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## RESEARCH ARTICLE

### Predominance of CTX-M Type Extended Spectrum $\beta$ -lactamase (ESBL) Producers Among Clinical Isolates of Enterobacteriaceae in a Tertiary Care Hospital, Kathmandu, Nepal

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#### Abstract:

#### Background:

ESBL problem is increasing worldwide and only limited studies on genes of ESBL are performed in Nepal.

#### Objectives:

We aimed to focus on the molecular detection of plasmid-mediated  $bla_{TEM}$ ,  $bla_{SHV}$  and  $bla_{CTX-M}$  genes among the ESBL producing Enterobacteriaceae from different clinical samples.

#### Methods:

A total of 550 clinical samples were processed and organisms of Enterobacteriaceae were identified using standard microbiological process. ESBL producers were screened and confirmed using the modified Kirby Bauer disc diffusion method by CLSI guidelines. Plasmids extracted from the confirmed ESBL positives were the template for PCR.  $bla_{SHV}$ ,  $bla_{TEM}$  and  $bla_{CTX-M}$  genes were amplified using specific primers of respective genes by uniplex PCR. The presence of these genes was confirmed by gel electrophoresis.

#### Results:

Among 550 different clinical samples 343 (62.36%) were culture positive. Of which, 157 (45.57%) belonged to Enterobacteriaceae. *Escherichia coli* (45.9%) was predominant. Of these 33.2% (52/157) isolates were ESBL positive. ESBL- *E. coli* (52.8%) were prevalent. All ESBL positive organisms were sensitive to imipenem. Of confirmed ESBL positives, 34.6% harbored  $bla_{TEM}$  gene, 30.8% harbored  $bla_{SHV}$  gene and  $bla_{CTX-M}$  genes were present in all ESBL producers. Twenty-eight out of 52 (53.9%) isolates harbored multiple  $bla$  genes, the most common combination being  $bla_{CTX-M} + bla_{TEM}$  (21.2%).

#### Conclusion:

We report 100% plasmid mediated CTX-M genotype among ESBL producers which might indicate rapid dissemination of  $bla_{CTX-M}$  genes from the community to the patients. Besides, there is a need for regular monitoring of antibiotic resistance in the country and de-escalate the use of antibiotics so as to preserve the antibiotics for future generation.

**Keywords:** ESBL, Enterobacteriaceae,  $Bla_{TEM}$ ,  $Bla_{SHV}$ ,  $Bla_{CTX-M}$ , Gel electrophoresis.

#### Article History

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## 1. INTRODUCTION

ESBLs are the enzymes able to confer resistance against

the broad spectrum cephalosporins (3<sup>rd</sup> generations) and monobactams; but not cephamycins and carbapenems and having a

minimum of 10% hydrolysis rates as that of benzylpenicillin, and these enzymes are inhibited by  $\beta$ -lactamase inhibitors such as clavulanate [1]. ESBLs are frequently detected in *Klebsiella pneumoniae* and *E. coli*; however, other ESBL producing isolates of Enterobacteriaceae (such as *K. oxytoca*, *Proteus mirabilis*, *Salmonella* spp. etc) and *Pseudomonas aeruginosa* are also identified [2, 3].

Initially, the resistance properties are attributed to the dissemination of TEM and SHV types, but nowadays CTX-M type has become predominant [1, 4]. Since they are plasmid mediated and transposon associated it takes no time for them to transfer to several members of Enterobacteriaceae [2, 3]. Plasmid mediated ESBL is of global concern because of its unfavorable clinical and economic outcomes [5, 6]. The resistance attributes of ESBL producers toward 3<sup>rd</sup> generation cephalosporins and other antibiotics make it difficult in the treatment of infections [4].

Pokhrel *et al.* [7] reported ESBL producers (24.27%) for the first time in Nepal. Since then, the rate of detection of ESBL producers is increasing rapidly. In the developing country like Nepal, where there is rapid irrational use of antibiotics [7], the problem of antimicrobial resistance is growing day by day. Many studies have been performed in the field of ESBL in Nepal. However, there is limited study on the major types of genes causing the wide spread of ESBL that explains the epidemiological features of ESBL producing microorganisms [8]. It is indeed important to study the prevalence of ESBL at the national and global level in order to know their resistance pattern and types of ESBL being circulated in those regions that would aid in the treatment of the infections promptly. The purpose of this study was to determine the frequency of ESBL producing Enterobacteriaceae and characterize plasmid mediated TEM, SHV and CTX-M types among the ESBL producing clinical isolates belonging to Enterobacteriaceae.

## 2. MATERIALS AND METHODS

### 2.1. Specimens and Identification of Organisms

A cross-sectional study was performed from November 2015 to June 2016 in Annapurna Neurological Institute and Allied Sciences, Kathmandu, Nepal. A total of 550 non-duplicate samples (209 urine samples, 81 sputum samples, 87 cerebrospinal fluid, 68 blood, 29 pus, 20 wound swab, 17 catheter tip, 6 central venous pressure line tips, 16 tracheal aspirates, 6 tracheostomy tube, 6 external ventricular drain tube and 5 endotracheal tube) sent to the microbiology laboratory were processed and cultured by standard microbiological techniques. The organisms were identified by Gram staining, cultural characteristics in MacConkey agar and Blood agar (Hi Media, India) and biochemical tests such as IMViC, catalase test, oxidase test, urease test and oxidative/fermentative test (Hi Media, India) [9]

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### 2.2. Antibiotic Susceptibility Tests

The antibiotic susceptibility testing of the isolates was done using Kirby-Bauer disc diffusion technique. The antibiotics tested were cefotaxime (30  $\mu$ g), ceftazidime (30  $\mu$ g), ceftriaxone (30  $\mu$ g), cefepime (30  $\mu$ g), aztreonam (30  $\mu$ g), imipenem (30  $\mu$ g), ofloxacin (5  $\mu$ g), gentamicin (10  $\mu$ g), amikacin (30  $\mu$ g), nitrofurantoin (300  $\mu$ g) and piperacillin/tazobactam (100/10  $\mu$ g) (Hi Media, India).

### 2.3. Screening and Confirmation of ESBL

The screening was done by disc diffusion technique using cefotaxime (30  $\mu$ g), ceftazidime (30  $\mu$ g), ceftriaxone (30  $\mu$ g), aztreonam (30  $\mu$ g). For confirmation, combined disc test was performed using cefotaxime (30  $\mu$ g) and ceftazidime (30  $\mu$ g) alone, and cefotaxime + clavulanic acid (20 $\mu$ g/10  $\mu$ g) and ceftazidime + clavulanic acid (20 $\mu$ g/10  $\mu$ g) [10].

### 2.4. Gene Identification

From confirmed ESBL producers, plasmids were extracted using standard alkaline hydrolysis method [11]. These plasmids served as the template for PCR. PCR amplification was carried out using *bla*<sub>TEM</sub> [12], *bla*<sub>SHV</sub> [13], *bla*<sub>CTX-M</sub> [13] specific primers (Macrogen, Korea). For gene amplification, 1.5  $\mu$ l plasmid DNA was added to 25  $\mu$ l mixture containing 13  $\mu$ l master mix (Solis Biodyne, Estonia), 10.5  $\mu$ l nuclease free water and 0.5  $\mu$ l each of reverse and forward primers. PCR was performed in 5 Prime/02 thermocycler, Bibby Scientific, U.K. using optimized condition.

*bla*<sub>TEM</sub> gene was amplified under the optimized condition of initial denaturation at 94°C for 5 minutes followed by 30 cycles each of denaturation (95°C for 45 seconds), annealing (50°C for 45 seconds) and extension (72°C for 30 seconds) respectively, and final extension at 72°C for 10 minutes. For amplification of *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes, same sets of conditions were used except for the annealing step which was performed at 56°C for 45 seconds and 62°C for 45 seconds respectively.

The amplified products were subjected to gel electrophoresis (2% gel stained with ethidium bromide) at 70V for 45 minutes. 100 bp DNA ladder (Zymoresearch, England) was used to estimate the molecular weight of the amplified products. The gel was then taken to the gel doc system (Uvitec Cambridge, UK) for photo documentation.

### 2.5. Controls

*E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603) were taken as a negative control and positive control respectively for ESBL test. For PCR already confirmed *E. coli* strains harboring *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub> (Mahidol University, Thailand) were taken as a positive control and nuclease free water as the negative control.

### 2.6. Statistical Analysis

Data were entered and analyzed by using SPSS version 16 software package.

3. RESULTS

A total of 550 clinical samples, 343 (62.36%) were culture positive and 157 isolates belong to Enterobacteriaceae family. *E. coli* (45.9%) was the most predominant. Bacteria belonging to Enterobacteriaceae isolated from the urine sample were predominant (63.7%), followed by those isolated from sputum (21.1%) and pus (5.1%). The prevalence of *E. coli* was high (68%) among the urine samples. Likewise, *K. pneumoniae* (57.6%) was a predominant organism in the sputum sample as well as in the tracheal aspirate (100%), catheter tip (66.7%) and wound swab (66.7%). Single CSF sample, EVD and tracheostomy tip was culture positive from which *K. pneumoniae* was isolated. High prevalence of *C. freundii* (62.5%) was reported from the pus sample. *S. Typhi* (66.7%) was predominantly isolated from the blood sample.

Of 157 bacterial isolates belonging to Enterobacteriaceae, 33.2% isolates were tested positive for ESBL. ESBL- *E. coli* (52.8%) were more prevalent followed by ESBL- *K. oxytoca* (27.3%) and ESBL- *K. pneumoniae* (14.6%) while only one each of *Providencia* spp. (1/3) and *C. koseri* (1/3) was ESBL positive. In addition to *E. coli* being the most prevalent ESBL

organism, it could be noted that there were more ESBL-*E. coli* than ESBL negative *E. coli*. This was not the case for the other organisms (Table 1).

Table 1. Prevalence of ESBL positive organisms.

Bacteria isolated	ESBL test	
	Positive No. (%)	Negative No. (%)
<b>Organisms</b>		
<i>E. coli</i> (n=72)	38 (52.8)	34 (47.2)
<i>K. oxytoca</i> (n=22)	6 (27.3)	16 (72.7)
<i>K. pneumoniae</i> (n=41)	6 (14.6)	35 (85.4)
<i>C. freundii</i> (n=14)	0 (0.0)	14 (100.0)
<i>C. koseri</i> (n=3)	1 (33.3)	2 (66.7)
<i>S. Typhi</i> (n=2)	0 (0.0)	2 (100.0)
<i>Providencia</i> spp. (n=3)	1 (33.3)	2 (66.7)
<b>Total (n= 157)</b>	<b>52 (33.2)</b>	<b>105 (66.8)</b>

All ESBL positive organisms were sensitive to imipenem. Of the ESBL producers 96.2%, 92.3% and 88.5% were resistant to cefotaxime, ceftazidime and ceftriaxone, respectively (Table 2).

Table 2. Antibiotic resistance pattern of ESBL producers.

Antibiotics used	ESBL producers (n= 48) No. (%)
<b>Nitrofurantoin</b>	1 (2.1)
	n= 52
<b>Amikacin</b>	2 (3.8)
<b>Piperacillin/tazobactam</b>	4 (7.7)
<b>Ofloxacin</b>	30 (57.7)
<b>Gentamicin</b>	4 (7.7)
<b>Aztreonam</b>	31 (59.6)
<b>Ceftazidime</b>	48 (92.3)
<b>Cefotaxime</b>	50 (96.2)
<b>Ceftriaxone</b>	46 (88.5)
<b>Cefipime</b>	26 (50)
<b>Imipinem</b>	0 (0)

Table 3. Overall Prevalence of bla genes.

Types of genes	ESBL producing bacteria (n= 52)					<i>Providencia</i> spp. (n=1)
		<i>E. coli</i> (n=38)	<i>K. pneumoniae</i> (n=6)	<i>K. oxytoca</i> (n=6)	<i>C. koseri</i> (n=1)	
<i>bla</i> <sub>SHV</sub>	Present	8 (21.1)	5 (83.3)	4 (66.7)	0 (0)	0 (0)
<b>No. (%)</b>	16 (30.8)					
	Absent	30 (78.9)	1 (16.7)	2 (33.3)	1 (100)	1 (100)
	36(69.2)					
<i>bla</i> <sub>CTX-M</sub>	Present	38 (100)	6 (100)	6 (100)	1 (100)	1 (100)
<b>No. (%)</b>	52 (100)					
	Absent	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	0 (0)					
<i>bla</i> <sub>TEM</sub>	Present	10 (26.3)	5 (83.3)	2 (33.3)	0 (0)	1 (100)
<b>No. (%)</b>	18 (34.6)					
	Absent	28 (73.7)	1 (16.7)	4 (66.7)	1 (100)	0 (100)
	34 (65.4)					

All ESBL positive organisms (100%) harbored *bla*<sub>CTX-M</sub> gene. Likewise, *bla*<sub>SHV</sub> genes were more prevalent among ESBL-*K. pneumoniae* (83.3%) followed by ESBL-*K. oxytoca* (67.7%), while 26.3% of the ESBL-*E. coli* harbored *bla*<sub>TEM</sub> gene (Table 3).

46.1% and 53.9% of total ESBL producers harbored single *bla* genes (*bla*<sub>CTX-M</sub> only) and *bla* genes in combination respectively. *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> were not present as single genotype but in combination. The most common combination was reported to be *bla*<sub>CTX-M</sub> + *bla*<sub>TEM</sub> (21.2%) followed by *bla*<sub>CTX-M</sub> + *bla*<sub>SHV</sub> (19.2%) and *bla*<sub>CTX-M</sub> + *bla*<sub>SHV</sub> + *bla*<sub>TEM</sub> (13.5%), whereas *bla*<sub>SHV</sub> + *bla*<sub>TEM</sub> genotypic combination was not detected (Table 4).

#### 4. DISCUSSION

This study provides essential data regarding the distribution of ESBL producing Enterobacteriaceae and their major types (SHV, TEM and CTX-M). The types of ESBL producing bacteria varies by country and CTX-M type ESBL producing Enterobacteriaceae is a predominant one in Nepal.

Out of 550 non-duplicate samples, 343 (62.36%) were culture positive and of these 157 (45.57%) isolates belonged to Enterobacteriaceae family which is in accordance with the study of Pokharel *et al.* [8]. Of these, 33.2% (52/157) were ESBL producers which were found to be in agreement with the previous study in Nepal [14]. ESBL producing strains were high among *E. coli* (52.8%) in this study which agrees with the previous study of Nepal [15]. However, another study from Nepal has reported that *Klebsiella* spp. were the predominant ESBL producers [16]. In our study, none of the isolates of *C. freundii*, and *S. Typhi* were ESBL positive which was contrary to other studies [17, 18]. It might be because of the less number of isolates [19].

This study showed imipenem to be the most effective drug against ESBL producers which is similar to other studies of Nepal [16, 20]. Similarly, amikacin (AK), Gentamicin (GEN) and piperacillin/tazobactam (PIT) also showed good activity against ESBL producers in this study. Different studies have shown different resistant pattern against these three drugs (AK 4.2%-65.4%, PIT 37.1%- 78.18%, GEN 8.3%-83.3%) [8, 21 - 23]. In our study, most of the ESBL producers were resistant against ceftazidime (92.3%), cefotaxime (96.2%) and ceftriaxone (88.5%) attributable to the definition of ESBL. These findings were similar to that of Segar *et al.* [21]. High resistance rate against the cefotaxime might be due to 100% CTX-M type. Some studies in Nepal have reported low resistance pattern towards these 3<sup>rd</sup> generation cephalosporins (CAZ 64.8%, CTX 64.4%, CTR- 63.6%) [20] while some studies showed 100% resistant ESBL producers [8, 16]. Similarly, our study showed that half of the ESBL producers were resistant towards 4<sup>th</sup> generation cephalosporins, the cefipime.

The genes encoding the ESBL are often present in large plasmids and hence largely responsible for disseminating genes *via* conjugation [24]. Many of the plasmid encoded  $\beta$ -lactamases like TEM-1, TEM-2, SHV-1, *etc* typically have the

chromosomal origin and Livermore [25], defined such enzymes as "secondary  $\beta$ -lactamase".

In this study, all ESBL producing isolates harbored a certain type of major ESBL genes which is in harmony with the study done in Amsterdam by Reuland *et al.* [26]. Hence the major ESBL genes *i.e.* *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> were responsible for the ESBL producing attributes of the isolates. However, Kaur and Aggarwal [27] have shown the absence of three major types of ESBL genes in the ESBL producing isolates of *E. coli* (4/59), *K. pneumoniae* (3/24) and *C. freundii* (2/2). The absence of the major genes in ESBL producing isolates indicated that the isolates might belong to the minor ESBL types, such as SFO, BES, BEL, TLA, GES, PER and VEB types [1].

PCR analysis showed 21.1% *E. coli*, and 83.3% *K. pneumoniae* and 66.7% *K. oxytoca* harbored *bla*<sub>SHV</sub> gene but this gene was absent in *C. koseri*. In total, 30.8% of the ESBL producing isolates harbored *bla*<sub>SHV</sub> gene. In Brazil [28] and the Netherlands [29] the prevalence of SHV type ESBL producing Enterobacteriaceae isolates was reported to be 24.2% and 11% (56/512), respectively. Babini and Livermore [30] concluded the ubiquity of *bla*<sub>SHV</sub> related gene in *K. pneumoniae*, in Europe, explained by the presence of the very gene in all confirmed isolates of *K. pneumoniae*. However, in a study in China, no SHV-type *K. pneumoniae* was detected [31]. In Nepal, 12.5% SHV type ESBL producing *E. coli* was reported [8] which is lesser than what we reported. Our result is in the line with the study of Yagi *et al.* [32] in Japan where they reported 16% SHV-type ESBL-*E. coli*, but the prevalence is quite less than that reported by Varkey *et al.* [13] in India (66% SHV type ESBL-*E. coli*).

In this study, 34.6% of the ESBL producers were TEM type. This prevalence is less than that reported in the study of Moosavian and Deiham [33] in Iran. The current study revealed 26.3%, 33.3% and 83.3% of tested *E. coli*, *K. oxytoca* and *K. pneumoniae* harbored *bla*<sub>TEM</sub> gene, whereas only tested *C. koseri* also harbored *bla*<sub>TEM</sub> gene. In Nepal, 29.2% TEM type ESBL producing *E. coli* was reported [8]. However, the proportion of TEM type ESBL-*E. coli* in this study was contrary to the study in Iran where 67% ESBL producing *E. coli* were reported to be TEM type [34].

All the isolates tested in this study harbored *bla*<sub>CTX-M</sub> gene (100%). It is in harmony with the recent study done in Sierra Leone [35]. A study from Nepal also reported a high prevalence of *bla*<sub>CTX-M</sub> gene (95.2%) in comparison to the other two *bla* genes [8]. Varkey *et al.* [13] reported high prevalence of TEM type *E. coli* than CTX-M type (75% vs 71%) but high prevalence of CTX-M type *K. pneumoniae* (85%) than other two types. The study in the USA showed a high prevalence of CTX-M type *E. coli* since 2005; however *K. pneumoniae* producing CTX-M type ESBL was reported to increase from 1.7% (2005-2009) to 26.4% (2010-2012) [36]. Though CTX-M type *E. coli* are community acquired, Nicholas-Chanoine *et al.* [37] showed that the origin of patients, their lifestyles and health care systems are independently associated with isolation of CTX-M type *E. coli*.

Table 4. Prevalence of *bla* genes either alone or in combination.

<i>bla</i> genes	ESBL producers					Total n= 52
	<i>E. coli</i> (n= 38)	<i>K. pneumoniae</i> (n= 6)	<i>K. oxytoca</i> (n= 6)	<i>C. koseri</i> (n=1)	<i>Providencia spp.</i> (n=1)	
Single <i>bla</i> gene ( <i>bla</i> <sub>CTX-M</sub> only)	22 (57.9)	0 (0.0)	1 (16.6)	1 (100.0)	0 (0.0)	24 (46.1)
<i>bla</i> genes in combination	16 (42.1)	6 (100)	5 (83.3)	0 (0.0)	1 (100)	28 (53.9)
<i>bla</i> <sub>CTX-M</sub> + <i>bla</i> <sub>TEM</sub>	8 (21)	1 (16.6)	1 (16.6)	0 (0.0)	1 (100)	11 (21.2)
<i>bla</i> <sub>CTX-M</sub> + <i>bla</i> <sub>SHV</sub>	6 (15.8)	1 (16.6)	3 (50.0)	0 (0.0)	0 (0.0)	10 (19.2)
<i>bla</i> <sub>CTX-M</sub> + <i>bla</i> <sub>SHV</sub> + <i>bla</i> <sub>TEM</sub>	2 (5.3)	4 (66.6)	1 (16.6)	0 (0.0)	0 (0.0)	7 (13.5)

In this study 28 out of 52 (53.9%) isolates harbored multiple *bla* genes, the most common combination type being *bla*<sub>CTX-M</sub> + *bla*<sub>TEM</sub> (21.2%) and 19.2% of the isolates harbored *bla*<sub>CTX-M</sub> + *bla*<sub>SHV</sub> gene, whereas 13.5% of the isolates harbored all three types of *bla* genes. *bla*<sub>SHV</sub> + *bla*<sub>TEM</sub> genotypic combination was not detected. Complex antibiotic resistance pattern might be the result of the presence of multiple genotypes [38]. Remaining isolates (24, 46.1%) were reported to harbor single *bla*<sub>CTX-M</sub> gene. *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> were not present as a single genotype. This result is not in agreement with the study of Kaftandzieva et al [39] where they reported *bla*<sub>TEM</sub> + *bla*<sub>SHV</sub> as the most common combination.

## CONCLUSION

Though this study does not provide the overall picture of the epidemiology of major ESBL types across the country, it gives fair data regarding the increased predominance of the major ESBL types in a tertiary care hospital in Kathmandu. In this study, 100% CTX-M type ESBL was noted which indicated the possibility of rapid dissemination of *bla*<sub>CTX-M</sub> genes from the community to hospitals. It warrants the regular monitoring and surveillance of antibiotic resistance and genes encoding them across Nepal to identify better treatment options and de-escalate the use of antibiotics to reduce the chance of spreading antibiotic-resistant organisms, and to preserve the antibiotics for their efficient use in future generation.

## LIST OF ABBREVIATIONS

ATCC	= American Type Cell Culture
<b>bla</b>	= $\beta$ -Lactamase Coding Gene
CLSI	= Clinical and Laboratory Standards Institute
CSF	= Cerebrospinal Fluid
CTX-M	= Cefotaximase, Munich
CVP	= Central Venous Pressure
ET Tube	= Endotracheal Tube
EVD	= External Ventricular Drain
PCR	= Polymerase Chain Reaction
SHV	= Sulfhydryl Variable
SPSS	= Statistical Package for Social Sciences
TEM	= Temoniera

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ethical approval was taken from the Ethical Review

Board of Nepal Health Research Council (NHRC), Kathmandu, Nepal (Reg. no. 315/2015).

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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