Extended spectrum β-lactamase producing multidrug resistant urinary isolates from children visiting Kathmandu Model Hospital

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ABSTRACT

A study was conducted to analyze the status of the multidrug resistant (MDR) isolates producing Extended Spectrum of β-lactamase (ESBL) among the uropathogens infecting children less than 15 years from November 2010 to April 2011 in the Bacteriology laboratory, Kathmandu Model Hospital. Urine samples received in the laboratory were processed for routine culture. The antimicrobial susceptibility of bacterial isolates was determined following Clinical and Laboratory Standard Institute (CLSI) recommended Kirby-Bauer Disc Diffusion method. The defining criterion in this study for an isolate to be multidrug resistant was resistance to two or more drugs of different structural classes. Isolates were confirmed for ESBL-production by performing the Inhibitor Potentiated Disk Diffusion (IPDD) Test/ Combined Disk Assay for ESBL confirmation. Out of 252 urine samples received in the laboratory, 59(23.41%) showed significant growth of which 54.23% (32/59) were MDR isolates. Additionally, 25 isolates (21 *Escherichia coli* and 3 *Citrobacter freundii* and single *Enterobacter aerogenes*) among them were ESBL producers. Among the first line drugs used against gram negative isolates, Nitrofurantoin was drug of choice; meanwhile among the second line drugs Cefoperazone/ Sulbactum was drug of choice, whereas, Cephotaxime, Ciprofloxacin, Norfloxacin and Gentamicin were the drug of choice for Gram positive isolates. Significant association was found between ESBL production and spectrum of drug resistance ($p<0.05$).

Keywords: UTI, MDR, ESBL.

INTRODUCTION

Urinary tract infection (UTI) is common bacterial infection causing illness in infants and children. It may be difficult to recognize UTI in children because the presenting symptoms and signs are non-specific, particularly in infants and children younger than 3 years.[1] Common pathogens that have been implicated in UTIs are primarily Gram-negative organisms with *Escherichia coli* having a more prevalence than other Gram-negative pathogens including *Klebsiella pneumoniae, Enterobacter spp., Proteus mirabilis, Pseudomonas aeruginosa* and *Citrobacter spp*.[2] In community and hospital settings the etiology of UTIs and the antimicrobial susceptibility of uropathogens have been changing over the years.[3,4] Patients with infections by resistant organisms are at an increased risk of treatment failure. Resistance is neither a new phenomenon nor unexpected in an environment in which potent antimicrobial agents are used.[5] Resistance to the expanded-spectrum β-lactam antibiotics due to β-lactamases called extended-spectrum β-lactamases (ESBLs) is one of the ways of the organisms to withstand the adversities they face.[6]

MATERIALS AND METHODS

The study was conducted prospectively in the Microbiology Laboratory of Kathmandu Model Hospital, Kathmandu from November 2010 to April 2011.

The study included urine samples collected from patients of age <15 years and both genders visiting Kathmandu Model Hospital, requesting for routine culture and antibiotic susceptibility testing. A total of 252 urine samples from patients suspected of urinary tract infection were collected for routine culture and were processed according to the standard laboratory methods.

The urine samples were cultured onto the MacConkey agar and Blood agar plates by the semi-quantitative culture technique using a standard calibrated loop. The identification of bacterial isolates was done using standard microbiological techniques which comprises of studying the colonial morphology, staining reactions and various biochemical properties.[7]

Susceptibility tests of the different isolates towards various antibiotics were performed by Kirby-Bauer disk diffusion method for the commonly isolated

Table-1: Profile of urine samples and status of MDR and ESBL isolates

Specimen	Total samples processed	Significant growth		MDR isolates		Suspected ESBL isolates	ESBL producers (%)
		n.	%	n.	%		
Urine	252	59	23.41	32	54.23	29	25 (86.20)

pathogens using Mueller Hinton Agar (MHA) as recommended by Clinical and Laboratory Standard Institute (CLSI).[8] MDR isolates in pure culture were alienated and preserved for three months in 20% Glycerol containing Tryptic Soya Broth and kept at -20°C until subsequent tests for the presence of ESBL was performed.

MDR isolates were screened for possible ESBL production using Cefotaxime, Ceftazidime (30µg) and Ceftriaxoneas recommended by Clinical and Laboratory Standard Institute (CLSI)**.**[8] According to the guidelines, isolates showing Cefpodoxime <17mm, Ceftazidime <22 mm, Aztreonam <27 mm, Cefotaxime <27 mm, and Ceftriaxone <25 mm are the possible ESBL producing strains. The suspected ESBL strains were tested for confirmation by Combined Disc Assay. This method compares the zones of cephalosporin discs to those of the same cephalosporin plus clavulanate. Greater than and equals to 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism.

The entire denouement were entered in the worksheet of Statistical Package for Social Science (SPSS) software (Version 16.0) and analyzed.

Table-2: Microbiological profile of urinary isolates

Organism isolated	Infection		Total
	Freq.	%	
Gram negative			
Escherichia coli	46	77.96	54
Citrobacter freundii	5	8.47	
Proteus mirabilis	2	3.38	
Enterobacter aerogenes	1	1.69	
Gram positive			
Coagulase Negative Staphylococci	3	5.08	5
Staphylococcus aureus	1	1.69	
Enterococcus fecalis	1	1.69	

RESULTS

Out of 252 urine samples, 59 (23.41%) samples showed significant growth, among them 32 (54.23%) were MDR-strains and 25 isolates were found to be ESBL-producers. The predominant bacteria causing UTI were the Gram negatives which constituted 91.52% (54/59) and among them 55.56% (30/54) were MDR-strains. Gram positive bacteria constituted only 8.47% (5/59) and of them 40% (2/5) were MDR-strains. Altogether 7 different species of bacteria were isolated from the growth positive cultures, *Escherichia coli* (N=46) was found to be the most predominant (77.96%). Among Gram positives, CoNS (N=3) was the most predominant with 5.08% of total isolates, 33.33% (1/3) of this organism was MDR. Of total 46 E. coli, 56.52% (26/46) were found to be MDR-strains; while the isolates of *Citrobacter freundii* (3/5), *Enterobacter aerogenes* (1/1), Coagulase Negative Staphylococci (1/3) and *Enterococcus faecalis* (1/1) were found to be MDR (Table-1 to 4).

Nitrofurantoin was found to be the most effective drug among the first line of drugs with susceptibility of 96.29% against the Gram negative bacteria. And Cefoperazone/Sulbactum was the most effective drug with 100% susceptibility against the Gram negative MDR isolates.

Of the total isolates, 45.65% (21/46) of *E. coli*; 60% (3/5) of *Citrobacter freundii*; and the *Enterobacter aerogenes* were found to be ESBL-producers. All these 25 ESBL-producers were also found to be MDR. Increasing spectrum of drug resistance seen among ESBL producers was found statistically significant (P<0.005) deducing the presence of significant association between ESBL production & spectrum of drug resistance (Fig. 1 and 2).

Table-3: ESBL production profile among different bacterial genera

Organisms	No. of suspected ESBL producers	No. of cases confirmed (%)	Negative cases on confirmation
E. coli	24	21 (87.50)	3
C. freundii	3	3 (100)	0
E. aerogenes	1	1 (100)	0
E. fecalis	1	0 (0)	1
Total	29	25 (86.20)	4

DISCUSSION

Out of the total 252 urine samples processed, only 59 (23.41%) showed significant growth, of which 32 (54.23%) were from male, while 27(45.76%) were from female. Of the total isolates, 32 (54.23%) were found to be multidrug resistance. The overall growth positive rate (23.41%) in our study was slightly lower than previous reports of 27-29% 9,10 in this age group from Nepal. On the contrary, in similar study,11 higher culture positivity of 57% in children of age below 15 years was found, which might be due to various exclusion criterions (neonates, children with pre-existing Vesico-Ureteric Reflux and Posterior Urethral Valves, acute coexisting renal diseases, recurrent UTI and previous antibiotic usage) of samples.

The presenting signs and symptoms are non-specific, particularly in infants and children younger than 3 years,[1] this is why the low rate culture positivity might have been observed in this age-group. The low growth positive rate observed in this study might also be due to inclusion of every urine samples for culture regardless of their illness, the referral of all the patients seeking intervention regarding problems of urinary tract to urine culture, the prior use of the antibiotics, or the possible presence of the fastidious bacteria.

In our study, Gram negative isolates (91.52%) were the predominant organism isolated from the urine samples of the children which was lower than 97.02% found in previous study[7] and slightly higher than 89.58% in study[11] in Nepal. *E. coli* was the most common organism isolated and constituted 77.96% of all positive samples. This was lower than 86-94% reported[9,10,12,13] earlier. In studies[14,15] conducted in children, *E. coli* has been found to be 73-78% which was in harmony with our study. Our finding was found to be much higher than 50-62% reported in previous studies.[9,11]

The predominance of *E. coli* was followed by *Citrobacter freundii* (8.4%), Coagulase Negative Staphylococci (5.08%), *Proteus mirabilis* (3.38%), *Enterobacter aerogenes, Enterococcus fecalis* and *Staphylococcus aureus* (1.69% each). In similar study,[9] the pattern of growth was found to be *E. coli* (93.3%) followed by *Proteus spp* (2.3%), *Klebsiella spp* (1.5%), *Citrobacter spp, S. aureus* and *Pseudomonas spp* (0.7% each), *Enterobacter spp* (0.6%) and *Salmonella spp* (0.2%). The most common organism isolated was *E.coli* (50%) followed by *Klebsiella pneumoniae* (16.66%), *Proteus vulgaris* (12.50%), *Enterococcus spp* (6.25%), *Citrobacter spp, Enterobacter spp, Staphylococcus aureus* (4.10% each) and *Pseudomonas aeruginosa* (2.08%) of cases in a hospital based study[11] in Nepal. In similar study in Tunisia,[16] the most frequent pathogens recovered were *E. coli* (71%), *K. pneumoniae* (10%), *P. mirabilis* (8%), *Staphylococcus spp* (1.6%), *P. aeruginosa* (1%) and others (2%).

Table-4: Spectrum of drug resistance among ESBL producing isolates

ESBL Production	Range of Drug resistance		p value
	4-8	8-12	
Positive	4	21	<0.005
Negative	4	2	

P value calculated from Karl Pearson's χ^2 with Yates's correction, at $\alpha = 0.05$ and d.f = 1

Of the total 59 isolates, 81.25% of total MDR organisms were found to be *E. coli*. The high level of drug resistance seen among *E. coli* is due mediated by β-lactamases, which hydrolyze the β-lactam ring inactivating the antibiotic. The classical TEM-1, TEM-2, and SHV-1 enzymes are the predominant plasmid-mediated β-lactamases of Gram-negative rods.[17] Mutations at the target site i.e. *gyrA*, which is a gyrase subunit gene, and *parC*, which encodes a topoisomerase subunit, confer resistance to fluoroquinolones.[18] In addition to this mechanism, there are more than seven efflux systems in *Escherichia coli* that can export structurally unrelated antibiotics; these multidrug resistance efflux pump (**MDR** pump) systems contribute to intrinsic resistance for toxic compounds such as antibiotics, antiseptics, detergents, and dyes.[19] It has also been reported that intracellular *E. coli* can develop into biofilms, creating pod-like bulges on the bladder surface. These pods contain bacteria encased in a polysaccharide-rich matrix surrounded by a protective shell of uroplakin.[20] This may contribute to persistence of bladder infections in the face of robust host defenses and long term antimicrobial treatment. Multidrug resistance seen in Coagulase negative staphylococci might also be due to the biofilms formation,[21] leading the treatment complicated.

In our study 3 out of 5 *C. freundii* were MDR. The higher level of drug resistance seen among *Citrobacter* spp. is mediated by the production of different kind of β-lactamases primarily ESBL,

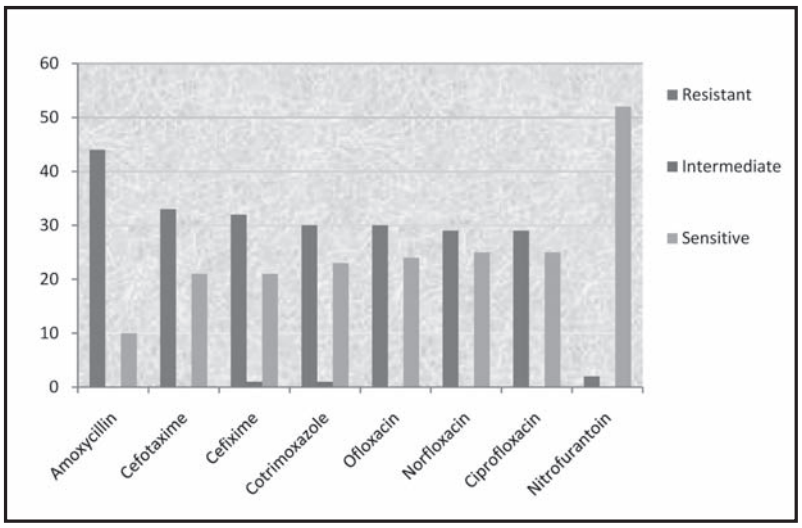

Fig. 1. Antibiotic susceptibility pattern of gram negative organisms

AmpC and Metallo β-lactamases. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for β-lactamases have had a great impact on the drug resistance character shown by these pathogenic bacteria.[22] The plasmid-specified *qnr* determinants mediates resistance presumably by protecting DNA gyrase and topoisomerase IV from the inhibitory action of the fluoroquinolones.[23] Variants of the *qnr* element, first identified in *K. pneumoniae*,[24] have been found in *Citrobacter freundii*.

Enterobacter aerogenes is a common agent of hospital-acquired infection. It exhibits a remarkable adaptive capability and easily acquires resistance to β-lactam antibiotics during therapy.[25] In the last 5 years, it has been shown that clinical isolates of this species, which are naturally resistant to aminopenicillins, often express an extended-spectrum β-lactamase, TEM-24, which gives rise to resistance to β-lactam antibiotics.[25,26] Moreover, *E. aerogenes* exhibits acquired resistance to other families of antimicrobial agents like quinolones, tetracycline, and chloramphenicol.[27]

Three different mechanisms account for the acquired resistance to Macrolide-lincosamide-streptogramin (MLS) antibiotics in Gram-positive bacteria: modification of the drug target (23s rRNA); inactivation of the drug, and active efflux of the antibiotic.[28] Two mechanisms of ß-lactam resistance in *E. faecalis*, the production of β-lactamase and the over production of penicillin-binding proteins (PBPs), have been reported. Studies[29,30] suggest that horizontal gene transfer is a factor in the occurrence of antibiotic resistance in clinical isolates and suggested that the high prevalence of resistance to a particular antibiotic does not always reflect antibiotic consumption.

Nitrofurantoin as an option for empirical therapy has been considered by many authors[31,32] since its multiple mechanisms of action seem to have enabled it to retain potent activity against *E. coli* despite nearly 50 years of use.[33] Similarly, in our study, Cefoperazone/Sulbactum and Amikacin were found to be the most effective drugs with susceptibility of 96.55% and 96.42% respectively among the second line drugs.

Of the total 29 suspected ESBL producers, 24 were of *E. coli*, 3 of *C. freundii*, 1 of *E. aerogenes* and 1 of *E. fecalis*. However, of the 24, 3 and 1 screening positive *E. coli*, *C. freundii* and *E. aerogenes*

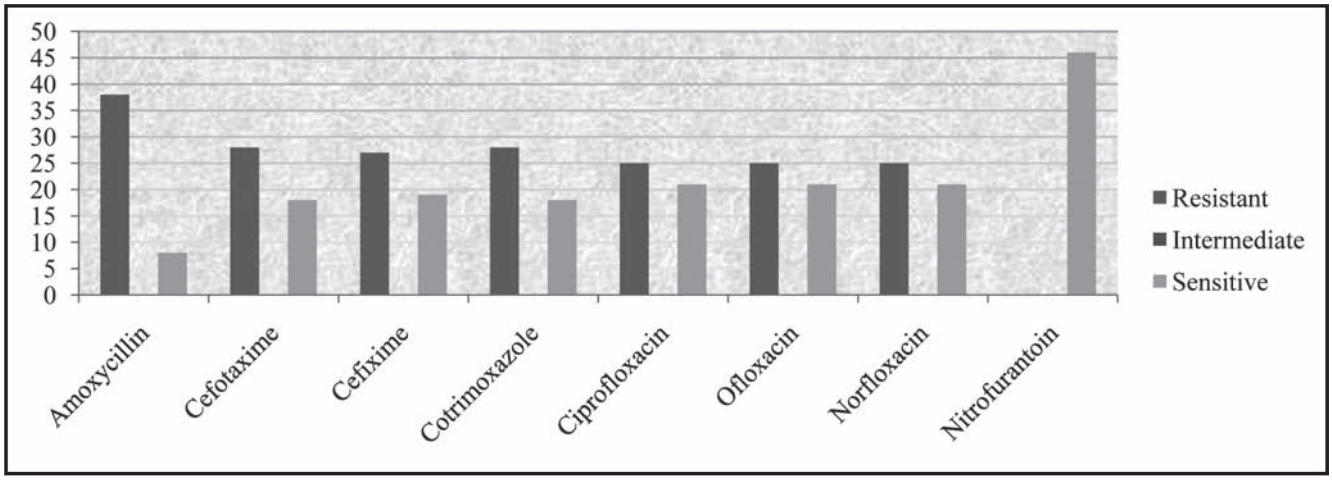

Fig. 2. Antibiotic susceptibility pattern of *E. coli* against first line of drugs

respectively, 21 *E. coli*, 3 *C. freundii* and the *E. aerogenes* were confirmed as ESBL producers and the *E. fecalis* suspected of ESBL production was found to be ESBL non producer by combined disc assay. In a similar study,[34] 62.7% bacterial isolates were found ESBL positive. Among which, *E. coli* (86.9%), *K. pneumoniae* (5.8%), *K. oxytoca* (2.9%), 1.4% each of *Acinetobacter spp., Proteus mirabilis* and *Citrobacter freundii* were found to be ESBL producers.

ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first, second, and third generation cephalosporins (such as cefazolin, cefuroxime, ceftazidime, cefotaxime, ceftriaxone etc) and aztreonam but not the cephamycins (eg cefoxitin and Cefotetan) and carbapenems (eg imipenem and meropenem) by hydrolysis of these antibiotics and which are inhibited by β-lactamase inhibitors such as clavulanic acid. ESBLs include the β-lactamases of Bush-Jacoby-Medeiros group 2be and those of group 2d whereas ESBLs except OXA-type have been grouped in class A in the classification scheme of Ambler.[35] ESBLs are able to hydrolyze penicillins, narrow spectrum and third generation cephalosporins and monobactams with hydrolysis rate for ceftazidime, cefotaxime, or aztreonam at least 10% that for benzyl penicillin.[35,36] ESBLs are harder to detect in those Enterobacteriaceae with inducible AmpC chromosomal enzymes e.g. *Enterobacter* spp., *Citrobacter freundii, Morganella morganii, Providencia* spp. and *Serratia* spp.[37]

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 four or more of the most commonly used antibiotics and was comparable to findings of other studies.[34,38]

E. coli were the major organism (77.96%) that was found infecting the urinary tract of the children. As the major organism, they were also the most frequent MDR (81.25%) and ESBL producers (84%). This may have been the result of resistance factors that are readily retained by *E. coli*, the easy acquisition of resistance factors outside the host, or significant sources of resistant bacteria not captured by this study. This type of precarious situation is quite quotidian after the trend of misuse and overuse of drugs as per the effect of competition for survival. On the contrary, humans need more elaborate study and more dimensions in new way outs, including strategies that target bacterial virulence in the battle against resistant organisms.

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