DETECTION OF METALLO- β -LACTAMASE PRODUCTION IN GRAM-NEGATIVE BACILLI ISOLATED FROM PATIENTS ATTENDING A TERTIARY CARE HOSPITAL IN BIHAR

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ABSTRACT

BACKGROUND

MBL (Metallo-beta-lactamase) production by GNB (Gram-negative bacilli) has recently emerged as one of the most worrisome resistance mechanisms. MBL producing GNB have been reported from different parts of the world. There is no effective MBL inhibitor developed to combat them in vivo, although in vitro metal chelators like EDTA can help augment their detection by phenotypic methods. Carbapenems are the last resort in treating infections caused by GNB, occurrence of MBL genes in these isolates leaves us with few therapeutic options like polymyxins which are potentially toxic compounds.

The aim of this study was to detect the production of MBL in GNB obtained from patients attending Katihar Medical College and Hospital in Katihar district of Bihar.

MATERIALS AND METHODS

GNB were identified as per standard protocol and were tested against different antimicrobial agents on MHA (Mueller-Hinton Agar) by modified Kirby-Bauer Disc Diffusion Technique. Carbapenemase production was detected by MHT (Modified Hodge Test). MBL detection was done with DDST (Double Disk Synergy Test) and DPT (Disk Potentiation Test) in all 103 GNB isolates included in our study.

RESULTS

Out of a total of 103 GNB isolated 18 were detected as MBL producers. Maximum MBL production was seen in *P. aeruginosa* isolates 13/22 (72.22%) followed by *E. coli* 3/51 (16.67%) and *K. pneumoniae* 2/19 (11.11%). All the three methods for detection of MBL were found equally useful.

CONCLUSION

MBL production was found to be fairly high in our region, especially in *Pseudomonas aeruginosa*, in which 59.09% were MBL producers. These findings are a cause for much alarm, as this college is situated in a rural area catering mainly to rural patients, as regards to the isolation of such a high number of MBL producing isolates. All three phenotypic tests performed equally. Out of the three methods tested, the DPT was the easiest to perform. It would be wise to perform any one of these tests routinely in diagnostic laboratories especially on strains showing resistance to any one of the carbapenem antibiotics.

KEYWORDS

Gram-negative Bacilli, MBLs.

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BACKGROUND

MBLs are enzymes that have the ability to hydrolyse β -lactam antibiotics which require zinc at active site. MBLs have recently emerged as one the most worrisome resistance mechanisms as MBL genes are located on integron structures

that reside on mobile genetic elements such as plasmids or transposons, which gives them ability for widespread dissemination.¹

Acquisition of MBL gene invariably mediates broad spectrum β -lactam resistance in *Pseudomonas aeruginosa*, but the level of in vitro resistance in *Acinetobacter* sp. and *Enterobacteriaceae* is less dependable. Their clinical significance is further embellished by their ability to hydrolyse all β -lactams and by the fact that currently there is no clinical inhibitor, nor is there likely to be for the foreseeable future.

The first indication of mobile MBLs was with the discovery of *Pseudomonas aeruginosa* strain GN17203 in Japan in 1988.²

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The belief that mobile MBLs genes were solely a distant Japanese problem was negated with the advent of bla-IMP-2 in 1997 and bla-IMP-5 in 1998 from Italy and Portugal, respectively.²

VIM was described first in Verona, Italy, from a *Pseudomonas aeruginosa* isolate. A clinical *Pseudomonas aeruginosa* isolate from 1997 from Sao Paulo, Brazil, was analysed as part of the SENTRY surveillance program and shown to contain a novel gene, designated bla-SPM-1 (Sao Paulo MBL).²

In 2002, five *Pseudomonas aeruginosa* isolates were recovered from different patients from a medical site in Dusseldorf, Germany and shown to possess a novel class-B β -lactamase designated GIM-1 (German imipenemase).²

New Delhi metallo-beta-lactamase-1 (NDM-1) has been reported from many countries and its origin has been traced to Asia. This MBL is plasmid borne and has propensity to spread between species through horizontal transfer.

The isolates produce zinc dependent metallo-beta-lactamases which hydrolyse beta-lactam ring of the antibiotics and render them inactive. They require zinc for their activity. The detection of MBL has become very important in all laboratories to determine the ultimate clinical outcome in all those patients who are infected with MBL producing strains. Even smaller laboratories must try and identify these MBL strains. Their wide spectrum of activity leaves us with few therapeutic options with no effective MBL inhibitor developed to combat them in vivo, although in vitro metal chelators like EDTA can help augment their detection by phenotypic methods. Clinical and Laboratory Standards Institute⁴ has not laid down any specific guidelines though there are several screening methods for detection and confirmation of MBL production in GNB. Currently the most accepted method is MBL E-Test. However, due to high cost many laboratories use alternative methods as the DDST and DPT.²

MATERIALS AND METHODS

Patients of both sexes and all age groups were included in this study after obtaining Institutional Ethical Committee clearance and informed consent from each and every patient. All samples were subjected to microscopic examination and cultured on standard laboratory media. GNB were identified as per standard protocol.³ Antibiotic susceptibility tests were put up by modified Kirby-Bauer's disc diffusion method⁴ using a panel of antibiotics disks obtained from HiMedia (Mumbai).

MBL Screening

Screening for MBL production was done in all isolates by MHT, DDST and DPT.

Modified Hodge Test⁵

A suspension of 0.5 McFarland standard of the indicator organism (*E. coli* ATCC 25922) was prepared. It was inoculated on the surface of a MHA plate. The test strain was heavily streaked from the centre to periphery of the plate. The plate was allowed to stand for 15 minutes. An imipenem 10 μ g disk was placed in the centre. 10 μ L of 50 mM zinc sulphate (140 μ g) was added to the imipenem disks. The plates were incubated overnight at 37°C. After overnight incubation the plates were observed for the presence of

"clover leaf" shaped zone of inhibition. The plates with such zones were interpreted as Modified Hodge test positive.

Disc Potentiation Test⁶

A 0.5 McFarland standard suspension from log phase growth of the test organism was prepared in Mueller Hinton broth or normal saline. This suspension was inoculated onto the plates of MHA. Two 10 μ g imipenem disks were placed on the plate and 5 μ L EDTA (750 μ g) was added to one of the disks. The plates were incubated at 37°C for 18 hours.

(Solution of 0.5 M EDTA was prepared by dissolving 186.1 g of disodium EDTA. 2H₂O in 1000 mL distilled water and pH was adjusted to 8.0 by using NaOH. The mixture was sterilized by autoclaving)

Reading

The inhibition zones of the imipenem and imipenem-EDTA disks were measured with the help of a scale. An increase in the zone diameter ≥ 7 mm in presence of EDTA indicated the presence of MBL. Figure 1.

Double Disk Synergy Test⁷

A suspension equivalent to 0.5 McFarland standards from a log phase of the test organism was prepared in Mueller-Hinton broth. This was inoculated on MHA plate and 30 μ g ceftazidime disk and 10 μ g imipenem disk was applied on the plates. The distance between the disks was kept at 4 to 5 cm. A blank filter paper disk was placed near the ceftazidime and imipenem disk with a centre to centre distance of 1.0 to 2.5 cm. 5 μ L of EDTA was added to the filter paper disk and plates incubated at 37°C overnight. After overnight incubation, even a small synergistic zone of inhibition was interpreted as positive. Figure 2.

RESULTS AND OBSERVATIONS

Out of a total of 3056 samples received in the laboratory from January 2015 to June 2016, 668 samples showed growth of different bacterial isolates. 223/668 (33.38%) showed growth of GPC in pure culture, 342/668 (51.20%) showed mixed growth and 103/668 (15.42%) showed growth of GNB in pure form which were processed for further study. Maximum number of cases was seen in the age group of 21 to 30 years 28/103 (27.18%) followed by the age group of 31 to 40 years 25/103 (24.27). In females, maximum number of GNB was isolated from age group 21-30 years 22/28 (78.57%) whereas in males, maximum number of GNB were isolated from age group 0-10 years 14/21 (66.67%). The overall male to female ratio was 1:1.15.

Majority of cases with GNB infections were from Obstetrics and Gynaecology department 35.92% (37/103) followed by Surgery 22.33% (23/103), Paediatrics 20.38% (21/103) and Medicine 19.42% (20/103).

Meropenem resistance was seen in 72/103(69.90%) of GNB isolates and 27/103(22.33%) isolates were found to be imipenem resistant.

The detection rates of carbapenemases production by three different phenotypic method namely MHT, DPT and DDST was 17.47% (18/103). All the three phenotypic methods of MBL detection showed 100% correlation. Table 1.

Maximum production of MBL was seen in *Pseudomonas aeruginosa* isolates 13/18 (72.22%). This was followed by *E. coli* 3/18 (16.67%) and *Klebsiella pneumonia* 2/18 (11.11%). Other Gram-negative isolates did not show the presence of MBLs. Table 2.

Majority of MBL positive strains 88.88% (16/18) were isolated from urine. None of the MBL producing isolates were from the out-patient department. Maximum numbers of MBL positive isolates were from Surgery in-patients 62.50% followed by Obstetrics and Gynaecology in-patients 50.00%.

GNB (other than *Pseudomonas aeruginosa*) showed maximum resistance seen to cefuroxime 73/81 (90.13%) followed by cefotaxime 71/81 (87.65%), ertapenem 58/81 (71.60%), meropenem 56/81 (69.14%). 18.82% of strains were resistant to imipenem. No resistance was seen with Colistin which showed 100.00% sensitivity.

Pseudomonas aeruginosa isolates showed 100.00% resistance to cefotaxime, 68.19% of strains showed resistance to amikacin, meropenem and netilmicin, followed by levofloxacin, gentamicin and ceftazidime 59.09% each. Resistance to imipenem was seen in 54.55% and no resistance was seen with Colistin which showed 100.00% sensitivity.

Resistance to amikacin, cefotaxime, ertapenem, meropenem, cefuroxime, levofloxacin, netilmicin, gentamicin and ceftazidime was 100.00% among MBL producers, followed by imipenem 83.33% and nitrofurantoin 60.00% which was tested against urinary isolates only. In MBL negative cases, the maximum resistance seen with cefotaxime 88.23% and cefuroxime 78.82% followed by meropenem 63.52%, levofloxacin 55.29%, netilmicin 28.57%, gentamicin 23.52% and amikacin 15.29%. Table 3.

Name of Test	Positive (%)	Negative (%)
Modified Hodge Test	18 (17.47)	85 (82.52)
DPT	18 (17.47)	85 (82.52)
DDST	18 (17.47)	85 (82.52)

Table 1. Carbapenemase Production by Gram-negative bacilli

GNB	MBL +ve (%)	MBL -ve (%)	TOTAL
<i>Escherichia coli</i>	3 (16.67)	48 (56.47)	51 (49.51)
<i>K. pneumoniae</i>	2 (11.11)	17 (20.00)	19 (18.44)
<i>P. aeruginosa</i>	13 (72.22)	9 (10.58)	22 (21.35)
<i>C. freundii</i>	0.00	4 (4.70)	4 (3.88)
<i>E. cloacae</i>	0.00	1 (1.17)	1 (0.97)
<i>P. mirabilis</i>	0.00	5 (5.88)	5 (4.85)
<i>A. baumannii</i>	0.00	1 (1.17)	1 (0.97)
Total	18	85	103

Table 2. MBL Production in Various Gram-negative bacilli

Antibiotic	MBL Positive n= 18		MBL Negative n=85	
	No.	%	No.	%
Amikacin	18	100.00	13	15.29
Cefotaxime	18	100.00	75	88.23
Meropenem	18	100.00	54	63.52
Cefuroxime‡	05	100.00	67	78.82
Imipenem	15	83.33	16	18.82
Levofloxacin	18	100.00	47	55.29
Colistin	00	00.00	00	00.00
Nitrofurantoin*	03	60.00	28	41.79
Netilmicin†	13	100.00	04	28.57
Gentamicin	18	100.00	20	23.52
Ceftazidime†	13	100.00	00	00.00

Table 3. Resistance Pattern of MBL-positive and MBL-negative Isolates

*Only for Urinary Isolates of GNB, † Only for *P. Aeruginosa*,

‡ Used only for GNB other than *Pseudomonas*

Figure 1. Showing Disk Potentiation Test of an MBL-Positive GNB Isolate

Figure 2. Showing Double Disk Synergy Test in case of MBL-Positive GNB Isolate

DISCUSSION

Maximum MBL production was seen in *Pseudomonas aeruginosa* isolates 72.22% followed by *Escherichia coli* 16.67% and *Klebsiella pneumoniae* 11.11%. MBL production was not seen with other isolates viz. *Citrobacter freundii*, *Enterobacter cloacae*, *Proteus mirabilis* and *Acinetobacter baumannii*. Out of a total of 22 *Pseudomonas aeruginosa* isolates, 13 were MBL producers, 59.09% as compared to 8.05% reported by other authors.⁸ This study; however, was conducted 8 years back and probably indicates an increasing trend in isolation of MBL producers. As far as Enterobacteriaceae is concerned out of a total 70 *E. coli* and *K. pneumoniae* isolates, only 7.14% (5/70) were found to be MBL producers. Few other authors on the other hand have reported that 25.53% of their Enterobacteriaceae isolates were MBL producers which is fairly high compared to the present study.⁹

In the present study, three different types of phenotypic tests were done for detection of carbapenemases viz. the MHT, DPT and DDST. All these tests showed 100% correlation with each other. However, as far as ease of performance of each of these tests is concerned the DPT was the easiest to perform. Other authors also emphasised that Imipenem-EDTA Combined Disk Test can be used as a convenient and cost effective MBL screening method in clinical Microbiology laboratory.¹⁰

A very high degree of resistance was seen with MBL positive strains. All 18 MBL positive showed 100% resistance

to amikacin, cefotaxime, meropenem, levofloxacin and gentamicin. In addition to this, all MBL positive *Pseudomonas aeruginosa* isolates were resistant to netilmicin and ceftazidime. Colistin was the only antibiotic to which all MBL producers were sensitive.

Other authors have also reported 100% resistance to gentamicin, co-trimoxazole, cefotaxime, ceftazidime in their *E. coli* isolates, which is similar to this study.¹¹

Other studies on NDM-1 producing enteric isolates reported that 61.64%, 69.23% and 76.92% of their isolates were resistant to amikacin, tobramycin and gentamicin respectively.¹²

CONCLUSION

MBL production was found to be fairly high in our region, especially in *Pseudomonas aeruginosa* in which 59.09% were MBL producers. MBL production was also relatively high in *Klebsiella pneumoniae* isolates. These findings are a cause for much alarm, as this college is situated in a rural area catering mainly to rural patients, as regards to the isolation of such a high number of MBL producing isolates. This poses a genuine threat, as far as patient care is concerned, as majority of these MBL were found to be resistant to almost all the antibiotics against which they were tested except Colistin.

As far as detection of MBLs by phenotypic methods is concerned, in our study all three phenotypic tests performed equally. Out of the three methods tested, the DPT was the easiest to perform and has the potential to be incorporated in even small diagnostic laboratories without any constraints on material, infrastructure and manpower. It would be wise to perform any one of these tests routinely in diagnostic laboratories especially on strains showing resistance to any one of the carbapenem antibiotics.

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