Original Article

DOI: 10.7860/JCDR/2013/5081.2872

Microbiology Section

Occurrence of the CTX-M, SHV and the TEM Genes Among the Extended Spectrum β-Lactamase Producing Isolates of Enterobacteriaceae in a Tertiary Care Hospital of North India

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ABSTRACT

Purpose: The present study was carried out to assess the prevalence of the Extended Spectrum Beta Lactamases (ESBLs) and to characterize the ESBL types which were prevalent in our hospital.

Material Methods: Five hundred gram negative isolates which belonged to the family, *Enterobacteriaceae,* which were isolated during the study period of 2009 to 2011, were investigated for ESBL production. Clinical isolates from urine (344), pus (109), blood (15), IV/ central line tip (10), sputum (12) and body fluid (10) specimens were processed. The organisms which were identified, included *E.coli* (351), *Klebsiella pneumoniae* (74), *Klebsiella oxytoca* (21), *Proteus mirabilis* (15), *Proteus vulgaris* (9) , *Enterobacter spp* (15) and *Citrobacterspp* (15). Antimicrobial susceptibility testing was done. The ESBL detection was carried out for all the isolates by the CLSI confirmatory method. The MIC of ceftazidime and ceftazidime plus clavulanic acid was determined by the E-test. Molecular typing of the ESBLs was performed by multiplex PCR among 93 ESBL isolates.

Results: 45.8% isolates were found to be ESBL producers by the CLSI confirmatory method and they were confirmed by the E-test ESBL strips. A majority of *E.coli* in the study possessed the CTX-M genes (59.32%). Among the *Klebsiella isolates*, a majority were co producers of the ESBL genes; either 2 or all the 3 genes co-existed together.

Conclusion: An indiscriminate use of the higher antibiotics should be restricted as far as possible. The infection control programmes should be monitored continuously in hospitals, to contain these ESBL producers. We are reporting the presence of all types of ESBL genes among the *Enterobacteriaceae isolates* from our hospital setting.

Key Words: Extended spectrum beta lactamase, *Enterobacteriaceae*

INTRODUCTION

The Extended Spectrum Beta Lactamases {ESBLs} are enzymes which are produced by gram negative bacilli, that mediate a resistance to the penicillins, cephalosporins and the monobactams and are commonly recognized in *Enterobacteriaceae*. Gram negative bacteria are the major pathogens in hospitalized patients with weakened host defenses, due to malignancy, malnutrition, debilitation, trauma, a prolonged antibiotic use and surgery. The most frequently bacteria are *E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter and Acinetobacter baumanii*. Once these organisms are established as the source of the infection, they are aggressively treated with antimicrobial drugs, which are thought to be the greatest contribution of the present century to therapeutics.

The first plasmid mediated beta lactamase in gram negative bacteria, TEM-1, was described in the early 1960s [1]. Being plasmid and transposon mediated, it quickly spread to various species of bacteria. SHV-1 was the next plasmid mediated beta–lactamase which was discovered [1]. Although most of the ESBLs are mutants of the TEM and the SHV enzymes, the CTX-M type beta lactamases have become more important. The CTX-M type of enzyme constitutes a distinct lineage of the molecular class A β lactamases, which are a rapidly growing group. Rather than arising by mutation, they represent examples of plasmid acquisition of the β lactamases genes which are found on the chromosomes of the *Kluyvera species*. CTX-M was 1st detected in *E.coli* strains in Germany (1988) and presently, there are more than 65 allelic variants which are known [2,3].

The detection of the common ESBL genes such as TEM, SHV and CTX-M by molecular methods in the ESBL producing bacteria and their patterns of antimicrobial resistance can provide useful information about their epidemiology and can aid a rational anti-microbial therapy.

The present study was therefore designed to investigate the prevalence of the ESBL producing gram negative organisms in the family, Enterobacteriaceae by phenotypic methods and to characterize the ESBL types which were prevalent in our hospital, by multiplex PCR.

MATERIAL METHODS

Samples

This study was done on 500 gram negative isolates which belonged to the family, *Enterobacteriaceae* in the Department of Microbiology

in our Institute. Clinical isolates from urine (344), pus (109), blood (15), IV catheter tip/ central line tip (10), sputum (12) and body fluid (10) specimens were processed.

Antimicrobial Susceptibility Testing

The isolates were tested for their antimicrobial susceptibilities by the disc diffusion technique according to the CLSI guidelines [4]. The following antibiotic discs (drug concentrations in µg) were used: ampicillin (10), amikacin (30), ceftazidime (30), cefotaxime (30), ceftriaxone (30), cefepime (30), gentamicin (10), imipenem (10), ciprofloxacin (5), netilmicin (30), norfloxacin (10) and nitrofurantoin (300).

The detection of ESBLs

The ESBL detection was performed as was recommended by the CLSI confirmatory procedure, by using cefotaxime (30ug) and ceftazidime (30ug) discs alone and in combination with clavulanic acid discs [4]. *E. coli* (ATCC-25922) and *K. pneumoniae* (ATCC-700603) were used as the controls throughout the study.

Confirmation of the ESBLs was done by performing the E-test with a two sided strip which contained ceftazidime on one side and ceftazidime+clavulanic acid on the other side (which was procured from Biomerieux, India). An isolate was considered as an ESBL producer if the ratio of the MIC of the combination to that of ceftazidime alone was ≥ 8 or if there was the development of a phantom zone [Table/Fig-1]. We used a modified method in which two strains were simultaneously swabbed on a single plate by dividing the plate into two halves, being separated by a 3mm wide uninoculated area where the E-test strip was placed.

[Table/Fig-1]: Modified E-test
(Tz/Ceftazidime, Tzl/Ceftazidime + Clavulanic Acid)

The Extended Spectrum β-lactamase Gene Identification

Multiplex PCR was carried out for the detection of the TEM, SHV and the CTX-M genes among 93 study isolates which were isolated from the ICU patient samples (which were determined as ESBL producers by phenotypic methods). PCR was used to characterize the type of ESBLs which were prevalent in our study, so that the strains that were found to be ESBL producers by both the CLSI confirmatory method and the E test method were genotyped. The DNA template preparation was performed as follows: 2 ml of an overnight broth culture (Luria Burtonni broth-Himedia) of the test organism was centrifuged at 8000rpm for 10 minutes. The supernatant was discarded and the pellet was mixed with 200 µl of sterile distilled water and it was vortexed. This was then boiled in a dry bath for 10 minutes and was immediately chilled by putting it on ice for 5 minutes. It was then centrifuged once again at 6000rpm and the supernatant which contained the nucleic acid material, was pipetted off and stored for genetic testing. The master mix for the PCR was prepared as follows: 2.5µL of PCR buffer, 2.5µL of 25mM $MgCl_2$, 2.5µLof10mM dNTP mix, 0.3µL of Taq DNA Polymerase , 9.2µL of MilliQ water and 1µL of each of the forward and reverse primers. Finally, after dispensing 23µL of the master mix in the individual amplification tubes, 2µL of the extracted DNA was added in the corresponding tubes to make up the total volume to 25µL.The PCR primers which were used have been indicated in [Table/Fig-2] [5,6]. PCR was performed according to the following protocol: prehold for 2 min at 95ºC, followed by 30 cycles, each consisting of a denaturation at 95ºC for 45 sec, annealing at 62ºC for 45 sec, extension at 72ºC for 1 min and followed by a post extension hold at 72ºC for 5min. The PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel in 0.5% of TAE buffer. The gels were stained with 0.5µg/ml Ethidium bromide and the PCR products were visualized in UV light.

RESULTS

A total of 500 isolates of *E.coli* (n=351), *Klebsiella pneumoniae* (n=74), *Klebsiella oxytoca* (n=21), *Proteus mirabilis* (n=15), *Proteus vulgaris* (n=9) , *Enterobacter spp* (n=15) and *Citrobacterspp* (n=15) were recovered from different clinical specimens.

Of the total 500 isolates, 315/500 (63%) isolates were found to be resistant to at least three cephalosporins (3rd or 4th generation). The resistance to the piperacillin tazobactam combination was 9.97 % in *E.coli* and it was 12.24 % in the *Klebsiella pneumoniae* isolates. Among the non beta lactams, the maximum resistance was shown to norfloxacin and ciprofloxacin (79.67% and 91.66% in *E.coli*, 59.45% and 71.42% in *Klebsiella pneumoniae*). The *E.coli* and *Klebsiella spp* exhibited comparatively less resistance to amikacin and nitrofurantoin. (13.75% and 18.35% in *E.coli*, 42.59% and 34.14% in *Klebsiella pneumoniae*).

Among the total 500 isolates, ESBL production was noted in 45.8% (229 isolates) by the CLSI confirmatory test. The maximum ESBL production was seen among the isolates of *Klebsiella pneumoniae* (52.27%), followed by those of *E.coli* (46.43%). The ESBL production was quite high among the pus samples (51.37%), followed by urine samples (45.63%). By the ESBL E-test method, 220 isolates were found to have an MIC ratio of TZ/TZL, as more than 8 and so were confirmed as ESBL producers. The E test which was done by using ceftazidime and ceftazidime plus clavulanic acid showed that the 220/229 isolates which were detected by the confirmatory method were also found to be ESBL producers by the E test and that the difference was not statistically significant. So, the CLSI confirmatory method is as sensitive and as specific as the E test. The ESBL producing *E.coli* and *K. pneumoniae* were found to be quite sensitive to Imipenem (the resistance was only 2.9%) and a combination of piperacillin and tazobactam .

In the present study, the rate of the drug resistance [Table/Fig-3] was higher in the ESBL producers than in the non-ESBL producers. As for the beta-lactam antibiotics, the drug resistance was higher for other antibiotics also, namely, ciprofloxacin (83.22%), norfloxacin

Target gene	Primers used
Bla_{TEM}	TEM-F(5'-3'): GTA TCC GCT CAT GAG ACA ATA ACC CTG TEM-R (5'-3'): CCA ATG CTT AAT CAG TGA GGC ACC
Bla_{SHV}	SHV-F(5'-3'): CGC CTG TGT ATT ATC TCC CTG TTA GCC SHV-R (5'-3'): TTG CCA GTG CTC GAT CAG CG
Bla_{CTX-M}	CTX-M (5'-3'): CGC TTT GCG ATG TGC AG ACC GCG ATA TCG TTG GT

[Table/Fig-2]: primer pairs used for amplification of the TEM, SHV and CTX-M ESBL types

S. No.	Anti-microbial Agent	ESBL producers	Non ESBL producers	P value
1.	Gentamycin	57.74%	30%	<0.001
2.	Amikacin	34.19%	16.31%	<0.001
4.	Norfloxacin	84.95%	50.72%	<0.001
5.	Levofloxacin	54.19%	31.05%	<0.001
6.	Ciprofloxacin	83.22%	60.52%	<0.001
7.	Netilmycin	50%	24.73%	<0.001
8.	Chloramphenicol	56.73%	30.76%	<0.05

[Table/Fig-3]: Antimicrobial resistance pattern among ESBL producers and non-ESBL producers

ESBL type	E.coli	Klebsiella pneumoniae	Proteus spp	Citrobacter	Total
TEM	2		8		
SHV		3			
CTX-M	35	6			
CTX-M +SHV	13	6			
CTX-M + TEM	5				
CTX-M + TEM + SHV		6			
Nil	4	3		2	
TOTAL	59	24	8	2	93

[Table/Fig-4]: Distribution of TEM, SHV and CTX-M ESBL types among 93 study isolates.

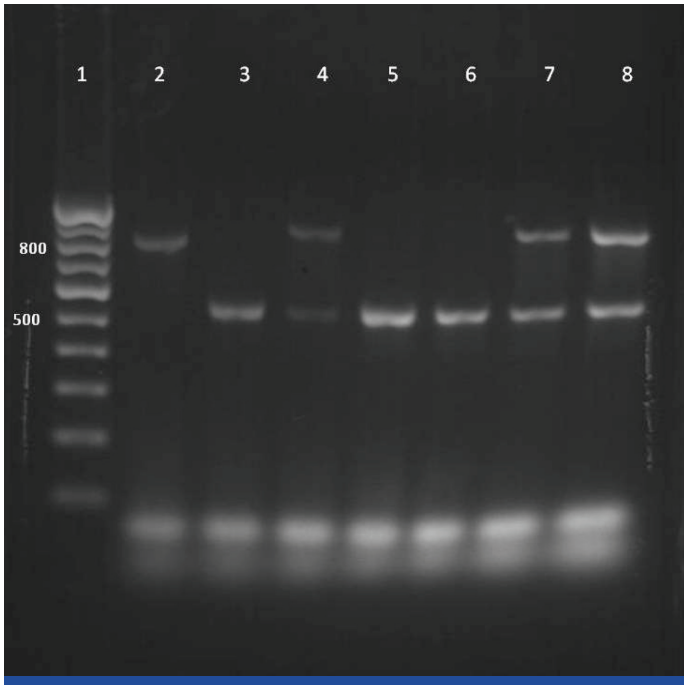
(84.95%) and gentamicin (57.74%). The statistical analysis showed a highly significant difference (a P value of less than 0.001) in the resistance for the non-beta lactam antibiotics like gentamicin, amikacin, norfloxacin, levofloxacin, ciprofloxacin and netilmicin among the ESBL producers and the non-ESBL producers.

Molecular Characterization

The 93 phenotypically confirmed ESBL producers were genotyped [Table/Fig-4]. The molecular characterization revealed that 44.08% isolates possessed the CTX-M genes alone, while the CTX-M and the SHV genes together were found in 20.43% isolates. 6.45% isolates which were genotyped, contained all the 3 genes, CTX-M, SHV and TEM. A majority of the *E.coli* (59.32%) isolates in the study carried the CTX-M genes and the *Klebsiella* isolates were found to be co producers of the ESBL genes; either 2 or all the 3 genes occurred together. The *Proteus* isolates showed the presence of the TEM genes only [Table/Fig-5].

DISCUSSION

The emergence of plasmid mediated ESBLs among the members of *Enterobacteriaceae* have increased worldwide. The incidence of ESBLs in the major hospitals of India has been reported to be as high as 6-87% [7-9]. We reported 45.8% (229) ESBL producers by the CLSI confirmatory test. The maximum ESBL production



[Table/Fig-5]: Agarose gel electrophoresis of PCR amplified products showing the presence of CTX-M (550bp), SHV (842bp) and TEM (918bp) genes in test and control strains.
Lane 1-DNA ladder
Lane 2- SHV positive control strain (Klebsiella pneumoniae ATCC 700603)
Lane 3 to lane 8-test strains
Lane 3,5,6- CTX-M
Lane 4,7,8- CTX-M + TEM

was seen among the isolates of *Klebsiella pneumoniae* (52.27%), followed by *E.coli* (46.43%). Among the phenotypic methods which were used to find the ESBL production, the CLSI confirmatory method was found to be equally sensitive and cost effective as compared to the E-test, which had limited use in resource limited settings.

The present study also documents that the ESBL producers are also resistant to other non beta lactam antibiotics like fluroquinolones and aminoglycosides and this fact was statistically significant.

It was interesting to note that specific ESBLs appeared to be unique to a certain country or region. Though the prevalence of ESBLs has been recognized in various parts of the country, there is only limited data on its genotypes in this part of northern India. Our finding emphasized the increasing roles of the multiple ESBL genes in antibiotic resistance and this led to the consideration of an empirical treatment for the infections which were caused by coliforms, especially in patients who were compromised by an underlying disease or who had a compromised immunological status. The data which has been presented here, demonstrates the presence of the CTX-M genes in this part of our country. A similar study from south India also reported the CTX-M genes as the predominant ESBL genes [10]. CTX-M was reported to be the most prevalent gene among the *E.coli* strains and the *Klebsiella* species in another similiar study, but their co-production was not reported [11]. Coproduction of all the three genes, which was also seen in our study, was also reported in *Klebsiella* species in a recent study from south India [12].

It can be concluded from this study, that microorganisms have been evolving towards an antimicrobial drug resistance and this has been considered as a major challenge in hospitals. The use of broad spectrum antibiotics such as quinolones , the second, third and fourth generation cephalosporins, beta lactams combined with

beta lactamase inhibitors and hospitalization are some of the risk factors for the infections which are caused by ESBL producing gram negative bacteria. There is a vicious circle between the antimicrobial resistance and the antimicrobial use. An indiscriminate use of the higher antibiotics should be restricted as far as possible. The infection control programmes should be monitored continuously in hospitals, to contain these ESBL producers.

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FINANCIAL OR OTHER COMPETING INTERESTS:
None.

Date of Submission: **Sep 13, 2012**
Date of Peer Review: **Oct 28, 2012**
Date of Acceptance: **Feb 16, 2013**
Date of Publishing: **Apr 01, 2013**