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bla_{CTX-M}, bla_{TEM}, and bla_{SHV} in *Enterobacteriaceae* from North-Indian tertiary hospital: high occurrence of combination genes

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ABSTRACT

Objective: To delineate the frequency of occurrence of bla_{CTX-M}, bla_{TEM}, and bla_{SHV} in *Enterobacteriaceae* from North-Indian tertiary hospital. **Methods:** A random collection of a subset of 45 *Escherichia coli (E. coli)* and 28 *Klebsiella pneumoniae (K. pneumoniae)* that was resistant to a third generation cephalosporin and obtained during 2007-2008 was selected for detailed screening for bla_{CTX-M}, bla_{TEM}, and bla_{SHV} by monoplex PCRs. The isolates demonstrating the presence of bla_{CTX-M} alleles were characterized for the specific CTX-M-genogroup by using a multiplex PCR. **Results:** Resistance to cefoperazone, ceftazidime, ceftriaxone, cefotaxime, cefoxitin and piperacillin was 100% each in *K. pneumoniae* isolates, whereas these resistance-rates for *E. coli* isolates were 93.1%, 83.8%, 91.9%, 93.6%, 97.3% and 97.1%, respectively. Concomitant resistance to aminoglycosides, quinolones and aztreonam was also noticed. Presence of any of the bla genes (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) was noticed in a total of 28 (38.4%) isolates of the 73 isolates studied. Many isolates demonstrated occurrence of these genes in various combinations. bla_{CTX-M}, bla_{TEM}, and bla_{SHV} were noticed in 28.8%, 10.9% and 13.7% isolates, respectively. Multiplex PCR in bla_{CTX-M} harboring isolates demonstrated the presence of CTX-M-Genogroup-1 and sequencing for the specific CTX-M-type revealed presence of CTX-M-15 type. RAPD typing showed wide diversity in isolates. **Conclusions:** This is amongst the premier report describing the simultaneous occurrence of bla_{TEM}, bla_{SHV}, and bla_{ampC} in Indian *Enterobacteriaceae* and that wider dissemination of these genes, as demonstrated by diversity of isolates, raises concern and emphasizes a need for extensive search for the presence of these gene pools in Indian subcontinent.

1. Introduction

β - lactamases are enzymes that are major cause of bacterial resistance to the β -lactam family of antibiotics such as penicillins, cephalosporins, cephamicins and carbapenems. These enzymes catalyzes the hydrolysis of the amide bond of four membered beta-lactam ring and render the antibiotic inactive against its original cellular target, the cell wall transpeptidase[1]. On the basis of their primary structure, beta-lactamases are grouped into four classes A, B, C and D. enzymes of classes A,C and D are active site serine enzymes, whereas the class B enzymes are Zn-metalloenzymes[1,2]. The bla_{CTX-M} type enzymes are a group of molecular class A extended spectrum β -lactamases that exhibit an overall preference for cefotaxime and ceftriaxone, than against ceftazidime, and a higher susceptibility to tazobactam than to clavulanate[3].

In recent years CTX-M ESBLs have very rapidly disseminated and are now frequently reported from various countries[3-7]. In India, the very first report of the presence of CTX-M came from New Delhi where only bacterial isolates from year 2000 were investigated and reported as CTX-M-15 type[8]. Subsequently, we performed a systematic survey studying a total of 130 non-duplicate third-generation cephalosporin-resistant Enterobacteriaceae collected during 2003-2005 and found high prevalence (73%) of CTX-M-15 and again all producing CTX-M[9].

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During those years, Walsh *et al* [10] also reported a similar high occurrence of bla_{CTX-M} in Indian *Enterobacteriaceae* and interestingly all were reported as CTX-M-15 type. Moreover, they also looked for the occurrence of bla_{TEM} and bla_{SHV} in the isolates and reported that TEM was the most common ESBL followed by CTX-M during that time. Those reports raised a high concern at our institution and institutional antibiotics prescribing policy was re-defined among clinicians. Following 3 years of implementation of re-defined policy we planned this study to find out its impact and existing prevalence of CTX-M in *Enterobacteriaceae*. Moreover, we also planned to look for the occurrence TEM and SHV beta-lactamases in the isolates since the systematic reports looking for the simultaneous occurrence of these ESBL genes are lacking in Indian literature.

2. Materials and methods

The present prospective study was carried out in the department of Microbiology of Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, U.P., India, over a period from July 2007 to July 2008.

2.1. Clinical samples and bacterial collection

During July 2007 – July 2008, a total of 9 423 clinical samples were subjected to our department for routine culture and antimicrobial susceptibility testing. Of these, 2 367 samples yielded growths of gram-negative bacterial species. A subset of 73 bacterial isolates (45 *E. coli* and 28 *K. pneumoniae*) that were found resistant to any of the third generation cephalosporins was selected for detailed screening for bla_{CTX-M}, bla_{TEM} and bla_{SHV}. All these 73 isolates were identified by the standard microbiological techniques[11,12].

2.2. Antimicrobial susceptibility and MIC testing

Antimicrobial susceptibility testing was carried out on Mueller Hinton agar (HiMedia, India) by the standard disk diffusion method as per Clinical Laboratory Standard Institute (CLSI; formerly NCCLS) guidelines[13]. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls. The antibiotics used and their concentrations (μg) were: cefotaxime (30), ceftazidime (30), cefoperazone (75), ceftriaxone (30), cefepime (30), cefoxitin (30), aztreonam (30), gentamicin (10), ciprofloxacin(1), imipenem (10), piperacillin (100), piperacillin/tazobactam (100/10), cephoperazone/sulbactam (75/10), ceftazidime-clavulanic acid (30/20), and amoxicillin-clavulanic acid (30/20). The antibiotics disks used were purchased from HiMedia Lab. Ltd., India. Minimum inhibitory concentrations (MICs) for ceftazidime and cefotaxime were noted according to CLSI guidelines[14].

2.3. Phenotypic detection of extended-spectrum beta-lactamases (ESBLs)

ESBL detection by disc synergy test using amoxicillin-clavulanate and piperacillin-tazobactam as ESBL inhibitors, in all 73 isolates, was performed as described previously[15]. Briefly, the test inoculum (0.5 McFarland turbidity) was streaked on Mueller-Hinton agar. A disc of amoxicillin-clavulanate (20/10 μg) was placed at a distance of 30 mm, center to center, from cefotaxime (30 μg) and ceftazidime (30 μg). A parallel experimentation was done using piperacillin-tazobactam, in place of co-amoxyclav disc, as described previously[15] for comparative analysis of phenotypic ESBL detection.

2.4. Detection of bla_{CTX-M} by PCR

All 73 isolates were initially screened for the presence of bla_{CTX-M} alleles by PCR using a set of universal primers responsible to yield an amplicon of 593 bp. The forward and reverse primers used for this monoplex PCR were 5′-ATGTGCAGYACCAGTAARGT-3′ and 5′-TGGGTRAARTARGTSACCAGA-3′, respectively and the cycling conditions were: initial denaturation at 94 ℃ for 7 min; 35 cycles of 94 ℃ for 50 sec; 50 ℃ for 40 sec and 72 ℃ for 1 min; and a final elongation at 72 ℃ for 5 min. Subsequently, all bacterial isolates demonstrating the presence of bla_{CTX-M} alleles were characterized for the specific CTX-M-genogroup by using a multiplex PCR protocol of Woodford *et al* [16].

2.5. Sequencing of representative isolates

Sequencing in the representative isolates was performed by courtesy of Chromous Biotech Lab Ltd, Bangalore, India.

2.6. Detection of bla_{TEM} and bla_{SHV} by PCR

The forward and reverse primers used for respective detection of bla_{TEM} and bla_{SHV} 5′-KACAATAACCCTGRTAAA-3′ &5′-AGTATATATGAGTAAACTTGG-3′, and 5′-TTTATCGGCCYTCACTCAAGG-3′&5′-GCTGCGGGCCGGATAACG-3′, respectively, as used in our previous studies[17]. The cycling conditions for detection of bla_{TEM} and bla_{SHV} were the same as described here: initial denaturation at 95 ℃ for 15 min; 35 cycles of 94 ℃ for 1 min; 58 ℃ for 2 min and 72 ℃ for 3 min; and a final elongation at 72 ℃ for 10 min.

2.7. RAPD-PCR typing

Epidemiological typing of the isolates by RAPD PCR was done, as described previously, to ascertain any specific clone circulating in the survey population[9, 18].

3. Results

3.1. Clinical specimens and bacterial isolates

During the study period of one year, a total of 2 367

specimens yielded growths of gram-negative bacterial species and a subset of 45 *E. coli* and 28 *K. pneumoniae* that were found resistant to any of the third generation cephalosporins was selected for detailed screening for bla_{CTX-M}, bla_{TEM} and bla_{SHV}. These 73 non-repeat cephalosporin-resistant bacterial isolates were obtained from pus (n=20), urine (n=19), blood (n=15), throat swabs (n=4), cervical swabs (n=7), stool (n=5), sputum (n=2) and cerebrospinal fluid (n=1).

3.2. Antibiotics resistance and MIC testing

The detailed antibiotic resistance rates and MIC-distribution results are shown in Table 1 & 2, respectively. It was interesting to note that all (100%) the *K. pneumoniae* isolates randomly selected for this study were found resistant to cefoperazone, ceftazidime, ceftriaxone, cefotaxime, cefoxitin and piperacillin. The above resistance-rates for *E. coli* isolates were 93.1%, 83.8%, 91.9%, 93.6%, 97.3% and 97.1%, respectively. Apart from the resistance to cephalosporins the isolates also demonstrated concomitant high resistance to aminoglycosides, quinolones and aztreonam (Table 1).

Table 1
Percent antibiotic resistance rates of *E. coli* and *K. pneumoniae* isolates against various antibiotics tested.

Antibiotics tested	%Resistance	
	E. coli (n=45)	*K. pneumoniae* (n=28)
Piperacillin	97.14	100.00
Piperacillin/Tazobactam	27.03	61.90
Cefoperazone-Sulbactam	44.44	64.71
Cefoperazone	93.10	100.00
Ceftazidime-clavulanic acid	16.67	27.78
Ceftazidime	83.78	100.00
Amoxyclav	72.97	85.71
Ceftriaxone	91.89	100.00
Cefotaxime	93.55	100.00
Cefoxitin	97.30	100.00
Cefepime	96.67	92.86
Aztreonam	80.00	89.30
Gentamicin	71.10	64.30
Ciprofloxacin	68.90	64.30
Imipenem	0.00	0.00

Table 2
MICs to ceftazidime and cefotaxime in *E. coli* and *K. pneumoniae* isolates.

MICs (mg/L)	% *K. pneumoniae* isolates resistant to		% *E. coli* isolates resistant to	
	Ceftazidime	Cefotaxime	Ceftazidime	Cefotaxime
>16	3.45	–	–	–
>32	3.45	–	5.71	4.65
>64	6.90	22.22	8.57	6.98
>128	31.03	11.11	31.43	18.60
>256	31.03	44.44	17.14	16.28
>512	17.24	22.22	8.57	9.30
>1024	6.90	–	8.57	44.11

3.3. Phenotypic ESBL detection

ESBLs were phenotypically detected in 61.7% (45/73) isolates using piperacillin/tazobactam in synergy test, however 51.2% (41/73) isolates were detected by using co-amoxyclav as an ESBL inhibitor.

3.4. Detection of bla genes

Out of 73 isolates studied, presence of any of the *bla* genes (bla_{CTX-M}, bla_{TEM}, and bla_{SHV}) was noticed in a total of 28 (38.4%) isolates. Occurrence of single bla gene viz. bla_{CTX-M}, bla_{TEM}, or bla_{SHV}, was noticed in 11, 2, & 3 isolates, respectively. However, many isolates demonstrated occurrence of these genes in various combinations viz. bla_{CTX-M} + bla_{TEM}, bla_{CTX-M} + bla_{SHV}, bla_{TEM} + bla_{SHV} in 4, 6, & 2 isolates, respectively. None of the isolates showed the presence of all these three bla genes (bla_{CTX-M} + bla_{TEM} + bla_{SHV}) in a single bacterial cell.

3.5. Detection of bla_{CTX-M}, bla_{TEM}, and bla_{SHV}

bla_{CTX-M} was noticed in a total of 28.8% (21/73) isolates whereas bla_{TEM} and bla_{SHV} was noticed in 10.9% and 13.7% isolates, respectively. The frequency of occurrence of these bla genes was higher in *K. pneumoniae* as opposed to *E. coli* (kindly refer to Table 3 for detailed description of results). Further analyses of the isolates demonstrating bla_{CTX-M} by monoplex PCR revealed the presence of CTX-M genoroup-1 by multiplex PCR.

Table 3
Distribution of various beta-lacatmases among Indian isolates of *E. coli* and *K. pneumoniae* screened in this study [n(%)].

Isolates (n)	Genotypes present in No. of isolates		
	CTX-M	TEM	SHV
E. coli (45)	10 (4.5)	3 (6.7)	3 (6.7)
K. pneumoniae (28)	11 (39.2)	5 (17.9)	7 (25.0)
Total (73)	21 (28.8)	8 (10.9)	10 (13.7)

3.6. Typing of isolates by RAPD-PCR

RAPD-PCR typing demonstrated great diversity in isolates and no predominant clone was identified (Figure 1). The isolates could not be grouped into clusters because of unique banding patterns. No gross banding difference was noticed between two RAPD-PCR experiments.

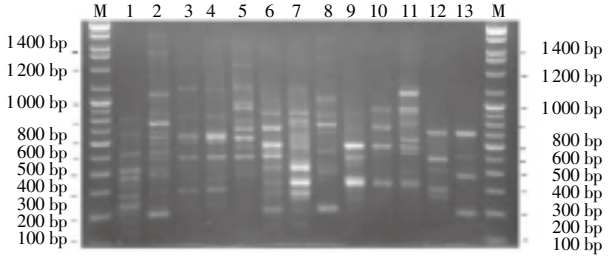

Figure 1. RAPD-PCR typing demonstrating diversity in isolates.

4. Discussion

The prevalence and type of ESBLs may vary from one geographic region to other. For e.g. in China TEM-type were found to be most prevalent ESBLs among the *E. coli* (ESBL producing strains) followed by SHV and then CTX-M-type enzymes[19]. Another report from Canada shows SHV as main group of ESBLs in *E. coli* and only 6% of ESBL producers were found to carry bla_{TEM} and bla_{CTX-M}[20]. Group 2 CTX-M enzymes are the most prevalent CTX-M enzymes in parts of South America and of Israel[21] while group 9 enzymes were found to be prevalent in Spain[22].

First CTX-M-9 (Group 9) producing *Klebsiella* was isolated in UK in 2000[23]. Then CTX-M-26 from group 25/26 was recovered from Birmingham[24]. Group 1 (CTX-M-9,-14 and -15) producing isolates were found in both community and hospitals in New York[25]. A surveillance study in U.K. showed wide scattering of CTX-M-15 producing *E. coli*[26]. In India, till date, CTX-M has been reported to be the most prevalent ESBL[9]. However, the reports simultaneously looking for bla_{CTX-M}, bla_{TEM}, and bla_{SHV} are fragmentary from India. In the present collection of Indian Enterobacteriaceae of 2007-2008, we found CTX-M as the most prevalent (28.8%) ESBL followed by SHV (13.7%) and TEM (10.9%). The frequency of occurrence of these bla genes was higher in *K. pneumoniae* than in *E. coli* isolates. In our earlier collection of 2003-2005[9], we already reported a higher occurrence of bla_{CTX-M} in Indian Enterobacteriaceae however, we couldn't look for bla_{TEM} and bla_{SHV} in those isolates. The present study is significant since it reports the presence of CTX-M enzymes as the most prevalent ESBL-types in contrast to Walsh *et al* [10]. who reported TEM as the most prevalent enzymes prior to year 2000. The present study clearly demonstrates the drastic change in the gene pool in Indian *Enterobacteriaceae*.

Infections caused by ESBL producing strains are increasing in frequency however only few PCR based surveys of ESBL producing strains have been performed and reported till now from India[9, 10]. Recently CTX-M-1-type was reported (with a prevalence of 58.3%) from South India[27] however in our previous study[9], in which we included 19 isolates from South India (Hubli), we got only CTX-M-15-type from South Indian isolates also.

A recent study also reported the presence of $bla_{CTX-M-28}$ for the first time from India[28]. In reaction to these reports describing the occurrence of new CTX-M types in Indian *Enterobacteriaceae* we also planned this study and screened our isolates by multiplex PCR to look for the presence of specific CTX-M-genogroups in our location and found all isolates belonging to genogroup-1. Sequencing of the representative isolates demonstrated the presence of CTX-M-15 type (GenBank Accession No. GQ117123).

To date, in addition to 150 OXA type, 55 SHV-type and 135 TEM-type, more than 70 CTX-M type ESBLs have been identified (http://www.lahey.org/studies/webt.htm).

Occurrence or association of more than one β-lactamase within the same isolate has been reported[8, 25]. In the present collection of Indian *Enterobacteria* not only we noticed the occurrence of these bla genes singly, but also in various combinations, which denotes their wider dissemination probably due to involvement of genetic elements in mobilization of these genes. In this study, only 38.4% of the isolates demonstrated the presence of bla genes as opposed to that of phenotypic detection in 61.7% isolates by piperacillin-tazobactam synergy test and we speculate that some other ESBL genes, probably OXA-type and others, are also prevalent in Indian bacteria which we even could not look for in this collection.

Bacteria are noteworthy for their remarkable ability to adapt changes in their environment. Although mutation has an important role to play in the evolution of antibiotic resistance, the predominant factor for escalation of antibiotic resistance is the acquisition of antibiotic resistance genes. The antimicrobial resistance of gram-negative organism has built up progressively during the last few decades, leading to increased incidence of outbreaks of infections due to existence of multi-resistant bacteria. Resistant bacterial strains are spreading rapidly, where they have chance to come in close contact with other bacteria, sharing resistance genetic material and thus spreading the antimicrobial resistance phenotypes.

In infections caused by the same bacterial clone (monoclonal outbreak) or by a few bacterial clones (oligoclonal outbreak), the pathogen usually passes horizontally (from patient to patient), while infections originated by various clones of the same species (polyclonal outbreaks) are usually caused by the intensive selective pressure imposed by antibiotic use[29]. On comparing the prevalence of ESBLs (CTX-M) in our collection of 2003-2005 with this collection of 2007-2008, we noticed drastic reduction from 73% to 28.8% which could be the impact of changes in the antibiotics prescribing habits at our institution. However, based on the RAPD-PCR typing, great diversity of the isolates was noticed and emphasize the need to re-analyze and implement strict national antibiotics prescribing policies to minimize the selective pressure.

In nutshell, CTX-M enzymes are the most prevalent ESBLs in our region (Aligarh) followed by SHV and TEM. More alarming is the detection of combination of these bla genes in recent collection. Moreover, wider dissemination of these genes as demonstrated by diversity of isolates raises concern and emphasizes a need for extensive search for the presence of these genes in Indian subcontinent and also to implement strict national antibiotics policies to restrict the spread of these resistance bugs.

Conflict of interest statement

The authors have no conflicts of interest

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