Phenotypic detection of Extended Spectrum Beta lactamase production from *E. coli* and *K. pneumoniae* in urinary samples among children

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ABSTRACT

Objectives: The main objective of this study was to detect antimicrobial drug resistance (AMR) and Extended Spectrum Betalactamase (ESBL) production phenotypically in *E. coli* and *K. pneumoniae* isolated from urines with significant bacteriuria.

Methods: This cross-sectional study was carried out in Microbiology laboratory of Kathmandu College of Science and Technology, Kamalpokhari. The urine samples from suspected urinary tract infected cases were collected from both genders of children below 15 years of age from Out and In-patient department of International Children Friendship Hospital, Maharajgunj and those with significant bacteriuria were cultured for isolating the bacterial etiology targeted as *E. coli* and *K. pneumoniae*. AMR for these two bacteria were tested and detected using Kirby Bauer Disc Diffusion technique. ESBL production was confirmed by Double Disc Synergy test (DDST) and Phenotypic Confirmatory Disc Diffusion Test (PCDDT) after screening for all the isolates showing resistance to third generation cephalosporin namely Cefotaxime and Ceftriaxone according to CLSI instructions.

Results: Out of 388 urine samples processed, 29.89% (116/388) showed significant bacterial growth. Five (5) different Aerobic Gram Negative bacterial species were detected and identified. *E. coli* topped the list (70.68%) followed by *K. pneumoniae* (15.52%), *K. oxytoca* (8.62%), *Proteus vulgaris* (3.45%) and *Pseudomonas aeruginosa* (1.73%). Among positively screened (44.82%) beta lactamase producers (36.2%) of total isolates were confirmed to produce ESBL. Among ESBL producing isolates, highest susceptibility was seen to Ceftazidime (23.80%) followed by Cefotaxime (16.67%). The ESBL producing isolates were least susceptible to Ceftriaxone (2.38%). AMR was detected using Kirby-Bauer Disc diffusion technique. Comparatively less resistance to amikacin and nitrofurantoin (19.1% and 9.53% respectively) was seen among ESBL producers. 40 out of the 42 (95.23%) ESBL producing strains showed susceptibility to the combination drug, piperacillin/tazobactam. The resistance to meropenem was observed to be less (9.53%) as compared to that to imepenem (7.15%).

Conclusion: This study concluded that there is high prevalence of multidrug resistant uropathogenic clinical strains of *E. coli* and *K. pneumoniae* with higher rates of ESBL production. A resistance to the carbepenems is also emerging. Appropriate antimicrobial regimen selection for empirical therapy is thus important for such cases. On managing the empirical antibiotics practice, one can reduce the risk of ESBL producers. There is an essence need of regular routine practice of ESBL detection.

Keywords: AMR, ESBL, *E. coli, K. pneumoniae*, PCDDT, DDST

INTRODUCTION

Antimicrobial resistance among bacterial strains causing Urinary tract infections (UTIs), one of the most common bacterial infections in humans both in the community and the hospital settings, is an emerging problem, worldwide. *E. coli* and *K. pneumonia*e are the two major pathogens commonly isolated in urine, with *E. coli* being the most prevalent type accounting

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for 75-90% of UTIs. Usually these infections are treated with a variety of antibiotics, including β-lactams, β-lactam/β-lactamase inhibitors, flouroquinolones, and carbapenems (Kariuki et al. 2007; Ullah et al. 2009; Hoban et al. 2011 and Briongos-Figuero et al. 2012). However, in recent times, these uropathogens have also developed resistance to commonly prescribed antimicrobial agents; this severely limits the treatment options of an effective therapy.

Primarily, these uropathogens exert their antimicrobial resistance against beta-lactams by producing extended spectrum beta-lactamases (ESBLs) enzymes that confers bacterial resistance to all beta-lactams except carbapenems, cephamycins and clavulanic acid (Coque et al. 2008). It is global matter of concern since infections associated with ESBL producing clinical isolates are found with higher mortality, length of stay, and health care cost and longer antibiotic therapy in comparison to that with non-ESBL producing pathogens (Schwaber and Carmeli 2007). Correct diagnosis and prompt treatment is crucial in order to prevent morbidity and mortality associated with the disease which is further backed up by frequent changing pattern of antimicrobial resistance with development of various resistant mechanisms like drug efflux, reduced uptake and production of hydrolytic enzymes like extended spectrum β-lactamases (Ghedira et al. 2004). The Infectious Disease Society of America has listed *E. coli* and *Klebsiella* species as two out of six pathogens for which new drugs are urgently needed in order to combat their growing resistance (Talbot et al. 2006) The prevalence of ESBL-positive isolates depends on a range of factors including species, geographic locality, hospital/ward, group of patients and type of infection, and large variations have been reported indifferent studies (Livermore et al. 2007).

The different studies from Nepal have indicated a variable rate of ESBL producing bacterial strains in Nepal, where Enterobacteriaceae were found 28% to 67% (Hammer et al. 2007). The acquisition and expression of ESBLs enzymes among Enterobactericeae have posed a serious public health problem in developing countries like Nepal that still lacks the facilities for urine culture and antimicrobial susceptibility testing; this clearly leads to missing ESBL isolates in our country. This might be one of the reasons that are creating deaths among urinary tract infected cases in Nepal. Thus, the regular surveillance of the drug resistance among the clinical isolates will be helpful to know the actual burden of the situation, which will help making the necessary policy to reduce the incidence of drug resistance among the bacteria primarily causing UTIs which are being difficult to treat nowadays. This study was carried out to detect antibiotic resistance and phenotypic detection of ESBL among multidrug resistance *E. coli* and *K. pneumoniae* to determine the prevalence and antibiotic resistance profile in clinically relevant urine isolates from children.

MATERIALS AND METHODS

This cross sectional study was carried out at microbiology laboratory of Kathmandu College of Science and Technology, Kamalpokhari, Kathmandu. During the study, 388 urine samples from suspected urinary tract infected cases were collected from both genders of children below 15 years of age from Out and In-patient department of International Children Friendship Hospital, Maharajgunj. For sample collection, In case of neonates and infants, genital area was first cleaned with sterile water and wiped from front to back until area is clean. For female, urine bag was affixed over genital area, starting from the perineum and working upwards. For male, urine bag was placed over the penis ensuring a tight seal all around the bag. Urine bag was checked frequently and removed as soon as the urine is passed. The parents of toilet trained children were suggested to collect midstream clean catch urine and were transported to our laboratory using ice box as soon as possible. However, improperly labeled, unlabeled and leaked sample were excluded from the study.

For processing of each sample, microbiological protocols were followed according to standard microbiological guidelines (Cheesbrough 2006 and Forbes et al. 2007). The collected urine samples were inoculated on MacConkey agar (MA) and Blood agar (BA) using a sterile calibrated loop. The inoculated plates were then incubated aerobically at 37˚C for 24 hours. Colony count was made and plates showing more than or equal to 10^5 colony forming units (cfu)/ml of urine was considered for positive result (Forbes et al. 2007). The plates showing significant bacteriuria were then cultured for presumptive identification of *E. coli* and *K. pneumoniae* that was carried out on basis of colonial characteristics, gram staining and biochemical tests.

The Kirby-Bauer disc diffusion method, according to the CLSI guidelines, was used to test the isolates for their antimicrobial susceptibilities using β-lactam antibiotics viz. cefpodoxime (30µg), cefotaxime (30µg), ceftrioxone (30µg), ceftazidime (30µg), cefepime (30µg), and non β-lactam antibiotics viz. gentamicin (10µg), ciprofloxacin (5µg), piperacillin/tazobactam (100/10µg), norfloxacin (10µg), amikacin (30µg), nitrofurantoin (100µg), cotrimoxazole (25µg), imipenem (10µg) and meropenem (10µg). All the antibiotic discs were procured from Hi-media, Mumbai (CLSI 2016).

In this study, if the isolates were resistant to at least one agent of three different classes of commonly used antimicrobial agents, they were regarded as multidrug resistant (MDR) (Magiorakos et al. 2012). If the zone of inhibition (ZOI) was ≤25mm for Ceftriaxone, ≤22mm for Ceftazidime, and/or ≤27mm for Cefotaxime, the isolate was considered a potential ESBL producer as recommended by CLSI and further tested by confirmatory methods.

For Double disc synergy test, Mueller Hinton agar was inoculated with the standard (0.5 McFarland) inoculum of the test isolate. Ceftazidime (30 µg) disc was placed on agar 15 mm away from the center of amoxicillin-clavulanic acid (20 µg/10 µg) disc. Extension of zone of inhibition towards amoxicillin-clavulanic acid was interpreted as ESBL producer.

For phenotypic confirmatory test, disks of third generation cephalosporins alone and disks of third generation cephalosporins plus clavulanic acid are required for the phenotypic confirmatory test that uses combination disk method according to CLSI guidelines. Ceftazidime (30 µg) disk alone and ceftazidime + clavulanic acid (30 µg + 10 µg) disk; and cefotaxime (30 µg) disk alone and cefotaxime + clavulanic acid (30 µg + 10 µg) disk were used in this study. The disks were placed at a distance of at least 25mm on a carpet culture of the isolate on MHA plate. Differences in zone diameters of cephalosporins alone and in combination with clavulanic acid were recorded after incubation for 16-18 hours at 37°C. The increase in zone diameter equal to or greater than (≥) 5mm around cephalosporin plus clavulanic acid disk compared to cephalosporin alone indicates ESBL production by the organism.

The data of the case record forms were entered in the worksheet of Microsoft Excel. Frequency and percentages were analyzed as descriptive findings.

RESULTS

Out of a total of 388 mid-stream urine specimens (202 from male and 186 from female child) screened for significant bacteriuria, a total of 116 (29.89%) were found to have significant growth from which bacterial isolates were obtained. Among total, 82/116 (70.68%) and 18/116 (15.52%) samples showed the growth of *E. coli* and *K. pneumoniae*, respectively. Rests of the bacterial isolates were *Proteus vulgaris* (4/116), *Pseudomonas aeruginosa* (2/116) and *K. oxytoca* (10/116). Initial screening of these isolates for ESBL production showed 42/82 of *E. coli* and 8/18 of *K. pneumoniae* strains to be ceftriaxone resistant. Confirmation test (PCDDT) revealed 34/82 (41.46%) of *E. coli* and 8/18 (44.44%) of *K. pneumoniae* isolates to be ESBL positive as shown in the following table.

Table 1: Distribution of bacterial isolates in urine sample

Bacterial isolates	Number(%)
E. coli	82(70.68)
K. pneumoniae	18(15.52)
K. oxytoca	10(8.62)
Proteus vulgaris	4(3.45)
Pseudomonas aeruginosa	2(1.73)
Total	**116(100)**

Table 2: Phenotypic confirmation of ESBL producer from potentially screened isolates

Isolates	Total Screened for ESBL	Confirmed ESBL producers (%)
E. coli	42	34(41.46)
K. pneumoniae	8	8(44.44)
K. oxytoca	2	0
Total	**52(44.82%)**	**42(36.20)**

Among total ESBL isolates, highest rate of ESBL producers were from age group below 5 years (66.66%) followed by age group 6-10 (23.80%) and 11-15 (9.52%). ESBL production was more (52.38%) from bacterial isolates of male child than female (47.62%).

Table 3: Distribution of ESBL producers according to age and gender of children

Age-Group (years)	Gender		Bacterial isolates	Gender		ESBL producers (%)
	Male	Female		Male	Female	
≤5	46	32	78	18	10	28(66.66)
6-10	16	8	24	4	6	10(23.80)
11-15	2	5	7	0	4	4(9.52)
Total	**68**	**45**	**116**	**22 (52.38)**	**20 (47.62)**	**42 (100)**

Among total bacterial isolates from Outpatient department, ESBL was produced from 46.87% isolates whereas from inpatient isolates only 23.07% produce ESBL.

Table 4: Distribution of ESBL producers from patients attending different departments

Patient department	Bacterial Isolates	ESBL producers(%)
Out-Patient	64	30(46.87)
In-Patient	52	12(23.07)
Total	**116**	**42**

Among total MDR isolates, 67.74% produce ESBL and among MDR *E. coli* 73.91 % produce ESBL and among MDR *K. pneumoniae*, 80% produce ESBL whereas *K. oxytoca* did not produce ESBL.

Table 5: Distribution of ESBL producers among MDR isolates

Bacterial isolates	No. of MDR isolates (%)	ESBL producer (%)
E. coli	46(74.19)	34(73.91)
K. pneumoniae	10(16.13)	8(80)
K. oxytoca	6(9.68)	0(0)
Total	**62(100)**	**42(67.74)**

Among ESBL producing isolates, highest susceptibility was seen to Ceftazidime (23.80%) followed by Cefotaxime (16.67%). The ESBL producing isolates were least susceptible to Ceftriaxone (2.38%)

Table 6: *In vitro* susceptibility of ESBL producers to β-lactam antibiotics

Antibiotics	*E. coli* n=34	*E. coli* n=34	Total N=42 Susceptible No. (%)
Ceftazidime	8	8	10(23.80)
Ceftriaxone	1	1	1(2.38)
Cefotaxime	6	6	7(16.67)
Cefepime	5	5	6(14.28)
Cefpodoxime	4	4	5(11.90)

Upon testing of Susceptibility pattern for ESBL and non ESBL producers to non β-lactam antibiotics, a co-resistance to the non-β lactam antibiotics was observed more with the ESBL producers. Comparatively less resistance to amikacin and nitrofurantoin (19.1% and 9.53% respectively) was seen among ESBL producers. 40 out of 42 (95.23%) ESBL producing strains showed susceptibility to piperacillin/tazobactam, a combination drug. The resistance to meropenem was observed to be less (9.53%) as compared to that to imepenem (7.15%) among ESBL producers while ESBL non-producers have shown absolute sensitivity towards meropenem, imipenem and piperacillin/tazobactam.

Table 7: Antibiotic susceptibility of ESBL producers and non-producers towards non β-lactam antibiotics

Antibiotics	ESBLproducers (n=42)		Non-producers (n=74)	
	Susceptible No. (%)	Resistant No. (%)	Susceptible No. (%)	Resistant No. (%)
Norfloxacin	2 (4.77)	40 (95.23)	45 (60.8)	29 (39.2)
Ciprofloxacin	5 (11.9)	37 (88.1)	48 (64.8)	26 (35.2)
Amikacin	34 (80.9)	8 (19.1)	69 (93.24)	5 (6.76)
Gentamicin	11 (26.19)	31 (73.81)	65 (87.83)	9 (12.17)
Co-trimoxazole	8 (19.04)	34 (80.96)	33 (44.59)	41 (55.41)
Nitrofurantoin	38 (90.47)	4 (9.53)	70 (94.59)	4 (5.41)
Piperacillin/Tazobactam	40 (95.2)	2 (4.8)	74 (100)	0 (0)
Meropenem	38 (90.47)	4 (9.53)	74 (100)	0 (0)
Imipenem	39 (92.85)	3 (7.15)	74 (100)	0 (0)

DISCUSSION

The occurrences of ESBLs among clinical isolates vary greatly worldwide and geographically and are rapidly changing overtime. The prevalence of ESBL producers was 36.20 % (*E.coli*= 41.46% and *K. pneumoniae* = 44.44%) in this study. It correlates with a study done in India (Babypadmini and Appalaraju 2004) which reported nearly 40% of urinary isolates of *E. coli* and *K. pneumoniae* were ESBL positive. Findings from other studies in Nepal have shown ESBL production ranging from 18% to 62.7 %. (Shrestha et al. 2011; Poudyal et al. 2011; Thakur et al. 2013). Variation might have occurred due to low number of samples studied from different geographical locations. Similarly, variation in prevalence of ESBL producing organisms was found in other countries. Significant increase in ESBL organisms were published from India (Sasirekha et al. 2010 and Sharma et al. 2012), Pakistan (Ullah et al. 2009) and Nigeria (Yusha'u et al. 2010).

Previous studies from Nepal have reported the prevalence of the ESBL producing bacteria ranging from 13.5% to 33.2%. Chander and Shrestha (2013) reported the ESBL prevalence rate to be 13.5% whereas, recently, Neupane et al. (2016) have reported 33.2% of ESBL producers in their study. Similarly, Ansari et al. (2015), Khanal et al. (2013), have reported 24% and 25% ESBL producers, respectively. These findings show less prevalence with those of our study. However, extremely lower rates of ESBL production have been documented from Japan, Korea, and United States (Paterson and Bonomo 2005; Yan et al. 2014). The differences in the ESBL rates may be attributable to the geographic difference, antimicrobial stewardship programme, and infection control practices.

In this study, ESBL production was more (52.38%) from isolates of male child than female (47.62%). However, Females showed a higher rate of isolation of ESBL producing *E. coli* (60%) and *K. pneumoniae* (62.5%), which discords the findings as reported earlier (Oladeinde et al. 2011 and Ahmed et al. 2012). The result is inconsistent because ESBL production and pathogenic nature may not differ according to gender. However, in our country, preference is given to male child and parents take care of them with so much love and take them to hospital in a higher frequency. This may be the reason behind higher inclusion of male child in this study.

The highest bacterial isolates were found in children less than 5 years age, including the prevalence of ESBL organisms which was above 66.66%. The reason for this may be due to the immunological status of the children below 5 years of age who are more vulnerable to infections, malnourished child, and child living in poor sanitation. Similar result was observed in a study done by Kayastha et al. (2020) in Nepal. The higher prevalence of bacterial growth in outpatients, in this study indicates the emergence of ESBL producing pathogens from community that may spread creating difficulties in treating the patients with drug resistance. However, this result is contrary to a study in Nepal (Kayastha et al. 2020) which showed greater prevalence in inpatients which may have been added by nosocomial infections associated with prolonged hospital stay, intensive care unit admission, extensive use of invasive medical devices, and overconsumption of antibiotic among inpatients.

MDR was found in 53.44% (62/116) of the urinary isolates, among them, major MDR producer was *E. coli.* Among total MDR isolates, 67.74% produce ESBL and among MDR *E. coli* 73.91% produce ESBL and among MDR *K. pneumoniae*, 80% produce ESBL. Production of different β-lactamase, hydrolyze β-lactam ring of

antibiotic, like TEM-1, TEM-2, SHV-1 and many other plasmid-mediated β-lactamases confers high level of resistance to drug in *E. coli*. Furthermore, different efflux pumps and target site mutation at *gyrA* and *parC* are responsible for fluoroquinolones resistance (Sharma et al. 2018).

Among ESBL producing isolates, highest susceptibility was seen to Ceftazidime (23.80%) followed by Cefotaxime (16.67%). The ESBL producing isolates were least susceptible to Ceftriaxone (2.38%). The high rates for non-ESBL mediated ceftriaxone resistant isolates may be due to their different mechanisms for resistance such as the production of ampC β lactamase, metallo-betalactamase, etc. (Dalela et al. 2012). This further limits the therapeutic options available to treat these infections. In our study, false susceptibilities to ceftazidime and cefotaxime were observed in 23.8% and 16.67% of the ESBL producers. This could be due to the reason that the optimal substrate profile varies from one ESBL enzyme to another (Wong 2001). Hence, the susceptibility panels with only one extended spectrum cephalosporin cannot predict the resistance to the other extended spectrum cephalosporins (Rice and Jao 1991).

Due to the difficulty in detection of ESBL by the current clinical methods, many of these strains have been falsely reported to be susceptible to the widely used broad-spectrum β-lactams, ESBLs constitute a serious threat to the β-lactam therapy (Mackenzi et al. 2002). The ESBL producers are intrinsically resistant to all the cephalosporins even if they exhibit an in vitro susceptibility. The ESBL production coexists with the resistance to several other antibiotics.

In the study, upon testing the susceptibility pattern for ESBL and non-ESBL producers to non β-lactam antibiotics, a co-resistance to the non β-lactam antibiotics was observed more with the ESBL producers. Comparatively less resistance to amikacin and nitrofurantoin (19.1% and 9.53% respectively) was seen among ESBL producers. In the study, 95.23% ESBL producing strains showed susceptibility to the combination drug, piperacillin/tazobactam. The resistance to meropenem was observed to be less (9.53%) as compared to that to imepenem (7.15%).

A co-resistance to the quinolones and the aminoglycosides is common. We found such an associated resistance of ESBL producers to co-trimoxazole (80.96%), gentamicin (73.81%) and the flouroquinolones (88.10-95.23%). Another study reported 91.17%, 100% and 94.91% resistances respectively to gentamicin, cotrimoxazole and ciprofloxacin in the ESBL producers (Gupta et al. 2007). The high resistance to the non β-lactam antibiotics of the ESBL producing strains poses a threat of treatment failure by these drugs and it also minimizes the therapeutic choice to the carbapenems. Hence, the emerging resistance to the carbepenems is a phenomenon of great concern for combating the infections of the multidrug resistant bacteria (Parveen et al. 2010). Although β- lactam/ β- lactamase inhibitor combinations have been suggested as the treatment option for ESBL producers, these drugs must be given in high doses in lower frequency, so that serum and tissue levels of these combinations are higher with a correspondingly higher clinical success rate. (Adam 2002).

CONCLUSION

This study concluded that there is high prevalence of multidrug resistant uropathogenic clinical strains of *E. coli* and *K. pneumoniae* with higher rates of ESBL production. These strains show lower rate of sensitivity to β-lactam antibiotics even they are capable of producing ESBL and higher rate of sensitivity to combination therapy. A resistance to the carbepenems is also emerging. Appropriate antimicrobial regimen selection for empirical therapy is thus important for such cases. On managing the empirical antibiotics practice, one can reduce the risk of ESBL producers. There is an essence need of regular routine practice of ESBL detection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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