Original Article

Beta-Lactamases in a Tertiary Care Hospital: "Biological Quake" Knocking at the Door

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ABSTRACT

Background: Antimicrobial resistance due to the production of extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases, and metallo-β-lactamases (MBLs) have emerged as a major health catastrophe limiting antibiotic treatment options. Therefore, this study was conducted to assess the current level of ESBLs, AmpC β-lactamases, and MBLs-producing bacteria and their antibiotic susceptibility profile in a Nepalese hospital.

Methods: This cross-sectional study was carried out among the inpatients of Medicare National Hospital, Kathmandu from April to September 2015. During the study period, a total of 589 specimens (urine, sputum, blood, pus, body fluids, throat swab, central venous catheter - CVC tip) collected aseptically from the admitted patients were selected in the study. The collected specimens were processed, and the isolated organisms were identified following the standard microbiological methods. ESBL was detected by standard combination disc method and double-disc synergy test. Tests for AmpC and co-production of ESBL and AmpC were done by using MASTDISCS™ ID AmpC and ESBL Detection Discs, and ESBL and AmpC detection Ezy MIC™ Strip. The Imipenem-EDTA combination disc method was done for the identification of MBL in Gram-negative bacteria.

Results: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Candida albicans* were the common microbial agents isolated from hospital-admitted patients. Among total 84 Gram-negative bacteria tested for ESBL-production; 23 (27.4%) isolates were ESBL-producers. ESBL production was seen in 32.3% of *Escherichia coli* and 28.6% of *Klebsiella pneumoniae* isolates. Similarly, MBL production was identified in 28.6% of *Pseudomonas aeruginosa*, and 6.5% of *Escherichia coli*. Likewise, 3.2% of *Escherichia coli* were AmpC β-lactamase-producers. The ESBL-producing bacteria showed less susceptibility to different antibiotics as compared to non-ESBL-producers. Consistent results were found with different methods like combination disk method, MASTDISKS™ ID AmpC and ESBL disk, Ezy MIC™ Strip (MIX+/MIX) method, and triple ESBL detection Ezy MIC™ strips employed for the detection of ESBL and AmpC.

Conclusions: ESBL was commonly seen in *Escherichia coli* while MBL in *Pseudomonas aeruginosa*. Routine monitoring of these kinds of resistance phenotypes following appropriate methods is essential for the proper treatment of patients.

Keywords: Antibiotic resistance, Beta-lactamases, ESBL, Hospital-admitted patients, MBL

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INTRODUCTION

The β-lactam antibiotics are the most frequently prescribed and preferred antibiotics for hospitalized patients worldwide because of their efficacy, broad spectra, and lower toxicity.[1] Degradation of β-lactam antibiotics by β-lactamase enzymes is the common mechanism of resistance for this class of antibacterial agents. There are at least 2,770 β-lactamases reported till date.[2] Extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases, and metallo-β-lactamases (MBLs) are the most common such enzymes responsible for conferring resistance to the cephalosporins. AmpC β-lactamases are poorly inhibited by clavulanate and confer resistance to cephalosporin, α-methoxy β-lactams (cefoxitin, cefotetan), and monobactams.[3,4] MBLs are responsible for making pathogenic bacteria resistant to penicillins, cephalosporins, and carbapenems.[5-7] Due to their broad hydrolysis profile that includes all β-lactam antibiotics, they are posing a therapeutic challenge to the clinicians and are hence regarded as remarkable but menacing enzymes.[4,5-8].

The recognition of some resistant pathogens may be difficult because they are falsely susceptible in routine antibiotic sensitivity tests that can result in the selection of ineffective antibiotics and give rise to the dissemination of the drug-resistant pathogens. The characterization and exploring antibiotic susceptibility profile of β-lactamase(s)-producing organisms can lead to the formulation of a successful infection control program involving antimicrobial stewardship and public health interventions.[9] Different studies from Nepal clearly depict that ESBL-, AmpC-, and MBL-producing multidrug-resistant (MDR) bacteria are giving a threat in Nepalese healthcare settings.[6,10-11] Therefore, this study was conducted to determine the current level of ESBL, MBL, AmpC-producing Gram-negative bacteria in the different specimens of hospitalized patients.

MATERIALS AND METHODS

Study design and setting

This cross-sectional study was carried out in the microbiology laboratory of Medicare National Hospital, Nepal from April 2015 to September 2015. All the specimens collected from patients admitted in the Medicare National Hospital for culture and sensitivity tests were selected for the study.

Identification of isolated organisms

A total of 589 different specimens (urine, sputum, blood, pus, body fluids, throat swab, central venous catheter (CVC) tip) were aseptically collected from the patients during the study period. The specimens were processed in the microbiology laboratory for isolation and identification of organisms following the standard methods.[12] The *Candida* species were identified by the germ tube test and growing on HiCrome™ Candida Differential Agar.[13,14]

Antibiotic susceptibility testing

The antibiotic susceptibility test (AST) of the bacterial pathogens against appropriate antibiotics was determined by the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar (MHA) as recommended by the Clinical and Laboratory Standards Institute (CLSI Document M100-S24). *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were also tested in parallel as a part of quality control.[15]

Screening of ESBL-, AmpC-, and MBL-producing isolates

The Gram-negative bacterial isolates showing zone of inhibition (ZOI) of ≤25 mm for ceftriaxone (30 µg) and ≤22 mm for ceftazidime (30 µg) were considered as potential ESBL-producers.[15] These isolates were further subjected to phenotypic confirmation of ESBL-production. The isolates either showing ZOI less than 18 mm for cefoxitin and/or resistant to third-generation cephalosporins were tested for AmpC β-lactamase.[16] The isolates were subjected to MBL detection if resistant to ceftazidime.[6]

Detection of ESBL by combination disk method

Ceftazidime (30 µg) and ceftriaxone (30 µg) disks alone and in combination with and clavulanic acid (10 µg) were used, and the disks were placed with 25 mm apart from each other. After incubation of 16-18 hours at 35±2^{0}C in ambient air, the bacterial isolates showing an increase in ZOI by ≥5 mm for ceftazidime+clavulanic acid (30/10 µg) and/or ceftriaxone+clavulanic acid (30/10 µg) compared to ceftazidime and/or ceftriaxone alone were confirmed as ESBL-producing isolates.[15]

Detection of ESBL and AmpC β-lactamases by MASTDISKS™ ID AmpC and ESBL disk

The lawn culture of the bacterial isolate was prepared on MHA plate, one each of four disks (A – Cefpodoxime 10 µg, B - Cefpodoxime 10 µg / ESBL inhibitor, C - Cefpodoxime 10 µg / AmpC inhibitor, and D - Cefpodoxime 10 µg / ESBL inhibitor / AmpC inhibitor) was placed onto an inoculated agar plate, and plates were incubated at 37°C. After overnight incubation, the results were interpreted based on the manufacturer's booklet. If the ZOI around disk B and D (but not C) was increased by ≥5 mm than that of disk A, the isolate was considered as ESBL producer. If the ZOI around disk C and D (but not B) was increased by ≥5 mm than that of disk A, the isolate was considered as AmpC β-lactamase producer. If the ZOI around disk D (but not B and C) was increased by ≥5 mm than that of disk A, the isolate was considered as both ESBL and AmpC β-lactamase producer.[17]

Detection ESBL and AmpC β-lactamases by Ezy MIC™ Strip (MIX+/MIX) method

A 0.5 McFarland standard inoculum of test organism was prepared on and inoculated as lawn culture on the MHA plate. The Ezy MIC™ strip was placed onto the inoculated agar plate and incubated at 35-37°C for 18 hours. The result was interpreted following interpretative guideline provided by the manufacturer.[18]

Detection of ESBL by Triple ESBL detection Ezy MIC™ Strip (MIX+/MIX)

A 0.5 McFarland standard inoculum of test organism was prepared and inoculated as lawn culture on MHA. The Ezy MIC™ strip was placed on the inoculated agar plate and incubated at 35-37°C for 18 hours. The result was interpreted following manufacturer's recommendation.[19]

Detection of MBL by imipenem-EDTA combination disk method:

A bacterial suspension equivalent to 1:10 dilution of 0.5 McFarland standard was prepared and was swabbed onto the MHA plate. One imipenem disk (10 µg) alone and another imipenem disk containing 10 µl of 0.1 M (292 µg) ethylenediamine-tetraacetic acid (EDTA) were placed 25 mm apart (center to center) on the inoculated plate. After overnight incubation at 35±2°C in ambient air, an increase in zone diameter by >4 mm around the imipenem-EDTA disk compared to that of the imipenem disk alone was considered positive for MBL.[6]

Data processing and analysis

The data generated during the study were analyzed by using SPSS version 16.0.

RESULTS

Age and gender-wise distribution of patients

Out of total 589 patients selected in the study, female was 314 (53.3%) and male were 275 in number (46.7%) with female to male ratio of 1.14. The age of patients ranged from 18-91 years. Among them, the highest number of patients was in the age group 21-30 years (26.0%) and the lowest was in >90 years.

Distribution of specimens and rate of microbial growth

During the study period, a total of 589 different specimens were collected from admitted patients. Urine was the most predominant specimen followed by sputum, blood, and pus. Significant microbial growth was seen in 23.4% of samples yielding a total of 138 non-duplicate microbial isolates. The growth rate was highest in pus and CVC tip samples (50.0%) whereas lowest in blood sample (13.0%) (Table 1).

Distribution of microbial isolates in various specimens

Among the total 138 microbial isolates, *Escherichia coli* was the predominant organism (44.9%) followed by *Candida albicans* (25.4%), and an equal number of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (5.1%). Based on systemic infection, urinary tract infection was the major type (60.1%) followed by lower respiratory tract infection (29.7%). Likewise, bloodstream infections and wound infections accounted for 2.2% and 5.0%. *Escherichia coli* (n=58) was the major isolate from the urine sample while *Candida albicans* (n=27) was the major isolate from the sputum sample (Table 2).

Ward wise distribution of microbial isolates

Among the different wards, a higher number of microbial species was isolated from intensive care unit (ICU) patients (62.3%) followed by surgical and medical wards (30.4% and 7.3% respectively).

Antimicrobial susceptibility profile of major bacterial isolates

The antibiotic susceptibility test showed a higher rate of resistance in *Escherichia coli* against amoxicillin, third-generations cephalosporins, gentamicin, fluoroquinolones and cotrimoxazole. Similarly, 57.1% of *Klebsiella pneumoniae* were resistant to ciprofloxacin and 85.7% resistant to cotrimoxazole. Nearly, seventy-one percent of *Pseudomonas aeruginosa* were resistant to piperacillin and 85.7% to ciprofloxacin. Amikacin, meropenem, and imipenem were found significantly effective against these Gram-negative bacterial isolates (Table 3).

Distribution of β-lactamases producing Gram-negative bacterial isolates

Out of 84 Gram-negative bacilli tested for ESBL production (except *Haemophilus influenzae*), 27.4% (n=23) isolates were ESBL-producer and 7.1% (n=6) isolates were MBL-producer. ESBL production was seen in 32.3% (n=20) *Escherichia coli* isolates, 28.6% (n=2) *Klebsiella pneumoniae* and 50.0% (n=1) *Acinetobacter* species. Similarly, 6.5% of *Escherichia coli* and 28.6% of *Pseudomonas aeruginosa* isolates were MBL-producer. Two *Escherichia coli* were AmpC β-lactamase producers co-producing the ESBL enzyme (Table 4).

Antibiogram of ESBL-producing and ESBL non-producing isolates

The susceptibility of ESBL-producing bacteria to different antibiotics was lower as compared to ESBL non-producers. Imipenem, meropenem, amikacin, and cefoperazone-sulbactam showed relatively better activity against ESBL-producers (Table 5).

Table 1: Distribution of specimens and rate of microbial growth

Specimen type	Number of specimens	Growth	
		Number	Percentage
Urine	427	83	19.4
Sputum	105	41	39.0
Blood	23	3	13.0
Pus	14	7	50.0
Body fluids	13	0	0
CVC tip	4	2	50.0
Throat swab	3	2	66.6
Total	589	138	23.4

Table 2: Distribution of microbial isolates in various specimens

Microbial species	Number of isolates (%)						
	Urine	Sputum	Pus	Blood	CVP tip	Throat swab	Total
Gram-negative isolates							
Escherichia coli	58	2	1	1	0	0	62 (44.9)
Klebsiella pneumoniae	3	3	1	0	0	0	7 (5.1)
Pseudomonas aeruginosa	4	2	0	0	1	0	7 (5.1)
Proteus vulgaris	2	0	0	0	0	0	2 (1.4)
Salmonella Typhi	0	0	0	2	0	0	2 (1.4)
Haemophilus influenzae	0	2	0	0	0	0	2 (1.4)
Acinetobacter species	1	1	0	0	0	0	2 (1.4)
Citrobacter freundii	0	0	1	0	0	0	1 (0.75)
Hafnia alvei	0	1	0	0	0	0	1 (0.75)
Gram-positive isolates							
Enterococcus faecalis	6	1	0	0	0	0	7 (5.1)

Staphylococcus aureus	0	0	4	0	1	1	6 (4.4)
Streptococcus pneumoniae	0	2	0	0	0	0	2 (1.4)
Staphylococcus saprophyticus	1	0	0	0	0	0	1 (0.75)
Streptococcus pyogenes	0	0	0	0	0	1	1 (0.75)
Candida albicans	8	27	0	0	0	0	35 (25.4)
Total	83 (60.1)	41 (29.7)	7 (5.0)	3 (2.2)	2 (1.5)	2 (1.5)	138 (100)

Table 3: Antimicrobial resistance rate of major bacterial isolates

Antibiotics	**Number of antibiotic-resistant isolates (%)**		
	E. coli (n=62)	*K. pneumoniae* (n=7)	*P. aeruginosa* (n=7)
Amoxicillin	55 (88.7)	NT	NT
Amoxicillin-clavulanate	39 (62.9)	4 (57.1)	NT
Piperacillin	NT	NT	5 (71.4)
Piperacillin-tazobactam	8 (12.9)	3 (42.8)	3 (42.8)
Ceftriaxone	34 (54.8)	3 (42.8)	NT
Ceftazidime	34 (54.8)	3 (42.8)	2 (28.6)
Cefoperazone-sulbactam	7 (11.3)	3 (42.8)	3 (42.8)
Gentamicin	36 (58.1)	3 (42.8)	3 (42.8)
Amikacin	9 (14.5)	2 (28.6)	1 (14.3)
#Nitrofurantoin	15 (25.9)	1 (33.3)	NT
#Norfloxacin	36 (62.1)	2 (66.7)	NT
Ciprofloxacin	36 (58.1)	4 (57.1)	6 (85.7)
Levofloxacin	32 (51.6)	3 (42.8)	6 (85.7)
Cotrimoxazole	51 (82.3)	6 (85.7)	NT
Meropenem	6 (9.8)	2 (28.6)	3 (42.8)
Imipenem	6 (9.8)	2 (28.6)	2 (28.6)

*NT: Antibiotics not tested/not recommended. #: Only applicable to urinary isolates [*E. coli* (n=58) and *K. pneumoniae* (n=3)].

Table 4: Distribution of β-lactamases producing Gram-negative bacterial isolates

Bacterial isolates	**Number and type of β-lactamases producers (%)**			
	ESBL	AmpC	MBL	ESBL+AmpC
Escherichia coli (n=62)	20 (32.3)	2 (3.2)	4 (6.5)	2 (3.2)
Klebsiella pneumoniae (n=7)	2 (28.6)	0 (0)	0 (0)	0 (0)
Pseudomonas aeruginosa (n=7)	0 (0)	0 (0)	2 (28.6)	0 (0)
Acinetobacter species (n=2)	1 (50.0)	0 (0)	0 (0)	0 (0)

Table 5: Antibiotic sensitivity rate of ESBL–producers and ESBL non-producers

Antibiotics	Rate of antibiotic susceptible isolates (%)	
	ESBL-producers (n=23)	ESBL non-producers (n=61)
Piperacillin-tazobactam	13 (56.5)	51 (83.6)
Cefoperazone-sulbactam	19 (82.6)	56 (91.8)
Meropenem	21 (91.3)	60 (98.4)
Imipenem	22 (95.7)	59 (96.7)
Gentamicin	10 (43.5)	41 (67.2)
Amikacin	20 (87.0)	58 (95.1)
Nitrofurantoin	7 (30.4)	45 (73.8)
Norfloxacin	5 (21.7)	39 (63.9)
Ciprofloxacin	5 (21.7)	39 (63.9)
Levofloxacin	7 (30.4)	44 (72.1)

DISCUSSION

The β-lactamases like ESBL, AmpC, and MBL have emerged as causes of antibiotic resistance among Gram-negative bacteria in recent years worldwide. Although β-lactamases have been discovered a few decades ago, failure to detect these β-lactamases in routine diagnostic laboratory has resulted in their unrestricted dissemination and sometimes to therapeutic failure.[3]

In this study, 60.1% of total isolates were recovered from urinary tract infection. A relatively similar rate of uropathogens (59%) was reported by Singh et al.[20] from India; however, higher growth (78%) was seen in Nigeria.[21] A study from India showed a relatively higher percentage of *Acinetobacter* species (48.78%), *Pseudomonas aeruginosa* (31.71%), *Staphylococcus aureus* (8.54%) whereas *Escherichia coli* (3.66%) was less frequent when compared to that in this study.[22] The variation in microbial growth from institution to an institution may be due to the clinical profile of patients, length of antibiotic therapy, infection control practice (including different diagnostic stewardship, antimicrobial stewardship and infection control stewardship programs adopted in different centers, or no such particular practices) of healthcare setting as well as whether there was use of indwelling medical devices.

This study showed that *Escherichia coli* were resistant to commonly used antibiotics, but relatively less resistant as compared to nosocomial isolates.[23] Likewise, *Klebsiella pneumoniae* showed increased resistance towards amoxicillin-clavulanate (57.1%), ciprofloxacin (57.1%), and cotrimoxazole (85.7%). Similarly, 71.4% of *Pseudomonas aeruginosa* isolates were resistant to piperacillin and 85.7% to ciprofloxacin. Meropenem, imipenem, ceftazidime, and amikacin showed promising efficacy against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa.* Another study by Parajuli et al.[24] from Nepal had reported higher resistance among Gram-negative bacteria where 19.3% *Escherichia coli*, 48.6% *Klebsiella* species, and 62.5% *Pseudomonas aeruginosa* were resistant to carbapenems.

In our study, ESBL production was seen in 32.3%, AmpC β-lactamase in 3.2%, and MBL in 6.5% of *Escherichia coli* isolates. MBL was also documented in 28.6% of *Pseudomonas aeruginosa*. In 2012, Mishra et al.[25] reported that 9.5% of *Escherichia coli* and 25.4% of *Klebsiella pneumoniae* isolates were ESBL-producers, and 1.6% of *Pseudomonas aeruginosa* MBL-producing. When MBL was first reported in Nepal by Mishra et al.[6] in 2008, the prevalence was very low (1.3%). An increasing trend of ESBL and MBL production was reported from Nepal in 2015 where 70.9% *Escherichia coli*, 59.4% *Klebsiella* species, and 33.4% *Acinetobacter* species were ESBL-producers and 16.1% *Escherichia coli* and 62.5% *Pseudomonas aeruginosa* isolates were MBL-producers.[24] This shows that ESBL- and MBL-producing bacteria are being problematic in

Nepalese hospitals as their prevalence has dramatically increased in the last decades. Different methods have been introduced for the phenotypic detection of emerging β-lactamases. For AmpC detection, the findings of the MAST ID™ method concurred with that of ESBL and AmpC E-test Ezy MIC™ strip (HI media).

In this study, we have also focused on the comparison of the antibiotic susceptibility profile of ESBL-producing and non-producing isolates. The ESBL-producing isolates showed significantly lower susceptibility than non-producing isolates towards ciprofloxacin (21.7% vs. 63.9%), levofloxacin (30.4% vs. 72.1%), gentamicin (43.5% vs. 67.2%) and amikacin (87.0% vs. 95.1%), meropenem (91.3% vs. 98.4%) and cefoperazone-sulbactam (82.6% vs. 91.8%). Luvsansharav et al.[26] and Mathai et al.[27] had also documented that ESBL-producers are more resistant to different antibiotics. The emergence of β-lactamase enzymes in Gram-negative bacteria is becoming problematic as these enzymes results in rapid hydrolysis of penicillins, cephalosporins, and carbapenems. Furthermore, the genes encoding these enzymes are present on the bacterial plasmid and can disseminate rapidly to Gram-negative bacterial species.[24]

CONCLUSION

Different types of β-lactamases-producing MDR bacteria were isolated from clinical specimens. Therefore, proper identification, characterization, and surveillance of antibiotic susceptibility profile of β-lactamase-producing Gram-negative bacteria along with the execution of a special strategy of antibiotic stewardship are recommended to mitigate the burden of antimicrobial resistance.

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