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Prevalence of metallo-β-lactamase NDM-1-producing multi-drug resistant bacteria at two Pakistani hospitals and implications for public health

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KEYWORDS
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Summary
Background: The rapid spread of metallo-β-lactamase producing clinical pathogens is a matter of great concern and with the addition of NDM-1 it poses more threat for public health as NDM-1 positive isolates show resistance to most of the antibiotics. The current study was carried out to determine the prevalence of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs), particularly NDM-1 in clinical multi-drug resistant isolates from two tertiary care hospitals in Pakistan.
Methods: A total of 356 clinical isolates were included in the study where 301 isolates were collected from the Pakistan Institute of Medical Sciences (PIMS), Islamabad and 55 were collected from the Mayo Hospital Lahore. The isolates were screened for ESBLs and MBLs production by phenotypic method and PCR was performed to detect the presence of bla_{VIM}, bla_{IMP} and bla_{NDM-1} genes.
Results: Out of 356 clinical isolates, 160 showed carbapenem resistance. Of these 160 isolates, 131 displayed MBLs production as accessed by combined disk method. In MBLs producing organisms, PCR amplification confirmed 31 (23.6%) isolates harboring bla_{NDM-1} gene, 33 (25.1%) isolates having bla_{VIM} gene and 2 (1.5%) isolates displaying bla_{IMP} gene. Plasmid profile analysis of NDM-1 positive organisms showed variable number of plasmids which were stable during serial passages in antibiotic free media. The prevalence of ESBL producing organisms was recorded to be 87.5%.
Conclusion: The results show a high level of NDM-1 positive organisms from variety of samples at both hospitals, implicating the spread of MBL genes in clinical isolates.

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Introduction

Carbapenem-hydrolyzing β-lactamases, known as carbapenemases, are an increasing concern for global health-care due to their association with resistance to β-lactam antibiotics. These enzymes are carried either on chromosome or acquired via plasmids. Ambler classification divides β-lactamases into four major classes; A, B, C and D. Metallo-β-lactamases (MBLs) fall in class B and are further divided into three subclasses: B1, B2, and B3 [1,2] where most acquired MBLs belong to the B1 class except AIM and SMB. The most common MBL enzymes include IMP, VIM, GIM, SIM [1] and NDM-1. The carbapenemase, New Delhi metallo-β-lactamase-1 (NDM-1), was first reported in 2009 in *Klebsiella pneumoniae* and *Escherichia coli* isolates from a Swedish patient who had sought medical care in New Delhi, India [3] though a retrospective study later reported that NDM-1 positive organisms were disseminated in Indian health care facilities in 2006 [4]. It was recently shown that NDM gene is a chimeric molecule and is a result of fusion of two different genes [5]. Like other acquired MBLs, NDM-1 can hydrolyze all β-lactam drugs except aztreonam, which is mostly inactivated by co-produced extended-spectrum β-lactamases (ESBLs) or AmpC β-lactamases [3]. The co-existence of bla_{NDM-1} with other resistance genes makes the majority of *Enterobacteriaceae* highly resistant against most of drugs except colistin and tigecycline. Since its discovery in 2009, NDM-1 has been disseminated in different countries and is being reported globally [6,7]. Because of its association with Indian sub-continent, a number of studies have included samples from Pakistan and India to evaluate the prevalence and spread of this enzyme. A study conducted on stool samples from patients at Military hospitals in Pakistan revealed an overall prevalence of 18.5% NDM-1 positive *Enterobacteriaceae* [8] with 27.1% from inpatients while 13.8% were from out patients. That study was conducted to evaluate a chromogenic media and was focused on fecal carriage of *Enterobacteriaceae* of NDM-1 hence included only stool samples. The present study was carried out to investigate the prevalence of ESBLs and MBLs carriage in different clinical samples. The aim was to isolate gram negative pathogens responsible for various infections, and investigate the antibiotic resistance pattern and the presence of bla_{NDM-1} along with bla_{VIM} and bla_{IMP} in these isolates in order to estimate the impact of MBLs producing pathogens on public health care.

Methods

Bacterial isolates

A total of 356 gram negative clinical isolates were collected from different specimens of hospitalized patients and outpatients. Fifty five isolates were collected from the Mayo Hospital Lahore while 301 isolates were taken from the Pakistan Institute of Medical sciences (PIMS), Islamabad, Pakistan. Of all isolates, 62% were from inpatients while 38% were from outpatients. Initial identification of the pathogens was done by gram staining and growth characteristics on McConkey agar and was later confirmed by conventional biochemical tests.

Phenotypic detection of carbapenemases

For the phenotypic detection of carbapenemase, Modified Hodge test (MHT) was performed on Mueller Hinton agar (MHA) using a 10 μg disk of meropenem according to Clinical Laboratory Standards Institute (CLSI) guidelines [9]. After 18 h incubation at 37 °C, a clover leaf-type indentation at the intersection of test organism showed a positive result (Fig. 1A). Extended spectrum β-lactamases (ESBLs) were detected by disk diffusion method [10] following Clinical and Laboratory Standards Institute (CLSI) recommendations. A 30 μg disk each of cefotaxime, ceftazidime, ceftriaxone and aztreonam were placed at 20 mm center to center from a 30 μg amoxicillin—calvulanic acid disk. After 18—24 h incubation, the plates were observed for an enhancement in zone diameter in the area between any of the four disks and amoxicillin—calvulanic acid disk (Fig. 1B). MBLs were detected by using combined disks diffusion method on MHA with 10 μg disk of meropenem and 0.5 M EDTA as an inhibitor. After 18—24 h incubation, the plates were observed for an increase of ≥7 mm in the zone of inhibition of combination disks compared to meropenem disks (Fig. 1C).

Molecular identification of MBL genes using PCR

DNAs were extracted from all MBLs producing strains by ethanol precipitation method. For bla_{NDM-1} detection, the extracted DNAs were subjected to single target PCR using forward primer; 5′-CAGCGCAGCTTGTCG-3′ and reverse primer; 5′-TCGCGAAGCTGAGCA-3′ sequences as already described in literature [7]. For bla_{IMP} and bla_{VIM} the primer sequences were used as already

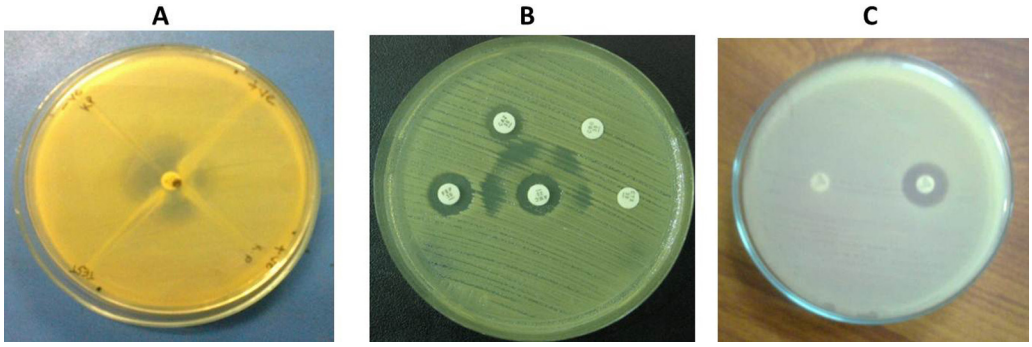

Figure 1 Phenotypic detection of carbapenemases. (A) Positive Modified Hodge test showing a clover leaf-type indentation at the intersection of test organism. (B) Disk diffusion method for extended spectrum β-lactamases (ESBLs) detection. (C) Combined disks diffusion method for MBL detection.

described in literature [11]. Briefly, for bla_{IMP}, forward primer; 5′-GAATAGAATGGTTAACTCTC-3′ and reverse primer; 5′-CCAAACCACTAGGTTATC-3′ were used while for bla_{VIM} detection forward primer; 5′-GTTTGGTCGCATATCGCAAC-3′ and reverse primer; 5′-AATGCGCAGCACCAGGATAG-3′ were used. Amplification products were visualized under UV light on 1% agarose gel electrophoresis.

Molecular identification of bla_{NDM-1} gene on plasmids

Plasmids DNA were isolated using plasmid isolation kit (Norgen BIOTEK) following the manufacturer's guidelines. The DNAs were separated on 0.7% agarose gel in TBE buffer along with DNA ladder of 1 kb (Fermentas) as size reference. The presence of bla_{NDM-1} on plasmids DNAs was detected by PCR using the method as described earlier.

Plasmid stability assay

The plasmids of NDM-1 positive strains were tested for stability. Serial passages of the isolates were performed on antibiotic (meropenem) free and 0.5 mg/L meropenem containing media. After every passage, the plasmid DNA was extracted and separated on agarose gel.

Antimicrobial susceptibility testing

The isolates were tested for antimicrobial susceptibility by using Kirby-bauer method according to CLSI guidelines [9]. *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 strains were used as control strains.

Results

Bacterial Strains

Out of 301 isolates from the Pakistan Institute of Medical Sciences (PIMS) Hospital, 122 were *E. coli*, 98 were *P. aeruginosa*, 69 were *K. pneumonia*, 9 were *Proteus sp.* and 3 were *Enterobacter sp.* Out of 55 isolates from the Mayo Hospital, 23 were *E. coli*, 14 were *P. aeruginosa*, 13 were *K. pneumonia* and 5 were *Proteus sp.*

Prevalence of ESBL and MBL producing gram negative clinical isolates

Among all isolates, 36.7% produced MBLs as shown by phenotypic test. Among 112 isolates of *P. aeruginosa*, 38 (33.9%) were MBL producers. Among 82 isolates of *K. pneumoniae*, 37 (45.12%) were positive for MBL production. Of 145 isolates of *E. coli*, 50 (34.48%) were MBL producers. The prevalence of ESBLs as accessed by phenotypic test and antibiotic resistance to third generation cephalosporins according to literature guidelines [12] was recorded to be 87.5%.

Prevalence of MBL genes in clinical isolates

Out of 356 isolates, 160 showed resistance to the antibiotic imipenem, out of which 131 were phenotypically positive for MBL production. Out of these MBL positive isolates, 31 (23.6%) were found to harbor bla_{NDM-1} gene by PCR amplification (Fig. 2) where 13 were *K. pneumoniae*, 3 were *E. coli* and 15 were *P. aeruginosa*. These isolates came from different patients, sample source, and hospital wards and 18 were from inpatients while 13 were from outpatients. The detailed information is given in Table 1. PCR for bla_{VIM} showed positive result for 33

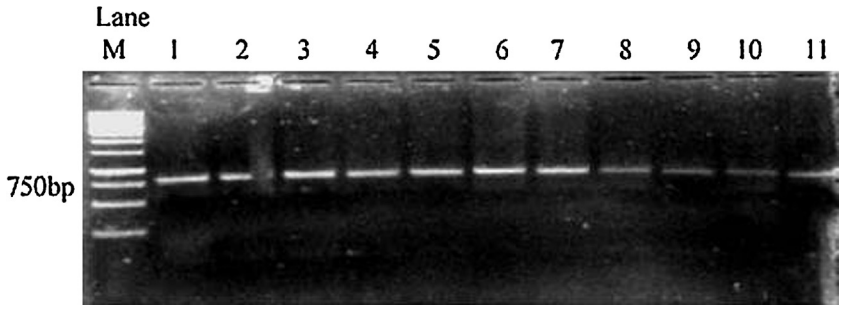

Figure 2 A representative gel showing PCR amplification product of bla_{NDM-1} gene at 784 bp in clinical isolates. The molecular size marker used is 1000 bp ladder.

(25.1%) of the total MBLs positive strains where 21 were from inpatient and 12 were from outpatient. Of total MBL producing *P. aeruginosa* 14 (36.8%) were positive for bla_{VIM} where 11 were from inpatients and 3 were from outpatients. Out of MBL positive *K. pneumoniae* 11 (29.4%) were positive for bla_{VIM} where 9 were from outpatients and 2 were from inpatients. Of all MBL positive *E. coli* 8 (16%) were found to carry bla_{VIM} gene and they all were from inpatient. Out of all MBLs positive isolates, only two were positive for bla_{IMP} gene which were *E. coli*.

Table 1 Data of patients, sample source and antibiotic resistance pattern for NDM-1 positive isolates.

Sample ID	Age	Gender	Specimen	Ward
2K.p	62 years	M	Pus	MW2
3K.p	30 years	M	CVP tip	Medical ICU
4K.p	18 years	M	Pus	SW1
5K.p	17 years	F	Blood	Gastroenterology OPD
8K.p	54 years	M	CVP tip	Medical ICU
9K.p	40 years	F	High vaginal swab	Emergency OPD
12K.p	45 years	M	Pus	SW6
22K.p	27 years	F	Blood	Medical ICU
27K.p	71 years	F	Urine	Nephrology OPD
29K.p	80 years	M	Urine	Plumnology OPD
42K.p	40 years	F	Pus	Emergency OPD
10E	40 years	F	Urine	MW1
9 P.a	34 years	F	Urine	MW1
10 P.a	55 years	F	Pus	Emergency OPD
12 P.a	85 years	M	Catheter tip	Emergency OPD
17 P.a	60 years	F	Urine	Neurosurgery OPD
19 P.a	75 years	M	Tracheal secretions	Emergency OPD
22 P.a	85 years	M	Tracheal secretions	Emergency OPD
26 P.a	30 years	M	Tissue	Medical ICU
29 P.a	29 years	M	Pus	SW1
55 P.a	14 years	M	Tracheal secretions	SW3
61 P.a	45 years	M	Pus	SW6
70 P.a	20 years	M	Tracheal secretions	Medical ICU
71 P.a	50 years	M	Catheter tip	Cardiology ward
46	35 years	M	Urine	OPD
44	36 years	F	Urine	OPD
48	40 years	M	Catheter tip	Medical ward
52	49 years	F	Urine	Medical ward
28	52 years	M	Catheter tip	Medical ward
23	46 years	F	Urine	Medical ward
25	60 years	F	Urine	OPD

ICU = intensive care unit, OPD = outpatient department, SW = surgical ward.

Table 2 Antibiotic resistance pattern of subsets of NDM-1 positive, NDM-1 negative but MBL positive, ESBL positive and ESBL negative isolates.

Antibiotic	NDM-1 positive ($n = 31$)	NDM-1 negative but MBL positive ($n = 53$)	ESBL positive ($n = 35$)	ESBL negative ($n = 42$)
Meropenem	96.7%	100%	20%	7.14%
Imipenem	96.7%	100%	20%	7.14%
Amoxicillin/calvulanic acid	90.3%	54.7%	94.2%	80.9%
Ceftriaxone	93.5%	71.6%	91.4%	69%
Ceftazidime	96.7%	81.1%	91.4%	85.7%
Cefotaxime	90.3%	78%	88.5%	80.9%
Aztreonam	90.3%	54.7%	82.8%	64.2%
Amikacin	67.7%	54%	NT	NT
Colistin	3.2%	NT	NT	NT

NT = not tested.

Plasmid profile analysis

Plasmid profile analysis showed that all NDM-1 positive isolates carried plasmids of different number and size with most of the plasmids displaying a size of >10 kb. Plasmids DNAs were amplified to check the presence of bla_{NDM-1} gene and 7 out of 31 showed positive result for bla_{NDM-1} gene. Of these 7 isolates, 3 were *P. aeruginosa*, 2 were *E. coli* and 2 were *K. pneumonia*.

Plasmid stability assay

We assessed plasmid stability by serial passage of NDM-1 positive isolates on antibiotic free and 0.5 mg/L meropenem containing media. Passages were done every 24 h up to 15 passages. All the plasmids remained stable up to passage 7. Toward the 8th passage, one plasmid of *Pseudomonas* was lost while at 10th passage; one plasmid of *E. coli* was lost. All other plasmids remained stable upto15 passages.

Antimicrobial resistance profile

The resistance against third generation extended spectrum cephalosporins was as following: ceftazidime (85.1%), ceftriaxone (83.5%), cefotaxime (87.3%). Antimicrobial resistance pattern of 31 bla_{NDM-1} positive isolates were as following: meropenem (96.9%), imipenem (96.9%), amoxicillin/calvulanic acid (90.3%), ceftazidime (96.9%), ceftriaxone (93.5%), cefotaxime (90.3%), aztreonam (90.3%), amikacin (67.7%), and colistin (3.2%). The antibiotic resistance pattern of a subset of NDM-1 negative but MBL positive isolates ($n = 53$) were as following: meropenem (100%), imipenem (100%), amoxicillin/calvulanic acid (54.7%), ceftazidime (81.1%), ceftriaxone (71.6%), cefotaxime (78%), cefuroxime (30.1%), aztreonam (54.7%), amikacin (54%), norfloxacin (35.8%), polymyxin B (0%), and piperacillin/tazobactam (5.6%). The comparative resistance pattern of different categories of isolates is given in Table 2.

Discussion

Since the early cases recorded from India and Pakistan [3,4,6], NDM-1 positive organisms have been reported widely [8,13—15]. We report here the prevalence of NDM-1 producing gram negative pathogens at two tertiary care hospitals in Pakistan. Of 356 isolates, 131 (36.7%) produced MBL, out of which 31 (23.6%) were found to be positive for bla_{NDM-1} by PCR. These isolates were taken from different types of specimen such as tracheal secretions, urine, blood, catheter tips, CVP tips, and pus and reflect the dissemination of NDM-1 in pathogens coming from different sources. The plasmid analysis shows that bla_{NDM-1} gene in tested isolates is carried by different plasmids with plasmid carriage of 22%. Most of the strains carried several plasmids which were of >10 kb in size.

It was also observed that bla_{NDM-1} carrying organisms came mostly from inpatients as compared to outpatients. This suggests that NDM-1 positive pathogens are more associated with carriage isolates than the infection ones. The emergence of NDM-1 positive strains presents major health problems as these strains show resistance against commonly used antibiotics, leaving the clinicians with limited options. In the present study, most of NDM-1 positive strains were found to be resistant to the effects of meropenem, imipenem, amoxicillin/calvulanic acid, ceftazidime, ceftriaxone, cefotaxime and azetreonam while highest susceptibility was observed for colistin which is in accordance with the prior studies [3,6,7]. The isolates showed 32.2% susceptibility to amikacin which

relates to the findings of Poirel et al., [16] where a single *E. coli* strain was found susceptible to the effects of amikacin. This is an interesting observation since most NDM-1 isolates also carry 16S methylase genes giving resistance to all aminoglycosides. This suggests this combination is not yet common in Pakistan and amikacin can be effective in treating such strains but it should be considered that its use is tightly regulated to avoid development of resistance against this antibiotic.

Besides bla_{NDM-1}, the study also detected the presence of bla_{IMP} and bla_{VIM} in MBL producing organisms and found 25.1% bla_{VIM} and 1.5% bla_{IMP} containing organisms. In a recent study from India, the prevalence of bla_{VIM} was reported to be 18.6% in carbapenem resistant gram-negative bacteria [17] and in another it was reported to be 61.8% in MBL producing bacteria [18]. The difference in prevalence rates could be due to the difference of sample size studied. The prevalence rate of bla_{IMP} correlated with the report from India [18].

The study also detected the production of ESBLs and MBLs by phenotypic tests. For an overall prevalence of MBLs, we found 33.9% MBL producing *P. aeruginosa* which is lower than another report from Pakistan where it was reported to be 59.5% [19]. On the other hand, in the same year, it was reported to be 8.05% in a study conducted in India [20]. The MBL producing *K. pneumonia* were 45% and sporadic MBLs producing *K. pneumonia* have been reported globally [21—23]. For *E. coli*, the prevalence of MBL was found to be 34.4% which is alarmingly high. This high prevalence requires immediate consideration. In another study, a high prevalence of up to 41% MBL producing *E. coli* strains was reported [24].

In our setup the high rate of MBL producing pathogens could be due to poor hygiene conditions and irrational use of broad spectrum antibiotics and this irrational use would have left only carbapenems to be the effective antibiotics which are starting to be ineffective as well. The data about the mortality rate in these health care facilities is not available so we cannot comment on whether the mortality rate has been affected by these resistant pathogens or not.

Conclusions

A considerable prevalence of bla_{NDM-1} carrying gram negative pathogens in patients coming to PIMS and Mayo Hospitals indicates the need for the countrywide screening of hospitals and community to evaluate the exact prevalence of bla_{NDM-1} carrying pathogens in Pakistan. A protocol should be introduced in hospitals if such a case may occur. Also, to avoid the risk of hospital acquired and patient to patient spread of these pathogens, proper hygiene should be implemented. Regular training of nursing staff should be practiced so the spread of pathogens can be decreased.

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Competing interests

None declared.

Ethical approval

Not required.

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