Extended Spectrum â-lactamase producing multidrug resistant clinical bacterial isolates at National Public Health Laboratory, Nepal

S Poudyal,[1] DR Bhatta,[1] G Shakya,[2] B Upadhyaya,[2] SP Dumre,[2] G Buda, [3]and BP Kandel [1]

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal, [2]National Public Health Laboratory, Teku, Nepal, [3] Department of Microbiology, National College, Khusibu, Nepal

Corresponding author: Shishir Poudyal, Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal; e-mail: zephyr_balak@hotmail.com

ABSTRACT

Extended Spectrum â-lactamase (ESBL) producing multidrug resistant bacteria complicate therapeutic management and limit treatment options**.** Therefore, detection of ESBL-producing multidrug resistant (MDR) pathogens has a paramount importance. Between April 2009 and January 2010, a prospective study was carried out in National Public Health Laboratory with an objective to determine the status of ESBL producing MDR bacterial isolates from different clinical samples. Identification of the isolates was done by standard microbiological techniques and antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method following Clinical and Laboratory Standard Institute (CLSI) guidelines. ESBL screening among MDR isolates was done using Ceftriaxone, Aztreonam, Cefotaxime, Ceftazidime and Cefpodoxime followed by confirmation using MASTDISCS™ ID ES²L Detection Discs (CPD10). Data analysis was done by SPSS 16 software. Of the 314 bacterial isolates from 1601 different clinical specimens, 199 (63.4%) were MDR. Cefotaxime was found the reliable screening agent for ESBL detection with sensitivity and positive predictive value of 98.6% and 76.4% respectively. Sixtey nine (62.7%) isolates of the 110 tested MDR isolates were ESBL positive with at least one of the Combined Disk (CD) Assays. *Escherichia coli* (80%) was the major ESBL producer followed by *Klebsiella pneumoniae* (5.8%). A statistically significant relationship was found between increasing spectrum of drug resistance and ESBL production ($p<0.05$). Thus it is concluded that a higher rate of ESBL production prevail among MDR clinical bacterial isolates underscoring the need for routine ESBL detection in clinical laboratories.

Keywords: Multidrug resistance, ESBL, combined disk assay, Nepal.

INTRODUCTION

The progressive emergence and rapid dissemination of antimicrobial resistance is one of the biggest challenges facing global public health.[1] Failure to adhere to proper infection control technique, unrationale use of antibiotics, unhygienic practices, increased uses of antibiotics in animal and plants and more so availability of antibiotics without prescription and counterfeit products of dubious quality in developing countries have resulted in spread of antimicrobial reistance[1,2] and selection of multidrug resistant bacterial pathogens.[3] The marked increase of the microbial resistance and frequent reports of establishment of polyantibiotic resistance; PAN drug resistant organisms in the hospital setting presents a significant challenges for the clinical microbiologists to decide about the inclusion of various antimicrobials in the routine and specialized susceptibility testing.[4]

β-lactamases are the major defense of Gram negative bacteria against β-lactam antibiotics[5] and are evolving dynamically with the production of enzymes with novel substrate profiles, reduced susceptibility to β-lactamase inhibitors, and the simultaneous production of multiple types of β-lactamases resulting in multiply and sometimes totally resistant pathogens.[6,7] Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated bacterial enzymes that confer resistance to the penicillins (except temocillin), first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) and are inhibited by β-lactamase inhibitors such as clavulanic acid.[8] Although ESBLs have been reported most frequently in *Escherichia coli* and *Klebsiella* species, they have been found in other bacterial species including *Salmonella enterica, Pseudomonas aeruginosa,* and *Serratia marcescens*.[9-11]

Antimicrobials remain the mainstay of empirical therapy; however, indiscriminate use of antibiotics in many developing countries including Nepal, has resulted in the outbreak of drug resistant microorganisms.[1] Several cases of multidrug resistant bacterial outbreaks of significant clinical concern have been frequently reported.[12-14]

The emergence and spread of antimicrobial resistance due to the production of ESBL represent a clinical threat

Table-1: Growth profile and distribution of MDR strains among different specimens

Specimen	No. of specimen	Growth		MDR strains	
		n.	%	n.	%
Urine	1404	237	16.9	153	64.6
Pus	86	42	48.8	23	54.8
Sputum	82	30	36.6	18	60
Body fluid	29	5	17.2	5	100
Total	1601	314	19.6	199	63.4

because of its unrivalled spectrum of activity, thus underscoring the need for continual surveillance of resistance, rapid identification of such organisms as they emerge using reliable methods, assess their potential impact on health and measure their prevalence in the hospital and community.

MATERIALS AND METHODS

This is a prospective study carried out in the Microbiology Department of National Public Health Laboratory (NPHL), Kathmandu between April 2009 to January 2010.

Specimen size and Specimen types: A total of 1601 different samples including urine (1404), pus (86), body fluids (29) and sputum (82) sent for routine culture and antibiotic susceptibility tests were processed during the study period.

Culture of the Specimens: Urine specimens were cultured by semi quantitative culture technique. A loopful of well mixed, uncentrifuged urine sample was inoculated onto Blood agar (BA) and MacConkey agar (MA) using sterile calibrated loop. The plates were incubated in ambient atmosphere at 37^0C for 24 hours.

Pus, sputum and body fluids were aseptically inoculated onto BA, MA and Chocolate agar (CA). In addition, for pus and body fluid specimens, enrichment was done by inoculating them into Mueller Hinton Broth. The BA and CA plates were incubated at 5-10% CO_2 enriched atmosphere whereas MA in ambient atmosphere at 37^0C for 24 hours.

Identification and Antibiotic susceptibility test: Identification of significant isolates was done by standard microbiological techniques.[15] Antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standard Institute (CLSI).[16] The zone of inhibition was measured and interpreted using the standard chart and organisms reported as susceptible, intermediate or resistant accordingly.

Criterion for Multidrug Resistance: In the present study, the defining criterion for an isolate to be Multidrug Resistant (MDR) was set as resistance to two or more drugs of different structural classes.[17]

Screening and confirmatory test for ESBL production: The test inoculum matching McFarland tube No. 0.5 turbidity was prepared and carpet cultured on Mueller-Hinton agar. The screening agents, viz. Aztreonam (30µg), Ceftriaxone (30µg), Cefpodoxime (10µg), Ceftazidime (30µg) and Cefotaxime (30µg) (Mast Diagnostics, UK) were placed onto the inoculated media and incubated at 37^0C for 18-24 hours. Isolates showing Cefpodoxime ≤17 mm, Ceftazidime ≤22 mm,

Table-2: Screening for ESBL production using different agents

Screening Agents	Screening Criteria	ESBL Screening		No. of confirmed ESBL producers	Sensitivity (%)	Positive predictive value (PPV)
Ceftriaxone (30µg)	≤25 mm	Screen positives	92	68	98.6	73.9
		Screen negatives	18	1		
Cefpodoxime (10µg)	≤17 mm	Screen positives	94	68	98.6	72.3
		Screen negatives	16	1		
Ceftazidime (30µg)	≤22 mm	Screen positives	77	62	89.9	80.5
		Screen negatives	33	7		
Cefotaxime (30µg)	≤27 mm	Screen positives	89	68	98.6	76.4
		Screen negatives	21	1		
Aztreonam (30µg)	≤27 mm	Screen positives	92	65	94.2	70.7
		Screen negatives	18	4		

Table-3: Pattern of ESBL production according to the use of various combination discs

Combined Disk (CD) Assay	Criteria for confirmation	No. of suspected ESBL producers	No. of confirmed cases	Total confirmed cases	Negative cases after confirmation
CPD (10µg) CPD (10µg) plus CV (1µg)	Increase in zone size of ≤5 mm with ≤1 of the combination disks	110	65	69	41
CAZ (30µg) CAZ (30µg) plus CV (10µg)			62		
CTX (30µg) CTX (30µg) plus CV (10µg)			69		

CPD-Cefpodoxime, CAZ-Ceftazidime, CTX-Cefotaxime, CV-Clavulanate

Aztreonam ≤27 mm, Cefotaxime ≤27 mm, and Ceftriaxone ≤25 mm were suspected as possible ESBL producers.

Regardless of screening reults, all the processed bacterial isolates were subjected to phenotypic confirmatory test using Combined Disks (CD) Assay; MASTDISCS™ID Extended Spectrum ² Lactamase Set (CPD10) consisting of Set 1: Ceftazidime (30µg) and Ceftazidime (30µg) plus Clavulanic acid (10µg); Set 2: Cefotaxime (30µg) and Cefotaxime (30µg) plus Clavulanic acid (10µg); Set 3: Cefpodoxime (10µg) and Cefpodoxime (10µg) plus Clavulanic acid (1µg). The zone of inhibition for the Ceftazidime, Cefotaxime and Cefpodoxime discs were compared to that of the Ceftazidime, Cefotaxime and Cefpodoxime plus Clavulanic acid combination discs; an increase in zone diameter of ≥5mm in the presence of Clavulanic acid from any or all of the combination dicscs confirmed the isolates as ESBL producers.

Data Analysis: All the information were entered in the worksheet of Statistical Package for Social Science (SPSS) software (Version 16.0) and analyzed accordingly.

RESULTS

Of the total 1601 specimens, urine 1404 (87.7%) consisted the most followed by pus 86 (5.2%), sputum 82 (5.1%) and body fluids 29 (1.8%). Of the 1404 urine specimens, 237 (16.9%) showed significant growth, among which, 153 (64.5%) were multi-drug resistant. Similarly, 42 (48.8%), 30 (36.6%) and 5 (17.2%) specimens of pus, sputum and body fluids respectively showed growth, among which 23 (54.8%) isolates from pus, 18 (60%) from sputum and all 5 (100%) from body fluids were multidrug resistant (Table-1). Altogether, 24 different bacterial species were isolated from different specimens processed, with *E. coli* 133 (42.4%), *K. pneumoniae* 42 (13.4%), *Staphylococcus aureus* 29 (9.2%), *Acinetobacter* spp. 22 (7%) being the most frequently isolated species. Eighty four (63.2%) *E. coli*, 31 (73.8%) *K. pneumoniae*, 16 (55.2%) *S. aureus* and 15 (68.2%) *Acinetobacter* spp. were the major contributor of the MDR bacterial strains (Results not shown).

A hundred and ten MDR isolates were subjected to ESBL screening using Ceftriaxone (30 µg), Aztreonam (30 µg), Cefpodoxime (10 µg), Ceftazidime (30 µg) and Cefotaxime (30 µg). Cefotaxime showed sensitivity and positive predictive value (PPV) of 98.6% and 76.4% respectively. Ceftriaxone and Cefpodoxime though had sensitivity of 98.6%, had lower PPV of 73.9% and 72.3% respectively. With Ceftazidime, the sensitivity was lowest of all i.e. 89.9% and a PPV of 76.4% (Table-2).

Table-4: ESBL production profile among different bacterial genera

Organisms	No. of suspected ESBL producers	No. of cases confirmed (%)	Negative cases on confirmation
E. coli	75	60 (80)	15
K. pneumoniae	7	4 (57.1)	3
K. oxytoca	4	2 (50)	2
Acinetobacter spp.	10	1 (10)	9
Ps. aeruginosa	2	0 (0.0)	2
P. mirabilis	1	1 (100)	0
P. vulgaris	1	0 (0.0)	1
C. freundii	3	1 (33.3)	2
Providencia spp.	1	0 (0.0)	1
S. aureus	6	0 (0.0)	6
Total	110	69 (62.7)	41 (38.3)

Cefotaxime-Clavulanate combination disk showed 69 (62.7%) isoaltes as ESBL producers while Cefpodoxime-Clavulanate combination disk missed four isolates i.e only 65 (59%) were correctly identified. Moreover, only 62 (56.4%) isolates were correctly identified by Ceftazidime-Clavulanate. It was further noted that all isolates classified as screen negative by Ceftazidime disc did not show any synergy with Ceftazidime-Clavulanate combination discs (Table-3).

Table-5: Spectrum of drug resistance among ESBL producing isolates

	Spectrum of drug resistance				Total	p value
		2-4 drugs	5-10 drugs	>10 drugs		
ESBL production	Positive	0	9	60	69	p<0.05
	Negative	7	15	19	41	

Of the total 110 multidrug resistant bacteria of different genera tested for ESBL production, 69 (62.7%) bacterial isolates were ESBL positive. Among the 69 ESBL positive isolates, *E. coli* i.e. 60/69 (86.9%), *K. pneumoniae* 4/69 (5.8%), *K. oxytoca* 2/69 (2.9%), 1 (1.4%) isolate each of *Acinetobacter* spp., *Proteus mirabilis* and *Citrobacter freundii* showed ESBL production. ESBL production was not detected in *Ps. aeruginosa, Providencia* spp., *P. vulgaris* and *S. aureus* (Table-4).

An increasing spectrum of drug resistance was observed among ESBL producers with majority of the isolates showing resistant towards >10 drugs. Out of 69 ESBL positive isolates, 60 (86.9%) isolates were resistant to ≥10 drugs (Table-5). The association between ESBL production and spectrum of drug resistance was found statistically significant (p<0.05).

DISCUSSION

In our study, of all the bacterial isolates, tow third of them were multidrug resistant with *E. coli* being the major contributor followed by *K. pneumoniae, S. aureus* and *Acinetobacter* spp. Other studies have demonstrated comparable findings in the similar clinical setting.[18] The classical TEM-1, TEM-2, and SHV-1 β-lactamases predominantly mediate resistance to β-lactam antimicrobial agents among gram negative rods.[19] In *E. coli*, mutations at the target site (*gyrA* and *parC* genes) confers fluoroquinolone resistance,[20] whereas, several multidrug resistance efflux pump (**MDR** pump) systems contribute to intrinsic resistance.[21]

Cefotaxime was found as a reliable agent for ESBL screening with the highest sensitivity and PPV. The results are consistent with other findings.[22] Cefpodoxime and Ceftriaxone were equally effective screening agents, however, had low PPV. Contrary to our findings, in a study of comparision of screening agents for potential ESBL producers, Cefpodoxime was categorized as the most efficient.[23]

Seven isolates classified as ESBL screen negatives by Ceftazidime, however, were found ESBL producers on confirmatory test suggesting the possible production of CTX-M type ESBL by these isolates. Since, CTX-M producing isolates have typical propensity towards Cefotaxime, however, are susceptible to Ceftazidime in vitro, diagnostic laboratories may fail to identify CTX-M positive isolates as ESBL producers if Ceftazidime resistance is used as the sole screening criterion.[10] CTX-M ESBLs differ from those derived from TEM and SHV enzymes by their preferential hydrolysis of Cefotaxime and Ceftriaxone compared with Ceftazidime.[24]

A higher prevalence of ESBL production was observed in *E. coli* followed by *K. pneumoniae* and *K. oxytoca*. The findings are in agreement with reports from SMART program in Asia-pacific regions[25] and ESBL producers in bloodstream infections,[26] however, contrary to the findings of MYSTIC program in Europe and United states where higher prevalence of ESBL was seen in *K. pneumoniae* isolates.[27] *E. coli* and *K. pneumoniae* isolates are known to produce SHV, TEM, CTX-M and PER types of ESBLs and show variable resistance to ²-lactam antibiotics resulting in therapeutic failure.[8]

In this study, ESBL production was also seen among two *K. oxytoca*, one each of *Acinetobacter* spp., *C. freundii* and *P. mirabilis* isolates. *K. oxytoca* isolates producing TEM or SHV-type ESBLs should be distinguished from isolates hyperproducing K1 (a type of chromosomal â-lactamase) to prevent their false categorization as ESBL producers; ESBL usually have Ceftazidime MICs ≥2 µg/ml (or equivalent zone diameters), while K1 hyperproducers do not.[28] Similarly, outbreaks with *Acinetobacter* spp. producing SHV and PER type ESBLs have been increasingly reported.[29]

In the present study, the increasing pattern of the drug resistance seen among ESBL producers was found statistically significant (p<0.05). All the ESBL producers were resistant to five or more of the most commonly used antibiotics and was comparable to findings of other studies.[30] ESBL represent a clinical threat because of their unrivalled spectrum of activity and moreover co-existence of bla_{ampC} with bla_{CTX-M} has been frequently reported and in a strain with decreased outer membrane permeability, such enzymes can provide resistance to carbapenems as well.[6]

ESBL-producing strains are creating significant therapeutic problems since these pathogens are resistant to a wide range of β-lactams, including third generation cephalosporins as well as have potential for plasmid-mediated quinolone and carbapanem resistance is creating significant therapeutic problems. As indicated by the present finding together with previous findings, it appears to be necessary to include ESBL detection in routine laboratory practice so as to limit the rapid spread of ESBL-producing organisms.

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