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Metallo-beta-lactamase Producing Gram-negative Bacterial isolates

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ABSTRACT

Background: The global emergence of metallo-β-lactamase (MBL) producing bacterial isolates causing lower respiratory tract infection (LRTI) has resulted in fewer therapeutic options in treatment modalities. However, to our knowledge no studies regarding MBLs had been done so far in Nepal. Therefore, this study was carried out to assess the current level of MBL producing bacterial isolates in our setup.

Methods: This was a cross-sectional study conducted over a period of six months (June to November 2008) at Bacteriology laboratory of a teaching hospital. A total of 1120 specimens representing lower respiratory tract (sputum, endotracheal secretion and bronchial washing) were processed from outpatients and inpatients, with suspected LRTI, at TUTH. The specimens were collected and processed according to the standard methodology. Combination disk method and Double disk synergy test methods were used for the detection of MBL producing isolates.

Results: Respiratory pathogens were recovered from 497 (44.4%) of suspected cases. Among these, gram-negative bacteria were observed in 448 (84.0%). Multidrug resistance (MDR) was found in 286 (53.7%) of the total bacterial isolates. MBL was present in 6 (1.3%) of the total 448 gram-negative isolates. MBL was detected by both DDST and CD methods in 3 isolates each of *Pseudomonas aeruginosa* and *Acinetobacter* spp. from inpatients. All MBL producers were MDR.

Conclusions: MBL-producing gram negative bacteria were detected from LRTI isolates in this study and this data can be used as base-line information of this novel type of β-lactamase in our setup.

Keywords: combination disk method; Gram-negative bacteria; lower respiratory tract infection; metallo-β-lactamase.

INTRODUCTION

Today, antibiotics remain the front-line therapy for conquering bacterial infections. Almost three-quarters of all antibiotic consumption are for respiratory tract infections.[1] Beta (β)-lactams remain a cornerstone for antimicrobial chemotherapy of a large number of bacterial infections, but their efficacy has been increasingly thwarted by dissemination of acquired resistance among pathogenic bacteria.[2] The exposure of bacterial strains to a multitude of β–lactams has induced mutation of β-lactamase in many bacteria, expanding their activity even against carbapenems by the production of metallo-beta-lactamase (MBL) resulting into fewer therapeutic alternatives.[3]

MBLs represent a formidable challenge to antimicrobial chemotherapy due to their extremely broad substrate specificity and mechanistic uniqueness: most β-lactams (including carbapenems and expanded-spectrum cephalosporins) are efficiently degraded by these

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enzymes, while conventional β-lactamase inactivators are useless against them.[2] Therefore, the detection of MBL producing bacteria is crucial. In our setup, since we are encountering many carbapenem resistant bacteria from patients suspected lower respiratory tract infection (LRTI), this study was intended to find out the status of MBL-producing bacterial isolates causing LRTI.

METHODS

A cross-sectional study was conducted at the Bacteriology Laboratory of Tribhuvan University Teaching Hospital (TUTH), Kathmandu from June 2008 to November 2008. This study was approved by Institutional Review Board of Institute of Medicine. Data were analyzed using Microsoft Excel 2007. A total of 1120 lower respiratory tract representing specimens (sputum, Endotracheal (ET) secretion and bronchial washing) received for culture and sensitivity in the laboratory from outpatients and inpatients, and which met the criteria as recommended by American Society for Microbiology (ASM) were included in the study.[4] The specimens were cultured on Chocolate agar (CHA), 5% Sheep Blood agar (BA) and MacConkey agar (MA) (Oxoid, UK) plates. On the CHA, bacitracin disk (10 Unit) and optochin disk (5 µg) (Oxoid, UK) were placed at primary and secondary inoculation to screen *H. influenzae* and *S. pneumoniae* respectively. The CHA plates were incubated in CO_2 incubator (10% CO_2) at 37 ^{0}C for 24 hours while BA and MA plates were incubated at 37 ^{0}C for 24 hours in aerobic atmosphere.

Identification of isolated organisms: Firstly, pure form of the culture was obtained from the primary culture by using purity plate and then it was processed for different biochemical tests following standard microbiological procedures.

Antibiotic susceptibility testing: The susceptibility test of the pathogens isolated from the clinical specimens against different antibiotics was done by the standard disk diffusion technique of Kirby-Bauer method as recommended by Clinical and Laboratory Standards Institute (CLSI).[5] CHA and BA were used for *H. influenzae* and *S. pneumoniae* respectively to perform sensitivity test. *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were also tested in every set of experiment, in parallel, as a part of quality control. In this study if the isolates were resistant to at least three classes of first-line antimicrobial agents, they were regarded as MDR.[6]

Tests for MBL-production in Gram-negative isolates

Screening test: The isolates were subjected for MBL detection when the zone of inhibition (ZOI) for ceftazidime (CAZ) (30µg) was <18mm. The sensitivity or resistivity pattern to imipenem (IPM) (10µg) and/or meropenem (MEM) (10µg) were not considered for MBL detection as bacteria might harbour "hidden MBL" and if only the carbapenem resistant phenotypes were considered, then such hidden MBL carrying isolates would be missed. A suspension of bacteria equivalent to 1:10 dilution of 0.5 McFarland standard was prepared and was swabbed on to two MHA plates. Then two methods were applied.

Combination disk (CD) method:[7] Two IPM disks (10 µg), one containing 10 µl of 0.1 M (292 µg) anhydrous Ethylenediamine-tetraacetic acid (EDTA) (Sigma Chemicals, St. Louis, MO), were placed 25 mm apart (center to center). An increase in zone diameter of >4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for an MBL.

Double disk synergy test (DDST) method: An IPM (10 µg) disk was placed 20 mm (center to center) from a blank disk containing 10 µl of 0.1 M (292 µg) EDTA. Enhancement of the zone of inhibition in the area between the two disks was considered positive for an MBL.

For MBL test standardization, *P. aeruginosa* ATCC 27853 and *P. aeruginosa* PA 105663 were used as negative and positive controls respectively.

RESULTS

Number of specimens and result pattern: A total of 1162 specimens from lower respiratory tract (LRT) were received in the bacteriology laboratory for culture and sensitivity from June to November 2008. Specimens processed in this study were sputum (n=1081), ET secretion (n=61) and bronchial washing (n=20). Out of total 1081 sputum specimens, only 1039 specimens were further processed while the remaining 42 specimens were rejected as they implied oral contamination.

Among the total processed specimens (n=1120), only 497 showed significant growth (44.4%) and among the different specimens, ET secretion showed the highest microbial growth as shown in Table 1.

Table 1. Pattern of growth in individual LRT specimens

Specimen	Significant growth n (%)	No significant growth n (%)	No growth n (%)
Sputum (N=1039)	454 (43.7)	574 (55.3)	11 (1.0)
ET secretion (N=61)	41 (67.2)	0 (0.0)	20 (32.8)
Bronchial washing (N=20)	2 (10.0)	0 (0.0)	18 (90.0)

Out of total 497 microbial growth, there was significant polymicrobial growth in 43 specimens (8.7%) while

predominant monomicrobial growth was seen in 454 cases (91.3%).

Among the 533 bacterial isolates, *Haemophilus influenzae* was the predominant organism, followed by *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus baumannii* complex, *Streptococcus pneumoniae* and others (Table 2). Moreover, six isolates of *Candida albicans*/*C. dublinensis* were also detected.

Table 2. Distribution of bacterial isolates from LRT specimens

Organisms	n (%)
Gram-Positive Bacteria	
Streptococcus pneumoniae	46 (8.6)
Staphylococcus aureus	33 (6.2)
Enterococcus spp.	5 (0.9)
Streptococcus pyogenes	1 (0.2)
Gram-Negative Bacteria	
Haemophilus influenzae	112 (21.0)
Klebsiella pneumoniae subspp. pneumoniae	101 (19.0)
Pseudomonas aeruginosa	69 (13.0)
Acinetobacter calcoaceticus baumannii complex	60 (10.9)
Escherichia coli	37 (6.9%)
Moraxella catarrhalis	22 (4.1)
Pseudomonas spp.	22 (4.1)
Citrobacter freundii	12 (2.3)
C. koseri	3 (0.6)
Morganella morganii	3 (0.6)
Enterobacter aerogenes	2 (0.4)
Serratia marcescens	2 (0.4)
A. lwoffii	2 (0.4)
K. pneumoniae subspp. ozanae	1 (0.2)
Total	533

Antibiogram of Pseudomonads

Even carbapenem was not much effective against Pseudomonads as around 15% isolates were resistant to it (Table 3).

Table 3. Antibiogram of Pseudomonads (n=91)

Antibiotics	Sensitive n (%)	Resistant n (%)
Ciprofloxacin	61 (67.0)	30 (33.0)
Gentamicin	60 (65.9)	31 (34.07)
Amikacin	71 (78.0)	20 (22.0)
Ceftazidime	43 (47.3)	48 (52.8)
Cefepime	38 (41.8)	53 (58.2)
Piperacillin	51 (56.0)	40 (44.0)
Piperacillin-Tazobactam	75 (82.4)	16 (18.0)
Cefoperazone-Sulbactam	76 (83.5)	15 (16.5)
Meropenem	75 (82.4)	16 (17.6)
Imipenem	77 (84.6)	14 (15.4)

Antibiogram of *Acinetobacter* spp.

For *Acinetobacter* spp. too, the antibiotic activity was very poor (Table 4).

Table 4. Antibiogram of *Acinetobacter* spp. (n=62), *Acb* complex (n=60) and *A. lwoffii* (n=2)

Antibiotics	Sensitive n (%)	Resistant n (%)	Intermediate n (%)
Amoxycillin	4 (6.5)	58 (93.6)	-
Ciprofloxacin	22 (35.5)	40 (64.5)	-
Cotrimoxazole	15 (24.2)	47 (75.8)	-
Gentamicin	23 (37.1)	38 (61.3)	1 (1.6)
Amikacin	28 (45.2)	33 (53.2)	1 (1.6)
Ceftriaxone	8 (12.9)	53 (85.5)	1 (1.6)
Ceftazidime	11 (17.7)	50 (80.7)	1 (1.6)
Cefepime	11 (17.7)	50 (80.7)	1 (1.6)
Amoxycillin-Clavulanate	7 (11.3)	55 (85.5)	-
Piperacillin	14 (22.6)	48 (77.4)	-
Piperacillin-Tazobactam	26 (41.9)	36 (58.1)	-
Cefoperazone-Sulbactam	28 (45.2)	34 (54.8)	-
Meropenem	31 (50.00)	31 (50.0)	-
Imipenem	40 (64.5)	22 (35.5)	-

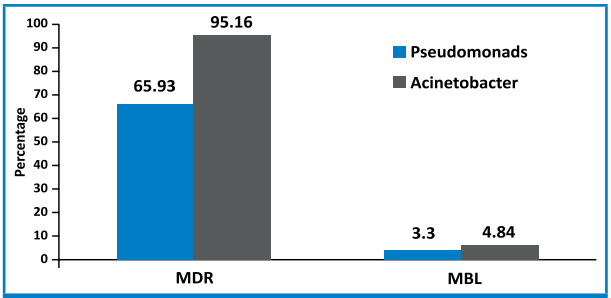

Figure 1. MDR and MBL production in Pseudomonads and *Acinetobacter* spp.

MDR and MBL-production in Pseudomonads and *Acinetobacter* spp.

MBL-producers accounted for 1.3% of gram-negative bacterial isolates. Of the total 91 *Pseudomonas* isolates, 60 (65.9%) were MDR. Similarly 3 (3.3%) were MBL-producers. All the MBL-producers were MDR. Around 95% of *Acinetobacter* isolates were MDR while 4.8% were MBL-producer (Figure 1). Non-fermentative bacteria were more MDR (77.8%) than *Enterobacteriaceae* (68.9%) isolates. MBL-production was observed only among non-fermentative bacteria (3.9%).

Distribution of MBL- producers in different wards

Isolates from Intensive care unit (n=4), Cardiac care unit and Medical wards (each n=1) were found to carry MBL-

encoded resistance property.

Comparison of DDST Method and CD method for MBL detection

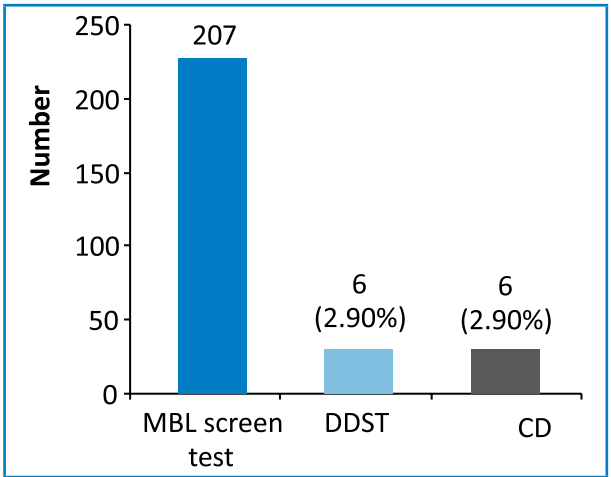

Figure 2. Comparison of DDST and CD methods for MBL detection

Among the 207 gram-negative bacterial isolates subjected to two different phenotypic MBL detection methods, both DDST and CD methods detected 6 (2.9%) MBL cases (Figure 2).

MBL production with respect to antibiotic resistance in *Acb* complex

When there was resistance to both imipenem and meropenem, 2 cases were found to be MBL-producing, while in 1 case MBL was present though the isolate appeared to be sensitive to imipenem and meropenem. Based on the finding of resistance to ceftazidime, when the isolates were subjected for MBL detection test, irrespective of their sensitivity to imipenem or meropenem, 3 MBL-producers were found (Table 5).

Table 5. MBL production with respect to antibiotic resistance in *Acb* complex

Resistance to	No. of cases	MBL-producer
CAZ + MEM	9	0
CAZ + IPM	0	0
CAZ + Both MEM and IPM	22	2
CAZ only (No MEM or IPM)	19	1
CAZ, with or without (MEM and/or IPM)	50	3

MBL production with respect to antibiotic resistance in Pseudomonads

When there was resistance to both imipenem and meropenem, 3 cases were found to be MBL-producing; MBL was not present in strains appearing sensitive to imipenem and meropenem (Table 6).

Table 6. MBL production with respect to antibiotic resistance in Pseudomonads

Resistance to	No. of cases	MBL-producer
CAZ + MEM	3	0
CAZ + IMP	1	0
CAZ + Both MEM and IMP	13	3
CAZ only (No MEM or IMP)	31	0
CAZ, with or without (MEM and/or IPM)	48	3

The only effective antibiotic in all the cases *in vitro* against MBL-producing bacteria were polymyxins (Table 7). Though one isolate was found to be susceptible to carbapenems in *in vitro*, it was reported as resistant as MBL-producers are resistant to carbapenems in *in vivo*.

Table 7. Antibiogram of MBL-producing bacterial isolates (n=6)

Antibiotics	Sensitive n (%)	Resistant n (%)
Amoxycillin	0 (0.0)	6 (100.0)
Ciprofloxacin	2 (33.3)	4 (66.7)
Cotrimoxazole	0 (0.0)	6 (100.0)
Gentamicin	3 (50.0)	3 (50.0)
Amikacin	3 (50.0)	3 (50.0)
Ceftriaxone	0 (0.0)	6 (100.0)
Ceftazidime	0 (0.0)	6 (100.0)
Cefepime	0 (0.0)	6 (100.0)
Amoxycillin-Clavulanate	0 (0.0)	6 (100.0)
Piperacillin	0 (0.0)	6 (100.0)
Piperacillin-Tazobactam	1 (16.7)	5 (83.3)
Cefoperazone-Sulbactam	1 (16.7)	5 (83.3)
Meropenem	1 (16.7)	5 (83.3)
Imipenem	1 (16.7)	5 (83.3)
Polymyxin B	6 (100.0)	0 (0.0)
Colistin-sulphate	6 (100.0)	0 (0.0)
Doxycycline	2 (33.3)	4 (66.7)
Tigecycline	3 (50.0)	3 (50.0)

DISCUSSION

In any nosocomial setting, carbapenems are used as the last resort for treatment of MDR gram-negative bacterial infection. However, since last 15 years, acquired resistance to this life saving antimicrobial has been increasingly reported not only in *Pseudomonas* and *Acinetobacter* spp,[8] but also among members of *Enterobacteriaceae*. This resistance is mainly mediated by MBLs.

Out of total 314 non-duplicate gram-negative bacteria, 206 isolates across 8 different genera of *Pseudomonadaceae*, *Moraxellaceae* and *Enterobacteriaceae* were resistant to ceftazidime. Of these, only 6 isolates were found to be MBL-producer; 4 isolates were from sputum and 2 from ET secretion specimens. All the MBL-producing isolates were nonfermentative. It consisted of 3.3% of *Pseudomonas* spp. and 4.8% of *Acinetobacter* spp. isolates. In some countries, *P. aeruginosa* possessing MBLs constitute nearly 20% of all nosocomial isolates, whereas in other countries the number is still comparatively small, but are in an increasing trend. In Italy, a SENTRY study for the years 2001 and 2002 found that 6.5% of *P. aeruginosa* isolates from three medical centers carried MBLs.[9] When compared with different studies in Asia, the prevalence of MBL in this tertiary care hospital of Nepal seems to be at the mediocre stage; it is lower than in India and China but higher than in some Japanese hospitals.

In this study, among the six MBL-producers, only one isolate was sensitive to both imipenem and meropenem and this was in *Acb* complex. It is noteworthy that all MBLs hydrolyze imipenem, but their ability to achieve this varies considerably. As seen with extended-spectrum-beta-lactamase (ESBL) and AmpC β-lactamases with cephalosporins, MBL carrying organisms may also appear susceptible to carbapenems using current CLSI breakpoints[5] but they may not be effective in *in vivo*.

Of the total 99 isolates of *Pseudomonas* spp. and *Acinetobacter* spp. showing reduced susceptibility to ceftazidime and/or carbapenems, 6.1% were positive for MBL which closely matches with the finding of Gupta et al (7.5%),[8] and Agrawal et al (8.05%).[10] When only carbapenem resistant isolates were considered, MBL-producers accounted for 9.7% of them. This finding is lower than that of Kim et al (13.4%),[11] Varaiya et al (20.8%),[12] and Dong et al (66.1%).[13]

In our setting, all of the pan-resistant gram-negative isolates and MBL producers were sensitive to polymyxin B and colistin sulphate. Because no fundamentally new anti-infective drugs are currently available, it has compelled to re-evaluate the 'old drugs' and fortunately they proved to be effective in this study too like in other studies.[14]

There was no aminoglycoside sensitive MBL-producing *P. aeruginosa* isolate in this study. The reason for this could be, in most instances, the gene cassettes involve the MBL gene and an *aacA4* gene which encode kanamycin, neomycin, amikacin and streptomycin resistance. Thus, both aminoglycosides and β-lactams will select clinical bacteria harboring these fused gene cassette, further compromising these antibiotic regimens.[15] The finding of lack of sensitivity of *P. aeruginosa* against amikacin is consistent with Varaiya et al.[12]

For *Acb* complex, all the MBL-producers were sensitive to tigecycline and aminoglycosides (gentamicin and amikacin). However, piperacillin-tazobactam and cefoperazone-sulbactam showed activity in only one case while ciprofloxacin was effective in two cases. Varaiya et al[12] found that 4.0% and 84.0% of MBL-producers were susceptible to ciprofloxacin and piperacillin-tazobactam respectively. Likewise, Taneja et al[16] showed that piperacillin and amikacin had the best in vitro activity against MBL. Complying with those findings, this study also found that MBL-producing *Acinetobacter* isolates were more sensitive to amikacin and gentamicin followed by piperacillin-tazobactam. However, in contrast to our finding, Gupta et al[8] and Navaneeth et al[17] reported all the MBL-producing strains to be uniformly resistant to piperacillin, cefoperazone-sulbactam and imipenem.

In this study, Peleg's modified DDST and CD methods[7] were employed for the detection of MBL phenotypes. The method, utilizing ceftazidime resistance as the selective criterion for the phenotype test to detect MBL, was fruitful. If only carbapenem resistant cases were selected, one MBL carrying isolate would have been missed. The use of lower concentration of EDTA, in contrast to that recommended by Yan et al,[18] lowered the inherent bactericidal activity of EDTA, thus facilitating the identification of MBL phenotypes. Use of 0.1 M EDTA has also been recommended by Zerrin A.[19] Likewise, this method which we practiced is simple to perform, and the materials used are cheap, nontoxic, and easily accessible, making it highly applicable to routine clinical laboratories. Though the DDST method had sensitivity of 79% and a specificity of 98% as compared to CD method with sensitivity of 100% and a specificity of 98%,[7] in this study both the methods detected all cases of MBL phenotypes .

CONCLUSIONS

To our knowledge, this is the first report of MBL-producing bacterial isolates from Nepal and this study documents that MBL has appeared in our country. The finding of this study could be taken as base-line information of MBL-producing isolates in Nepal so that the trend of MBL-producers can be studied in the future. Considering the need to institute correct antibiotics to the patients infected with MBL-producer, and to prevent spread of such organisms, all clinical microbiology laboratories must routinely identify MBL-producer. If carbapenem is not found to be effective in vivo, the possibility of MBL should be considered. Moreover, when an isolate is resistant to ceftazidime, it should be subjected for the test of phenotypic detection of MBL. The MBL isolates

should be subjected for genetic study to identify which classes of MBLs are predominant in our setting.

CONFLICT OF INTEREST

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