla*_{NDM-1}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{KPC} by following steps:

1. DNA extraction – DNA extraction was done from all isolates with QIAamp DNA mini kits (QIAGEN, Str. 1, 40724 Hilden, Germany) as per manufacturer's instructions for DNA extraction from fresh cultures. Filtrate containing the DNA was taken up for PCR
2. Multiplex PCR – Multiplex Tandem PCR kits provided by Aus Diagnostics Pty Ltd.

(205 Victoria Street Beaconsfield NSW 2015 Australia) were used for two sequential PCR steps.

Step 1 consisted of 15 cycles of multiplexed pre-amplification using primers homologous to all targets in the panel. Step 2 utilized the product from Step 1 diluted into individual wells for real time PCR reactions. Primers for Step 2 were 'nested inside' the primers used for Step 1. Till this point, the procedure was automated by the Mark II Easy-Plex™ 72 liquid handling robot system (AusDiagnostics Pty Ltd.)

Step 2 real time PCR reaction was performed in the Rotor-Gene of the Mark II Easy-Plex™ 72 system. Eva-Green™

fluorescent dye was incorporated into the DNA being formed in the specific amplification reaction. DNA amplification was then measured by the increase in fluorescence produced by the dye.

Step 1 was carried out in a 7-well tube strip (for seven samples), amplifying the five targets (including SPIKE, an internal control). A 72-well Easy-Plex™ ring is provided for performing Step 2.

Internal control – An artificial DNA target called SPIKE acted as an internal control for integrity of reagents (polymerase, primers, etc.), equipment function (thermal cyclers) and the presence of inhibitors in the sample and was contained in each Step 1 tube, in addition to target primers.

Reaction mixture for Step 1

5 µL of sample (template DNA) + 5 µL deionized water
+ 10 µL of Mastermix solution 1

Cycling conditions for Step 1

Reaction mixture for Step 2

2 µL of sample (template DNA) + 100 µL deionized water
+ 50 µL of Mastermix solution 2

Cycling conditions for Step 2

Primers for both steps were patented by AusDiagnostics Pty Ltd and not revealed.

Interpretation

Results were read based on fluorescent signal produced by detection and amplification of any of the target genes, *bla*_{NDM-1}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{KPC}. Results were generated both in tabular form as well as in the form of amplification curves.

Control group

A total of 30 nosocomial *E. coli* and *K. pneumoniae* isolates from similar clinical samples but sensitive to imipenem were also collected and tested during the same study period for detection of MBL genes (*bla*_{NDM-1}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{KPC}) which might be harbored without phenotypic expression of the resistance mechanism.

Results

Phenotypic studies

Maximum samples were urine (59) followed by blood (13), wound swabs (8), pus (5), body fluids (ascitic, bile, drain fluid)

Fig. 1 – Distribution of isolates amongst Enterobacteriaceae.

Fig. 2 – Comparison of distribution of isolates vs NDM-1 producers.

(5), respiratory specimens (BAL, sputum, tracheal aspirate) (4), high vaginal swabs (2), tissue specimens (2), urethral swab (1) and stool (1). These were mainly from Medical (45) and Surgical wards (40) while 15 samples were from ICU (adult and neonatal). *E. coli* (57%) and *Klebsiella* spp. (22%) were the commonest isolates. Distribution of isolates is shown in Fig. 1.

MICs of carbapenems determined by Vitek 2 Compact automated system were 8–64 µg/ml. Among the 100 isolates studied, 17 isolates were found to be imipenem resistant of which 12 (70.6%) belonged to *Klebsiella* spp. and 5 (29.4%) were *E. coli*. Rest all *Enterobacteriaceae* isolates were susceptible to imipenem (Fig. 2).

Imipenem resistance was maximum among the respiratory isolates (75%) followed by pus (40%) and wound swabs (25%). Urine isolates showed a low prevalence of imipenem resistance (15.25%) followed by blood (7.69%) (Fig. 3).

Molecular methods

All 17 imipenem-resistant isolates were subjected to nested, multiplex PCR for rapid detection of resistance genes *bla*_{NDM-1}, *bla*_{VIM}, *bla*_{IMP} and *bla*_{KPC} as described above.

All imipenem-resistant isolates in our study were found to be carrying the *bla*_{NDM-1} gene (100%). *bla*_{VIM} was detected in 4 isolates (23.5%) but all these isolates were positive for *bla*_{NDM-1} as well. One isolate co-harbored *bla*_{IMP} with *bla*_{NDM-1}. *bla*_{KPC} was not detected in any of the isolates (Table 1).

Thus, imipenem resistance as well as NDM-1 production was found to be predominant amongst *Klebsiella* isolates at our hospital (Table 2).

Out of the 100 nosocomial *Enterobacteriaceae* isolates studied, majority (85/100) were from medical and surgical wards while 15 came from the ICU. However, 12 of the 15 ICU

Fig. 3 – Distribution of imipenem resistance in various specimens.

Table 1 – Distribution of genes among carbapenem-resistant isolates.

Gene	Total no. of imipenem resistant isolates	No. of positive by multiplex PCR	%
<i>bla</i> _{NDM-1}	17	17	100%
<i>bla</i> _{VIM}	17	4	23.5%
<i>bla</i> _{IMP}	17	1	5.88%
<i>bla</i> _{KPC}	17	0	0

Table 2 – Distribution of NDM-1 producing organisms.

Organism	Total no. of imipenem resistant isolates	MBL producers		
		Number	%	Gene
<i>Klebsiella pneumoniae</i>	12	12	100%	<i>bla</i> _{NDM-1}
<i>E. coli</i>	5	5	100%	<i>bla</i> _{NDM-1}

isolates (70.6%) turned out to be carbapenem-resistant and NDM producers whereas the wards had only 5 such isolates (5.9%) (Fig. 1).

Imipenem-resistant urinary isolates harbored majority of NDM producers (53%) followed by respiratory specimens (17.6%), pus and wound swabs (11.76% each) and blood cultures (5.8%). These results are depicted in relevant tables/figures (Figs. 4 and 5).

None of the imipenem-susceptible control isolates harbored any of the target genes.

Discussion

Carbapenem resistance in clinical *Enterobacteriaceae* isolates has been reported worldwide. Imipenem resistance was detected in 17% of isolates in our study, 12 belonging to

Klebsiella spp. (70.6%) while the remaining 5 isolates were *E. coli* (29.4%).

MIC values for imipenem, meropenem and ertapenem ranged from 8–64 µg/ml for all isolates.

Indian studies report a range of 15–29% of imipenem resistance from different specimens from ICU and various wards.^{5–7} The *bla*_{NDM-1} gene was detected in all imipenem-resistant isolates in our study (100%) and was most prevalent in *Klebsiella* spp. Recent studies from other Indian tertiary hospitals corroborate our findings of carbapenem resistance and carriage of MBLs, especially NDM (40–92%) amongst carbapenem-resistant *Enterobacteriaceae* (CRE)^{8–16} with researchers from various regions of the country utilizing conventional or multiplex, real time PCR formats similar to the one used in our study. Deshpande et al reported 91.67% carriage of *bla*_{NDM-1} gene among the carbapenem-resistant isolates in her study from Mumbai⁸ similar to studies from Pune^{9–11} including a recent study on NDM producing extra-intestinal pathogenic *E. coli* (ExPEC) strains, where NDM-1 carriage was 69%. *bla*_{NDM-1} in studies from South Indian tertiary care hospitals ranged from 40% in Vellore, Tamil Nadu^{12,13} to 81% in Mangalore, Karnataka.¹⁴ Wattal et al. in their study in a tertiary care hospital in North India found 100% NDM-1 carriage in CRE (Carbapenem Resistant *Enterobacteriaceae*) isolates from ICU blood cultures.¹⁵ Bora et al. in their study from the Northeast detected 71.42% of *E. coli* isolates harboring *bla*_{NDM-1}.¹⁶

These findings are also supported by the data from Europe and North America.^{17,18} The SMART study⁷ detected NDM producers in 50% of all *Enterobacteriaceae* isolates with reduced susceptibility to carbapenems. Other studies also show a wide range of prevalence of NDM-1 producers amongst CRE isolates. A study of carbapenemase-producing *Enterobacteriaceae* from Russia and other Baltic countries detected *bla*_{NDM-1} in 15 out of 77 CRE isolates (19.48%).¹⁹ A study on an outbreak of NDM-1 producing *Enterobacteriaceae* in Italy in 2011 analyzed 44 isolates, out of which 6 isolates harbored the *bla*_{NDM-1} gene (13.63%).²⁰ Also, maximum number of NDM-1 producers belonged to *Klebsiella* spp.

NDM-1 producers in samples

Fig. 4 – Distribution of NDM-1 producers in various specimens.

No.	Colour	Sample	Gene	Call	Normalised Expr.
1		184 - A22541DM S470014 B22565M R468914	KPC		
2		184 - A22541DM S470014 B22565M R468914	NDM-1	Present	3440787
3		184 - A22541DM S470014 B22565M R468914	pan-vim	Present	872
4		184 - A22541DM S470014 B22565M R468914	pan-IMP		
5		184 - A22541DM S470014 B22565M R468914	SPIKE	Present	10000

(a) Tabulated version

(b) Cycling curves

Fig. 5 – Multiplex Tandem PCR report for isolate carrying both *bla*_{NDM-1} and *bla*_{VIM}. (a) Tabulated version and (b) cycling curves.

High prevalence of NDM-1 producers in our study can be attributed to limitation of sampling bias. Ours is a tertiary care hospital with superspeciality departments such as liver, renal and bone marrow/stem cell transplant, malignant diseases treatment center and departments dealing with conditions such as hematological malignancies, joint replacement and reconstructive surgery. Moreover, cases are referred from most of northern India, already subjected to various antimicrobial regimens in primary and secondary medical care. Thus, sample population is limited with known risk factors of multidrug resistance acquisition (general debility, immunosuppression, malignancy and antibiotic exposure). This is further corroborated by the finding of maximum NDM producers being isolated from the ICU.

This was a pilot study conducted to validate the utility of rapid and robust molecular detection of carbapenem resistance amongst hospital-acquired *Enterobacteriaceae* isolates at

a premier tertiary care hospital. Early detection of such isolates not only facilitates patient care but also proves invaluable in infection control and prevention of spread/emergence of multidrug resistance.

Various phenotypic methods of carbapenemase/MBL detection are also available, namely the modified Hodge test, agar diffusion tests like E-test, disk enhancement tests utilizing Zn and EDTA or commercially available combined disk tests like the KPC + MBL confirm ID pack and Neo-Sensitabs (Rosco Diagnostica) as well as chromogenic tests such as the Carba NP described by Nordmann et al and Dortet et al^{21,22} which has recently been introduced as a confirmatory test for carbapenemase producers by CLSI M100-S25.²³ however, there are conflicting reports as regards sensitivity and specificity of these tests in detecting carbapenemases/MBLs, further varied by the specific resistance-determinant gene. In addition, the turnaround time of >24 h of these tests is an added deterrent.^{13,23–28}

Conclusion

Our study demonstrated a high prevalence of NDM-1 in carbapenem-resistant nosocomial *Enterobacteriaceae* isolates. This shows that the emergence of this enzyme is a major threat in the field of microbial drug resistance. Early detection is desirable for patient benefit and to avoid spread of such isolates. However, routine detection is controversial and difficult. Molecular detection with multiplex PCR remains the gold standard with high sensitivity and specificity but techniques are demanding and not available at most laboratories. Hence, detection of pan-resistant organisms by phenotypic methods in smaller peripheral hospitals assumes extreme importance as such organisms are likely to harbor NDM-1 gene. A timely warning conveyed to treating clinicians in such cases would avert preventable calamities, especially in acute cases in high output areas such as the ICUs and Burns Center and Long-term care areas such as Orthopedic wards by timely institution of appropriate treatment and infection control practices such as isolation.

Our study also highlights the undeniable importance of strict infection control practices and judicious use of antibiotics especially in setting of ICU and critical care wards, not only in preventing emergence and spread of such organisms but also in extending longevity of carbapenems, the last resort antibiotics in many life-threatening infections.

Conflicts of interest

The authors have none to declare.

Acknowledgements

This paper is based on Armed Forces Medical Research Committee Project No 4401/2013 granted by the Office of the Directorate General Armed Forces Medical Services and Defence Research Development Organization, Government of India.

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