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Extended spectrum β-lactamases in *Escherichia coli* & *Klebsiella pneumoniae* & associated risk factors

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***Background & objectives*: Extended spectrum β-lactamases (ESBLs) have emerged as a major threat worldwide with limited treatment options. The genotypes of ESBL producing strains largely remain unknown in India; hence the present study was aimed to determine the occurrence of ESBLs in *Escherichia coli* and *Klebsiella pneumoniae*, their molecular types and associated risk factors in a tertiary care hospital.**

***Methods*: Total 200 consecutive clinical isolates of *E. coli* (n=143) and *K. pneumoniae* (n=57) collected between February and July 2006 at Sanjay Gandhi Postgraduate Institute of Medical Sciences, a tertiary care hospital in north India, were examined phenotypically for ESBL production. ESBL strains were further typed for the $bla_{TEM/SHV/CTX-M}$ genes by PCR using specific primers. The bla_{CTX-M} cluster was identified by restriction analysis and genotype by sequencing of PCR product. Resistance to other antimicrobial agents was also studied. Various risk factors associated with ESBL infections were analyzed by logistic regressions.**

***Results*: ESBLs were found in 63.6 per cent *E. coli* and 66.7 per cent *K. pneumoniae* isolates. Majority of the typeable isolates harboured two or more ESBL genes (57.3%). Overall bla_{CTX-M} was the commonest genotype (85.4%) followed by bla_{TEM} (54.9%) and bla_{SHV} (32.9%) either alone or in combination. All CTX-M enzymes in *E. coli* and 87.5 per cent in *K. pneumoniae* belonged to the CTX-M-1 cluster. Sequencing was done for randomly selected 20 bla_{CTX-M} PCR products and all were identified as CTX-M-3. Resistance of ESBL isolates to other antibiotics was amikacin 14.7 per cent, gentamicin 66.7 per cent, trimethoprim/sulphamethoxazole 79.1 per cent and ciprofloxacin 93.8 per cent. Prior antibiotic exposure, use of intravenous device and urinary catheter, renal insufficiency and ICU admission were associated with ESBL infection on univariate analysis. On multivariate, antibiotic exposure (*P*=0.001) and use of urinary catheter (*P*<0.001) were identified as risk for ESBL infection.**

***Interpretation & conclusions*: Our study showed high ESBL occurrence with CTX-M as the emerging type in our hospital and CTX-M-3 being reported for the first time in India. High co-resistance to other non-β-lactam antibiotics is a major challenge for management of ESBL infections.**

Key words CTX-M - drug resistance - ESBL - *Escherichia coli* - *Klebsiella pneumoniae* - risk factor

Due to extensive use of β-lactam antibiotics over the last several decades in the clinical practice, various β-lactamases have emerged. Extended spectrum β-lactamases (ESBLs) are the enzymes produced by Gram-negative bacilli that have the ability to hydrolyze β-lactam antibiotics containing an oxyimino group (third generation cephalosporins and aztreonam) and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam[1]. ESBLs are usually plasmid-mediated β-lactamases, most commonly found in *Klebsiella pneumoniae*, *Escherichia coli* and other Gram-negative bacilli[2]. Since 1983 the number of ESBL variants has been constantly growing; at present more than 300 different ESBL variants are known[3]. These have been classified into nine distinct structural / evolutionary families based on their amino acid sequences. TEM and SHV enzymes form major families. However, during the past decade CTX-M type has emerged in many countries of the world[4]. Currently the CTX-M family includes more than 40 β-lactamases, which are grouped on the basis of sequence similarity into five distinct clusters (subtypes) epitomized by CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25[3]. The present study was carried out to explore the occurrence of ESBLs in *E. coli* and *K. pneumoniae* strains isolated from various clinical specimens by phenotypic methods. Commonly prevalent molecular genotypes of ESBL ($bla_{\text{TEM/SHV/CTX-M}}$) were detected by PCR; $bla_{\text{CTX-M}}$ clusters were identified by restriction analysis and genotype was confirmed by sequencing. An attempt was also made to detect the resistance of ESBL strains to other antimicrobial groups and to analyze various risk factors associated with the occurrence of infections by ESBL producing strains.

Material & Methods

Bacterial isolates, specimens and patient populations: A total of 200 consecutive non-duplicate clinical isolates of *E. coli* (n=143) *and K. pneumoniae* (n=57) from various clinical specimens [blood=18, respiratory tract specimens=23, infected surgical wounds n=56, body fluids (bile, peritoneal dialysis fluid, CSF) n=30 and urine n=73] were studied prospectively for ESBL production between February and July 2006 at Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), a tertiary care hospital at Lucknow, north India. The following factors were analyzed as risk for infections by ESBL producing strains: use of broad-spectrum antibiotics within previous one month, surgical intervention, use of urinary catheter and intravenous device, ventilator support, diabetes, corticosteroid therapy, renal insufficiency (serum creatinine level >2 mg/dl), intensive care unit (ICU) admission, duration of hospital stay and malignancy.

Phenotypic methods for detection of ESBL and resistance to other antimicrobials: All *E. coli* and *K. pneumoniae* isolates were screened for ESBL production by disk diffusion method using ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg) and cefpodoxime (10 μg) antibiotic disks (Hi-Media, Mumbai, India) as recommended by Clinical and Laboratory Standards Institute (CLSI)[5]. ESBL production was confirmed by disk potentiation test using ceftazidime (30 μg) and cefotaxime (30 μg) antibiotic disks with and without clavunalic acid (10 μg)[5] and by double disk approximation method[6]. ESBL producing strain *K. pneumoneae* ATCC 700603 and non-ESBL producing strain *E. coli* ATCC 25922 were used as positive and negative controls (Ranbaxy Research Laboratory, Gurgaon, India) respectively in each phenotypic test. Minimum inhibitory concentration (MIC) of ESBL producing strains to cefotaxime and ceftazidime was determined by agar dilution method[5]. Co-resistance to other antimicrobial agents such as meropenem, amikacin, gentamicin, trimethoprim/ sulphamethoxazole and ciprofloxacin was determined by disk diffusion method following CLSI guidelines[5]. All the antibiotic discs and pure powder used in the study were obtained from Hi-Media, Mumbai, India.

Detection of ESBL types by PCR: Plasmid DNA from ESBL producing strains was extracted from overnight bacterial growth at 37°C by alkali lysis method[7]. Extracted DNA of all phenotypic confirmed ESBL producing strains was subjected to amplification by PCR using $bla_{\text{TEM/SHV/CTX-M}}$ specific primers. The primers to amplify the targeted genes were chosen from earlier published studies and were able to recognize all the known TEM, SHV and CTX-M variants[8,9]. The primers were procured from OPERON Biotechnologies, Germany. Final reaction volume of 25 μl was prepared with H_2O (Mili-Q grade), 20 pmol of both primers, 1 mM of each dNTP, 1 unit of Taq polymerase, 2.5 μl of 10X PCR buffer, 1.5 mM $MgCl_2$, 100 ng DNA template. Amplification reactions were carried out in a thermocycler (PTC-100, MJ Research, USA) under the following conditions: initial denaturation at 96 ºC for 3 min, followed by 35 cycle of denaturation at 96ºC for 30 sec, annealing as specified and elongation at 72ºC for 30 sec. The final elongation step was extended to 3 min at 72ºC.

Subtypes of bla$_{CTX-M}$ by PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing: Amplified 544-bp *bla*$_{CTX-M}$ products were digested by two restriction enzymes (*Pst* I and *Pvu* II) to distinguish the clusters as described earlier[8]. Randomly selected amplified products from 20 strains (*E. coli* n=16 and *K. pneumoniae* n=4) were purified using QIA quick spin columns (QIAGEN Inc., Valencia, USA) and directly sequenced using the CTX-M/F/ and CTX-M/R/ primers on an automated genetic analyzer. The nucleotide sequencing of the PCR products was done by ABI 3100 automated genetic analyzer (Bangalore Genei, India) based on Sanger's sequencing method[10], which uses fluorescent label dye terminator technique. The nucleotide sequences were aligned by Clustal W multiple alignment tool and BLAST tool available at *www.ncbi.nlm.nih.gov*.

Statistical methods: The risk factors were analyzed using SPSS statistical software, version 12.0 (SPSS Inc., Chicago, IL, USA) by univariate logistic regression analysis and the set of probable identified by univariate were subjected to multivariate logistic regression analysis.

Results

The mean age of infected patients was 42.02 yr (range- 1 to 76 yr) and male: female ratio was 1.86:1. The duration of median hospital stay was 16.95 days (range 1-150 days). The numbers of clinical specimens received were almost similar from medical (97) and surgical wards (103).

Disk diffusion method detected 110 (55%) isolates as potential ESBL producers. Phenotypic confirmatory methods detected ESBL production in 129 isolates (64.5%); additional 19 (9.5%) ESBL producing isolates were detected by confirmatory methods. Occurrence of ESBL in our isolates was as follows: *E. coli* 63.6 per cent and *K. pneumoniae* 66.7 per cent. MICs of ESBL producing isolates ranged from 8 to ≥ 512 μg/ml for both ceftazidime and cefotaxime; majority (94.0%) of the isolates had MIC between 32 μg/ml and ≥ 512 μg/ml.

Plasmid DNA isolated from 109 ESBL producing organisms (phenotypic confirmed) were subjected to PCR using TEM, SHV and CTX-M specific primers. DNA from 82 (75.2%) isolates could be amplified either for *bla*$_{TEM}$, *bla*$_{SHV}$, or *bla*$_{CTX-M}$. Two or more genes for ESBL were present in 47 (57.3%) of 82 ESBL typeable isolates, *bla*$_{TEM}$ + *bla*$_{CTX-M}$ being the most common combination (28.1%) followed by *bla*$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CTX-M}$ (15.9%), *bla*$_{SHV}$ + *bla*$_{CTX-M}$ (11%) and *bla*$_{TEM}$ + *bla*$_{SHV}$ (2.4%). Among the isolates harbouring single ESBL gene, *bla*$_{CTX-M}$ was present in 30.5 per cent, *bla*$_{TEM}$ in 8.5 per cent and *bla*$_{SHV}$ in 3.7 per cent isolates. Overall, *bla*$_{CTX-M,}$ *bla*$_{TEM}$ and *bla*$_{SHV}$ was detected in 85.4 per cent (70/82), 54.9 per cent (45/82) and 32.9 per cent (27/82) isolates either alone or in combination (Table I). All CTX-M enzymes in *E. coli* and 87.5 per cent in *K. pneumoniae* belonged to the CTX-M-1 cluster by restriction analysis. On sequence analysis, CTX-M gene PCR products from all 20 randomly selected isolates were identical to CTX-M-3.

The overall resistance to various antibiotics was as follows: amikacin 14.7 per cent (*E. coli* 10.9% and *K. pneumoniae* 23.7%), gentamicin 66.7 per cent (*E. coli* 68.1% and *K. pneumoniae* 63.1%), trimethoprim-sulphamethoxazole 79.1 per cent (*E. coli* 78.0% and *K. pneumoniae* 81.5%) and ciprofloxacin 93.8 per cent (*E. coli* 94.5% and *K. pneumoniae* 92.1%). All isolates were sensitive to meropenem.

Various risk factors associated with the occurrence of ESBL on univariate regression analysis are shown in Table II. When these factors were further subjected to multivariate analysis, antibiotic exposure (therapy) in last 1 month (Odds ratio 3.082; 95% confidence interval 1.629-5.831; *P*=0.001) and inpatient use of urinary catheter (Odds ratio 4.280; 95% confidence interval 2.210-8.290; *P*<0.0001) were found to be significantly associated with infections by ESBL producing isolates.

Table I. Extended spectrum β-lactamase (ESBL) genotypes in *E. coli* and *K. pneumoniae* strains

Positive by PCR for ESBL genes	Number amplified		
	E. coli (n= 61)	*K. pneumoniae* (n=21)	Total n=82 (%)
A. Two or more ESBL genes	35	12	47 (57.3)
bla$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CTX-M}$	9	4	13 (15.9)
bla$_{TEM}$ + *bla*$_{CTX-M}$	17	6	23 (28.1)
bla$_{TEM}$ + *bla*$_{SHV}$	2	0	2 (2.4)
bla$_{SHV}$ + *bla*$_{CTX-M}$	7	2	9 (11.0)
B. Single ESBL gene	26	9	35 (42.7)
bla$_{TEM}$ only	3	4	7 (8.5)
bla$_{SHV}$ only	0	3	3 (3.7)
bla$_{CTX-M}$ only	23	2	25 (30.5)

Table II. Risk factors associated with ESBL infection on univariate analysis

Variables	ESBL occurrence		Statistical significance	
	Present (%)	Total isolate	*P* value	Odd Ratio (95% CI)
Antibiotic exposure in last 1 month				
No	39 (48.5)	80	Ref	
Yes	90 (75.0)	120	<0.001	3.154 (1.727-5.761)
Use of urinary catheter				
No	53 (50.5)	105	Ref	
Yes	76 (80.0)	95	<0.001	4.360 (2.299-8.269)
Use of intravenous devices				
No	39 (48.1)	81	Ref	
Yes	90 (75.6)	119	<0.001	3.041 (1.668-5.545)
Use of ventilator				
No	115 (62.5)	184	Ref	
Yes	14 (87.5)	16	0.063	4.200 (0.927-19.04)
Renal insufficiency (creatinine >2 mg/dl)				
No	86 (59.3)	145	Ref	
Yes	43 (78.2)	55	0.006	2.824 (1.349-5.911)
Admission in intensive care unit				
No	81 (63.8)	127	Ref	
Yes	38 (79.2)	48	0.050	2.158 (0.984-4.731)
Diabetes mellitus				
No	106 (63.5)	167	Ref	
Yes	23 (73.1)	33	0.776	1.121 (0.509-2.471)
Corticosteroid use				
No	113 (65.3)	173	Ref	
Yes	16 (59.3)	27	0.541	0.772 (0.337-1.770)
Malignancy				
No	110 (63.2)	174	Ref	
Yes	19 (73.1)	26	0.590	1.277 (0.525-3.105)

Discussion

We confirmed ESBL producing isolates by two phenotypic methods- disk potentiation recommended by CLSI[5] and double disk approximation[6]. Confirmatory methods detected 9.5 per cent additional isolates as ESBL producers emphasizing that clinical laboratories should not depend solely on screening method for the detection of ESBL. ESBLs were detected 63.6 per cent in *E. coli* and 66.7 per cent in *K. pneumoniae* (overall 64.5%). Other studies from India had reported a high prevalence of ESBL production ranging from 41.0 to 63.6 per cent in *E. coli* and 40 to 83.3 per cent in *K. pneumoniae*[11-13]. In the West, ESBL production in *Enterobacteriaceae* varied from 5 to 52 per cent and in other Asian countries from 10 to 46.5 per cent[3]. We also determined MIC of ESBL isolates to third generation cephalosporins to confirm the resistance. All ESBL isolates had MIC >8 µg/ml to both ceftazidime and cefotaxime; majority had high level of resistance to both the drugs.

Using specific primers for TEM, SHV and CTX-M, only 82 (75.2%) of 109 ESBL isolates could be typed for one or more genes. The negative amplification in the remaining isolates may be due to presence of other ESBL genes, which we did not explore further. Two or more ESBL genes were present in 57.3 per cent typeable isolates. The $bla_{\text{CTX-M}}$ was the most common and was present either alone or in combination with other ESBL type(s). Our findings support the hypothesis that CTX-M is emerging as the dominant ESBL type in clinical isolates[3]. Pournaras *et al*[14] reported 87 per cent prevalence of CTX-M enzyme among ESBL producers in a tertiary care hospital of Greek. In a multi-centric study from Russia, CTX-M gene was reported in 35.9 per cent of *E. coli* and 34.9 per cent of *K. pneumoniae* ESBL strains[8]. Brenwald *et al*[15] reported an outbreak of CTX-M harbouring ESBL in UK. In a nationwide survey in Italy, CTX-M producing strains were reported by 10 of the 11 participating centers, with remarkably variable rates among the centers (1.2 to 49.5% of the ESBL producers)[16]. When CTX-M gene products from our ESBL producing isolates were further subjected to restriction analysis, all *E. coli* and majority of *K. pneumoniae* (87.5%) harboured CTX-M-1 cluster. On sequence analysis, CTX-M gene PCR products were identical to CTX-M-3 (Gene bank accession no. EF675749). To the best of our knowledge, this is the first study to report the presence of $bla_{CTX\text{-}M\text{-}3}$ gene in ESBL strains from India. An earlier report from India showed 73 per cent *E. coli* and 72 per cent *K. pneumoniae* isolates resistant to third generation cephalosporin carried $bla_{CTX\text{-}M\text{-}15}$[17]. In another study from India, CTX-M-15 was reported in four isolates of *E. coli*, one strain each in *K. pneumoniae* and *Enterobacter aerogenes*[18]. Countrywide spread of CTX-M-3 (cluster-1) β-lactamase producing organisms had been reported in Poland[19]. In Italy, all CTX-M determinants were of cluster 1, with CTX-M-15 and CTX-M-1 being the most prevalent variants (60% and 35%, respectively)[16]. Studies from Argentina and Japan have reported CTX-M-2 and CTX-M-3 as predominant ESBL types[20,21]. Some of our isolates harboured bla_{TEM} and bla_{SHV} but these genes were not analyzed further for type identification. In a study from another tertiary care hospital of north India, 95 randomly selected isolates of *Klebsiella* species from 170 samples were subjected to PCR for TEM/SHV; 67.3 per cent of them were positive for both TEM and SHV, followed by 20 per cent for TEM and 8.4 per cent for SHV alone[13]. Some authors have reported SHV-1 hyperproduction in *E. coli* and *K. pneumoniae* that may cause an increased MIC (>1 mg/l) to ceftazidime[22].

All our ESBL producing isolates were susceptible to meropenem was similar to other Indian studies[12,23]. The resistance pattern to other non β-lactam drugs. A study from Italy, showed[24] that carbapenems were active against all ESBL-positive enterobacteria and susceptibility to other drugs was as follows: amikacin 84.7 per cent, piperacillin-tazobactam 84.4 per cent, gentamicin 48.0 per cent, and ciprofloxacin 32.8 per cent. High resistance to other non β-lactam antibiotics by ESBL producing strains poses a threat to treatment failure by these drugs and also minimizes the therapeutic choice to carbapenem especially in patients with systemic sepsis.

We also analyzed various risk factors to determine their association with infection by ESBL producing organisms. Inpatient use of intravenous device, use of urinary catheter, antibiotic therapy in last 1 month, ICU admission and renal insufficiency (creatinine >2 mg/dl) were significantly associated with infections by ESBL producing isolates on univariate analysis. ESBL infection was more frequent in patients who required ventilator support; however, the difference was not significant. Significant association with prior antibiotic therapy supports the hypothesis that selection pressure related to overuse of broad spectrum antibiotics, especially third generation cephalosporins play important role for emergence of high level resistance in the family *Enterobacteriaceae.* Graffunder *et al*[25] found that use of third generation cephalosporins, aminoglycosides and trimethoprim/sulphamethoxazole were independently associated with infections by ESBL producing strains and patients on prolonged use of ventilator were at the greatest risk of having an ESBL organism. Several other studies had also shown strong association between infection with ESBL-producing *E. coli* or *K. pneumoniae* and antibiotic use[11,26]. Reduction in use of ceftazidime or all cephalosporins decreased the occurrence of infection by ESBL producing strains[25, 26].

In conclusion, high occurrence of ESBL in our hospital emphasized the need to adopt appropriate control measures to reduce the ESBL burden. Majority of our harboured two or more ESBL genes and the most common genotype being the CTX-M (CTX-M-1 cluster). Our observations emphasize the need of an antibiotic policy in hospital settings and appropriate infection control measures to overcome the problems associated with infections by ESBL producing strains.

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