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ORIGINAL RESEARCH ARTICLE

Phenotypic Characterization of Beta-lactamases Producing Gram-Negative Bacteria in a Tertiary Hospital, Nepal

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Abstract

Infections caused by beta-lactamases producing Gram-negative bacteria are increasing, thus posing a challenge to the management of such infections. The surveillance data of such bacteria is limited in Nepal so this study aimed to detect the beta-lactamase producing Gram-negative bacteria in a tertiary setting. A total of 604 clinical samples, including urine, blood, sputum and body fluids, were cultured and identified by the routine standard laboratory protocols. Antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method following Clinical and Laboratory Standard Institute guidelines (2014). Extended-spectrum beta-lactamases (ESBL) producers were identified by combined disk method and metallo-beta-lactamases (MBL) producers were identified by Imipenem- EDTA combined disk method. Out of 604 samples, 282 (46.7%) samples showed significant growth, of which 229 (81.2%) were Gram-negative bacteria. Of 229 Gram-negative bacteria, 200 (87.3%) were multidrug resistant, 67 (29.3%) were ESBL producers and 16 (7.0%) were MBL producers. *Klebsiella pneumoniae* were among higher ESBL producers and *Pseudomonas aeruginosa* were among higher MBL producers. The findings suggest higher antibacterial resistance among Gram-negative bacteria with the added burden of beta-lactamase production. Imipenem was effective against 125 of 229 Gram-negative bacteria tested. Thus, imipenem can be the drug of choice for empirical management. The higher multidrug resistance and higher beta-lactamases production among Gram-negative bacteria warrant the continuous monitoring, surveillance, early detection, and infection control practices of such bacteria.

Keywords: Antibacterial resistance, extended-spectrum beta-lactamases, ESBL, Gram-negative, metallo-beta-lactamase, MBL

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Introduction

Antibacterial resistance (ABR) is an increasingly serious threat to human health, challenging the effective management of infections. ABR increase the health care cost as a result of prolonged illness, additional tests and pricier drugs [1]. Annually, more than 750,000 deaths are caused by resistant bacteria. The median overall increased cost to treat a resistant bacterial infection is around 700 USD [2].

ABR is a natural genetic change, thus newer mechanisms are emerging and spreading, raising multidrug-resistant (MDR) bacteria [1]. One of the most worrisome resistance mechanisms is the production of beta-lactamases, of which extended-spectrum beta-lactamases (ESBLs) and metallo-beta-lactamases (MBLs) are the most impacting ones. ESBLs can hydrolyze all beta-lactam antibiotics including cephalosporins except cephamycins or carbapenems [3]. ESBLs are often plasmid-mediated. Since it was first reported from Germany in 1983, it has then spread worldwide [4]. In Nepal, ESBLs were first reported in 2006 [5]. Similarly, MBLs can hydrolyze all broad-spectrum beta-lactams including cephalosporins, and carbapenems, except monobactams [6]. Since its first report from Japan in 1991, it has also been reported

worldwide [7]. MBLs were first reported in Nepal in 2009 [8].

The acquisition, expression, and dissemination of beta-lactamase producing genes in pathogens have posed the major public health concern today [9]. Limited data are available on surveilling beta-lactamase producing clinical isolates in Nepal. ABR surveillance data can guide the physician in choosing appropriate therapy for effective management of infectious disease without extensive testing. Thus, this study aimed to produce updated data on surveilling beta-lactamase producing clinical isolates in a tertiary healthcare setting. This would help to formulate antimicrobial stewardship policy to circumvent the rising threat of ABR.

Materials and Methods

Study setting, design and study population

A prospective hospital-based study was conducted in the Department of Microbiology at Annapurna Neurological Institute and Applied Science, Kathmandu, Nepal from March to November 2014. A total of 604 clinical samples from patients of all ages and both sexes, visiting the hospital and requesting a routine investigation, was included in the study. The samples include 263 urines (clean catch urine and catheter tip), 140 sputum samples, 73 blood samples and 128 body fluids (pus, pus swab, CSF, bile fluid, pleural fluid, peritoneal fluid, synovial fluid, and tracheal secretion). Contaminated samples and repeated samples from the same patient were excluded to avoid selection bias.

Laboratory processing of the samples

The samples were subjected to the standard microbiological procedures for isolation and identification of bacteria. In short, the samples were inoculated onto MacConkey agar (HiMedia, India) and blood agar (HiMedia, India) plate by streaking, followed by incubation at 37°C. Growths of bacteria were observed after 18-48hrs. The identification of Gram-negative bacteria was done by Gram's stain morphology, cultural characteristics, and conventional biochemical tests. Biochemical tests employed were catalase test, oxidase test, indole test, citrate utilization test, methyl red test, Voges-Proskauer test, Christensen's urease test, triple sugar iron test, decarboxylase test, and phenylalanine deaminase test.

Antimicrobial susceptibility testing

The antimicrobial susceptibility test of all identified bacteria against antibiotics of various classes was done *in vitro* by Kirby-Bauer disc diffusion method [10]. A zone of inhibitions (ZOI) for each disc was measured and the results were interpreted as per CLSI guideline M100-S24 [11]. Control strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used for the quality control of the test.

Detection of extended-spectrum beta-lactamase producing strain

ESBL producing isolates were identified by a phenotypic method as described in CLSI guideline M100-S24 [11].

Screening test for ESBL:

Isolates exhibiting resistance against ceftriaxone (30µg) (ZOI ≤25mm) and/or ceftazidime (30µg) (ZOI ≤22mm) and/or cefpodoxime (10µg) (ZOI ≤17mm) and/or cefotaxime (30µg) (ZOI ≤27mm) were screened for ESBL production.

Combined disc (CD) method as a confirmatory test for ESBL:

ZOIs of isolates against ceftazidime disc (30µg) and cefotaxime (30µg) were compared against ZOIs of isolates against ceftazidime disc (30µg) containing clavulanic acid (10µg) and cefotaxime (30µg) containing clavulanic acid (10µg) when placed 25mm apart (center to center). The isolates showing the difference of 5mm or more between either of the two ZOIs of the disc and clavulanate added disc was confirmed positive for ESBL production. *Klebsiella pneumoniae* ATCC 700603 (ESBL positive) was used as control strains.

Table 1. Sample and significant growth pattern

	Total samples	Culture positive	Gram-negative bacteria	Gram-positive bacteria
Gender (M: F ratio=1.45:1)				
Male	357 (59.1%)	174 (61.7%)	143 (62.4%)	31 (58.5%)
Female	247 (40.9%)	108 (38.3%)	86 (37.6%)	22 (41.5%)
Departments of patients				
Inpatients	471 (78%)	180 (63.8%)	157 (68.6%)	23 (76.7%)
Outpatients	133 (22%)	102 (36.2%)	72 (31.4%)	30 (56.6%)
Age groups (years): Infants and children, adolescents, adults, elders				
≤9	19 (3.2%)	3 (1.1%)	2 (0.9%)	1 (1.9%)
10-19	56 (9.3%)	29 (10.3%)	26 (11.4%)	3 (5.7%)
20-59	313 (51.8%)	129 (45.7%)	99 (43.2%)	30 (56.6%)
≥60	216 (35.8%)	121 (42.9%)	102 (44.5%)	19 (35.8%)
Total	604 (100%)	282 (100%)	229 (100%)	53 (100%)

Detection of metallo-beta-lactamase producing strain

Since during the study no standard protocol was available for the detection of MBL, MBL producing isolates were identified by the commonly used phenotypic method.

Screening test for MBL:

Isolates exhibiting resistance against ceftazidime (30µg) (ZOI<18mm) were screened for MBL production. The resistance against imipenem (10µg) and/or meropenem (10µg) was not used as a screening tool so as to avoid missing the detection of hidden MBL in bacteria. Bacterial suspension equivalent to 1:10 dilution of 0.5 McFarland was used for lawn culture in Mueller-Hinton agar before incorporating antibiotic discs [12].

Combined disc (CD) method as a confirmatory test for MBL:

ZOI of isolate against imipenem disc (10 µg) was compared against ZOI of isolates against imipenem disc (10µg) containing 292µg (10µl of 0.1M) ethylenediamine-tetraacetic acid (EDTA) when placed 25mm apart (center to center). The isolates showing difference of 4mm or more between two ZOIs was confirmed positive for MBL production [12, 13]. *P. aeruginosa* ATCC 27853 (MBL negative) and *P. aeruginosa* PA 105663 (MBL positive) was used as control strains.

Results

Distribution of samples and isolates

Of 604 samples, only 282 (46.7%) were culture positive. Higher number of samples from males were culture positive 174 (61.7%). Similarly, a higher number of samples from the inpatient department were culture positive, 180 (63.8%). Culture positivity increased with the age of patients. Among 282 culture positive, 229 (81.2%) were Gram-negative bacteria (**Table 1**). Eight different species of Gram-negative bacteria were identified. Among these, *E. coli* were the predominant, 78 (34.1%). *E. coli* was also predominant in urine samples, 61(55.0%) (**Table 2**).

Table 2: Distribution of Gram-negative bacteria in different clinical samples

Bacteria	Sample				Total
	Urine	Blood	Sputum	Body Fluid	
ACB*	2 (1.8%)	0 (0%)	4 (5.5%)	2 (4.9%)	8 (3.5%)
Citrobacter spp.	1 (0.9%)	0 (0%)	0 (0.0%)	0 (0.0%)	1 (0.4%)
E. coli	61 (55.0%)	0 (0%)	9 (12.3%)	8 (19.5%)	78 (34.1%)
K. pneumoniae	16 (14.4%)	0 (0%)	33 (45.2%)	22 (53.7%)	71 (31.0%)
K. oxytoca	15 (13.5%)	0 (0%)	6 (8.2%)	1 (2.4%)	22 (9.6%)
P. aeruginosa	12 (10.8%)	2 (50%)	20 (27.4%)	7 (17.1%)	41 (17.9%)
Proteus spp.	4 (3.6%)	0 (0%)	1 (1.4%)	1 (2.4%)	6 (2.6%)
Salmonella spp.	0 (0.0%)	2 (50%)	0 (0.0%)	0 (0.0%)	2 (0.9%)
Total	**111 (100%)**	**4 (100%)**	**73 (100%)**	**41 (100%)**	**229(100%)**

*Acinetobacter calcoaceticus-baumannii Complex

Antibiotic susceptibility of Gram-negative bacteria

Of 229 Gram-negative bacteria, *E. coli* showed higher resistance against ampicillin, cefpodoxime, ceftriaxone, and cefixime, while imipenem and chloramphenicol were found effective. *P. aeruginosa* showed higher resistance against cefixime and cefpodoxime, while imipenem was found effective. Similarly, other Gram-negative bacteria showed higher resistance against ampicillin, cefpodoxime, and ceftriaxone, while imipenem was found fairly effective (**Table 3**).

Distribution of beta-lactamase producers

Of 229 Gram-negative bacteria, 200 (87.3%) were found MDR. Of these bacteria, 67 (29.3%) were ESBL producers and 16 (7.0%) were MBL producers. ESBL production was higher among sputum isolates i.e. 35 (52.2%) and MBL production was higher among urine isolates i.e. 8 (50%) (**Table 4**).Distribution of beta-lactamase production among Gram-negative bacteria

Of 229 Gram-negative bacteria, MDR was found higher among *Klebsiella* spp. isolates. ESBL production was higher among *K. pneumoniae* 25 (37.3%), *E. coli* 16 (23.9%) and *P. aeruginosa* 16 (23.9%). Similarly, MBL production was higher among *P. aeruginosa* 9 (56.3%) and *E. coli* 3 (18.8%) (**Table 5**).

Discussion

Rise of beta-lactamases among pathogens has now been a prime threat to global health. Beta-lactamase production has been reported in *P. aeruginosa*, *Acinetobacter* spp. and Enterobacteriaceae [14]. The evidence has shown that ABR has a significant adverse impact on clinical outcomes and increases costs due to the consumption of health care resources [15]. Of 229 Gram-negative bacteria, 29.3% were found ESBL producers. Similar results were reported by previous studies as 25% in 2013 [16], 25.8% in 2014 [17], 24% in 2015 [18], 26.9% in 2015 [19] and 34.5% in 2017 [20]. ESBL producers are increasingly disseminating in Nepal, 0.6% in 2006 to 40% in 2017 [5, 16-22]. ESBL production was higher among *K. pneumoniae* (37.3%), *E. coli* (23.9%) and *P. aeruginosa* (23.9%).

Table 3: Antibiotic susceptibility of Gram-negative bacteria

Antibiotics	*E. coli* (n=78) (%)			*P. aeruginosa* (n=41) (%)			Other GNB (n=110) (%)		
	S	I	R	S	I	R	S	I	R
Amikacin	30.8	14.1	55.1	58.5	17.1	24.4	39.1	11.8	49.1
Ampicillin	5.1	0.0	94.9	12.2	0.0	87.8	4.5	0.9	94.5
Aztreonam	10.3	15.4	74.4	34.1	19.5	46.3	20.0	10.0	70.0
Ceftriaxone	5.1	2.6	92.3	19.5	0.0	80.5	5.5	0.9	93.6
Cefixime	2.6	5.1	92.3	2.4	0.0	97.6	8.2	0.0	91.8
Cefotaxime	3.8	7.7	88.5	17.1	2.4	80.5	10.0	0.9	89.1
Cefpodoxime	5.1	0.0	94.9	12.2	0.0	87.8	5.5	0.0	94.5
Ceftazidime	9.0	2.6	88.5	17.1	4.9	78.0	10.0	0.9	89.1
Ciprofloxacin	14.1	1.3	84.6	19.5	2.4	78.0	11.8	0.0	88.2
Chloramphenicol	38.5	9.0	52.6	58.5	17.1	24.4	30.9	18.2	50.9
Co-trimoxazole	11.5	2.6	85.9	14.6	0.0	85.4	11.8	2.7	85.5
Gentamicin	21.8	5.1	73.1	41.5	4.9	53.7	20.9	2.7	76.4
Imipenem	39.7	15.4	44.9	78.0	0.0	22.0	56.4	4.5	39.1
Nitrofurantoin	18.0	4.9	77.1	NT	NT	NT	NT	NT	NT
Norfloxacin	12.8	1.3	85.9	9.8	4.9	85.4	13.6	0.0	86.4
Piperacillin/ Tazobactam	NT	NT	NT	63.4	9.8	26.8	NT	NT	NT

*GNB=Gram-negative bacteria, S=sensitive, I=intermediate, R=resistance, NT=not tested

Similar results were reported in *E. coli* as 25.8% by Pokhrel et al in 2014 [17], 22.4% by Raut et al in 2015 [23], 26.9% Yadav et al in 2015 [19]. Similarly, comparable results were reported in *K. pneumoniae* as 22.4% by Raut et al in 2015 [23] and 43.3% Nepal et al in 2017 [22]. ESBL production was higher among sputum isolates i.e. 35 (52.2%).

Of 229 Gram-negative bacteria, 7% were found MBL producers. Similar results were reported by previous studies as 7.1% Khanal et al in 2013 [16], 3.2% Pokhrel et al in 2014 [17] and 4% Nepal et al in 2017 [20]. But a lower rate of 1.3% was reported by Mishra et al in 2012 [12] and a higher rate of 15% was reported Ansari et al in 2015 [18]. MBL producers are increasingly disseminating in Nepal [12, 16-18, 20]. MBL production was higher among *P. aeruginosa* isolates, 56.3%. Besides *P. aeruginosa* and *Acinetobacter* spp., MBL production was also found in *E. coli* and *K. pneumoniae* as well. MBL in Enterobacteriaceae was reported by similar studies reported [16-18, 20].

However, this contrast with the result of similar previous studies [12] which reported MBL production only in *P. aeruginosa* and *Acinetobacter* spp. MBL production was higher among urine isolates i.e. 50% of total MBL producers.

Genotypic methods are considered superior tools for surveillance of such ESBL and MBL producers. But the evolution of newer beta-

Table 5. Distribution of Gram-negative isolates producing ESBL and MBL

Bacteria	Total isolates	MDR bacteria	ESBL producers	MBL producers
ACB*	8	4	4 (6.0%)	2 (12.5%)
Citrobacter spp.	1	1	0 (0.0%)	0 (0.0%)
E. coli	78	65	16 (23.9%)	3 (18.8%)
K. oxytoca	22	21	5 (7.5%)	0 (0.0%)
K. pneumoniae	71	66	25 (37.3%)	2 (12.5%)
P. aeruginosa	41	36	16 (23.9%)	9 (56.3%)
Proteus spp.	6	6	0 (0.0%)	0 (0.0%)
Salmonella spp.	2	1	1 (1.5%)	0 (0.0%)
Total	229	200 (100%)	67 (100%)	16 (100%)

*Acinetobacter calcoaceticus-baumannii Complex

lactamase genes would make use of such tools tough, owing to the lack of primers. Moreover, using such expensive tools in resource lacking settings, like Nepal, would be a fairy tale scenario. A combined disc phenotypic method using imipenem and EDTA for MBL, ceftazidime/clavulanate or cefotaxime /clavulanate for ESBL offers a cheaper alternative. Also, phenotypic method proves not only the presence of beta-lactamases producing genes but also their expression. This method has been reported to have a sensitivity of 100% and specificity of 98%, thus reliable [12, 24].

Of 229 Gram-negative bacteria, *E. coli* showed higher resistance against ampicillin, cefpodoxime, ceftriaxone, and cefixime while imipenem and chloramphenicol were found effective. *P. aeruginosa* showed higher resistance against cefixime and cefpodoxime while imipenem was found effective. Similarly, other Gram-negative bacteria showed higher resistance against ampicillin, cefpodoxime, and ceftriaxone while imipenem was found fairly effective. Imipenem was found as an effective drug against most of the tested Gram-negative isolates. This concords with similar reports which reported imipenem as the most sensitive drug [25, 26].

As per the expert consensus, isolates resistant to two or more classes of antibiotics are considered MDR strains [27]. Of 229 Gram-negative bacteria, 87.3% of the isolates were found MDR strains. MDR was found higher among *Klebsiella* spp. isolates (93.5%). Similarly, 87.8% of *P. aeruginosa* and 50% of *Acinetobacter* spp. isolates were found MDR strains. Some studies reported higher MDR in these bacteria [16, 18, 20].

In our study, nearly all ESBL and MBL producers were MDR strains. This limits physicians with therapeutics for the management of infections. Such ESBL producing strains can be inhibited by the use of beta-lacatamase inhibitors like clavulanate, but MBL producers are resistance to these inhibitors and MBL inhibitors are yet to be trialed in human. The reserve drug for MDR Gram-negative bacteria is emptied with the evolution of newer beta-lactamases. Colistin has always been the ultimate weapon in the fight against MDR superbugs in case all other therapeutic options fail. However, resistance against colistin has been reported in recent times, rendering our drug arsenal completely empty for such superbugs [28-30].

ABR is a crisis that must be managed with the utmost urgency to contain it. Such surveillance that generates updated data is required for the implementation of sound strategies and public health actions to contain ABR. ABR requires concerted cross-sectional action by governments

and society as a whole. Currently, 'the global action plan on antimicrobial resistance-2015', as formulated by WHO [31], must be implemented as envisioned to tackle the ABR.

Conclusion

The findings suggest higher MDR, ESBL, and MBL not only among *P. aeruginosa* and *Acinetobacter* spp. but also among *Enterobacteriaceae* family, including *E. coli* and *K. pneumoniae*. Imipenem showed promising sensitivity against most of the Gram-negative isolates, thus it can be the antibiotic of choice for management of such infections. Evolving beta-lactamases against newer generation beta-lactams have posed a serious threat to public health. Only the continuous monitoring, surveillance, early detection, and infection control practices can ensure the effective management of such resistant strains.

Conflict of Interest

The authors declare no conflict of interest.

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Consent to publish

Not applicable

Ethical approval and consent to the participant

No patient-related data were collected and thus ethical approval was not required. The study was a laboratory-based study and a part of the study was a routine patient care investigation. Oral informed consent was taken from all patients or from a guardian on behalf of the patients.

Availability of data and materials

All data generated or analyzed during this study are included in the article. Raw data can be made available upon request to the corresponding author.

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Authors' Contributions

All authors made substantial contributions to the study. EM, DS and PS conceived and designed the study. EM and PS collected sample, investigated and recorded the laboratory findings at the hospital. DS, BB and BPB curated the data and analyzed the data. EM and DS administered the project, reviewed literature, and drafted the manuscript. DS helped with critical review and revision of the manuscript by compiling, formatting, editing and writing the final version of the manuscript. All authors read and approved the final manuscript.

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