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Comparative study on occurrence of class A and class C β-lactamase genes and their co-occurrence in Indian *Enterobacteriaceae* during years 2009 and 2010

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ABSTRACT

Objective: To determine the occurrence of class A and class C β-lactamase genes and their co-occurrence in Indian *Enterobacteriaceae*. **Methods:** 52 third generation cephalosporin resistant isolates were phenotypically detected by combination disk method and screened by PCR to identify class A and class C type β-lactamase genes. **Results:** Of the 52 isolates, 94.2% (49) were found harboring any of the bla_{ESBLs}. bla_{CTX-M}, bla_{SHV} and bla_{TEM} were present in 82.6% (43/52), 59.6% (31/52), and 42.3% (22/52) isolates, respectively. Of the 49 ESBL positive isolates 57.1% (28/49) showed co-occurrence of bla_{ampC} with bla_{ESBLs}. On the contrary, the collection from 2009 showed their co-occurrence in 81.4% isolates. **Conclusions:** The comparative study shows a downward trend for co-existence of bla_{ESBLs} with bla_{ampC} from 2009 to 2010. Further large scale studies are needed to address the co-occurrence of class A and class C β-lactamases in India and the resistance trend occurring over a period of time.

1. Introduction

The introduction of oxyimino-cephalosporins into clinical practice for the treatment of resistant gram-negative bacterial infections was soon followed by the development of extended-spectrum β-lactamases (ESBLs). ESBLs are usually plasmid-encoded enzymes derived from TEM- and SHV- type β-lactamases by one or more amino acid substitutions which confer different levels of resistance to ceftazidime, cefotaxime, monobactams and to other broad-spectrum cephalosporins[1]. The CTX-M-type enzymes, which are non-TEM and non-SHV derivatives, represent a rapidly growing family of ESBLs. CTX-M type ESBLs have become the most prevalent family of ESBLs among *Enterobacteriaceae* since their first report in 1986[2].

Co-existence of class A and class C type β-lactamases in *Enterobacteriaceae* is frequently reported from many countries[3-8], however the molecular reports from India are fragmentary regarding the occurrence of class A and class C β-lactamases; especially looking for the co-occurrence[9]. Therefore, the present study was planned to look for the occurrence of *bla* genes of class A ESBLs (bla_{CTX-M}, bla_{TEM}, bla_{SHV}) and class C β-lactamase gene (bla_{ampC}). The isolates were also analyzed for the simultaneous occurrence of class A and class C β-lactamases. Moreover the comparative analysis in isolates obtained during year 2009 and 2010 was

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performed to find out the frequency and trend of these *bla* genes over a period of time.

2. Material and methods

2.1. Bacterial isolates

A total of 52 nonduplicate isolates of *Enterobacteriaceae* (39 *E.coli* and 13 *Klebsiella* spp.) reported resistant to any of the third generation cephalosporin and obtained from various clinical specimens (pus, urine, sputum, cervical swab, drain and abdominal fluid) of inpatients and outpatients from JNMC&H, Aligarh Muslim University, from January 2010 to June 2010 were studied. Antibiotics susceptibility testing was performed on Mueller-Hinton agar by the standard disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines[10]. Following antibiotic disks were used cefoperazone (75 μg), cefixime (5 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefpirome (30 μg), cefepime (30 μg), ofloxacin (5 μg), gentamicin (10 μg), amikacin (30 μg), gatifloxacin (5 μg) and aztreonam (30 μg). Antibiotic disks were procured from HiMedia Laboratories, Ltd., India.

2.2. Phenotypic Detection of ESBL

All isolates were screened for ESBL production by combination disk method using disks of cefotaxime (30 μg) *versus* cefotaxime-clavulanate (30/10 μg), and ceftriaxone (30 μg) *versus* ceftriaxone-sulbactam (30/15 μg). An increase in zone diameters of ⩾5 mm and ⩾8 mm in combination discs containing clavulanate and sulbactam, respectively, in comparison to their respective antibiotics was taken as a positive result for ESBL-producers[11].

2.3. Molecular characterization of SHV, TEM and CTX-M ESBLs by multiplex PCR

All isolates were screened for the presence of geno-groups of CTX-M by multiplex PCR using specific primers for CTX-M-1, -2, -8, -9 and -25/26 groups, and PCR conditions as described by Woodford *et al*[12]. These isolates were also characterized to determine bla_{TEM} and bla_{SHV} with the universal primer sets as described previously[13]. *E. coli* isolates (D1, D2, and D3), kindly provided by Prof. Daniel Jonas, Germany, and was used as positive controls for SHV, TEM and CTX-M-15, respectively.

2.4. Detection of ESBL positive isolates for co-existence of bla_{ampC}

All genotypically ESBL positive isolates were characterized for the co-existence of bla_{ampC} using method as given by Feria *et al*[14] with some modification as described previously[13].

2.5. Comparative analysis

A subset of 76 isolates of *Enterobacteriaceae* collected during 2009 and previously characterized for class A ESBLs (CTX-M, TEM, SHV) was included for comparable study with the collection of 2010 for occurrence of bla_{CTX-M}, bla_{SHV}, and bla_{TEM} and also to determine the co-existence of these bla_{ESBLs} with bla_{ampC}.

2.6. Screening of PCR results

After amplification, PCR products were analyzed in 2% agarose gel in 1× TAE buffer and for staining ethidium bromide (EtBr) at 0.1 μL/mL was used as nucleic acid intercalating agent for the visualization of amplified products under an UV light. High range DNA rular (Genei) was used to determine the size of DNA products.

3. Results

3.1. Antibiotic resistance

All 52 isolates included in the study were found resistant to cefotaxime, cefoperazone and aztreonam. The detailed results for antibiotics resistance rates are given in Table 1. 100% resistance was found with the following antibiotics cefoperazone, cefotaxime and aztreonam.

Table 1
Antibiotic resistance rates of *Enterobacteriaceae* isolates (*n*=52).

Antibiotics used	% Resistance (*n*)
Cefoperazone	100.0 (52)
Cefixime	67.3 (35)
Cefotaxime	100.0 (52)
Ceftazidime	92.3 (48)
Ceftriaxone	92.3 (48)
Cefepime	57.6 (30)
Cefpirome	42.3 (22)
Ofloxacin	28.8 (15)
Gentamicin	63.4 (33)
Amikacin	53.8 (28)
Gatifloxacin	94.2 (49)
Aztreonam	100.0 (52)

3.2. Phenotypic ESBL detection

Phenotypically, 80.4% and 86.5% isolates were found as ESBL producers by combination disk method using cefotaxime/cefotaxime-clavulanate and ceftriaxone/ceftriaxone-sulbactam, respectively.

3.3. Identification of SHV, TEM and CTX-M-genogroups by PCR

Out of 52 isolates, 49 (94.2%) were found positive for ESBL

genes, PCR assays revealed the occurrence of bla_{CTX-M}, bla_{SHV} and bla_{TEM} in 82.6% (43/52), 59.6% (31/52), and 42.3% (22/52) isolates, respectively. Of the 49 ESBL positive isolates, 30.6% (15/49), 4% (2/49), and 4% (2/49) isolates were found harboring single *bla* genes *viz.* bla_{CTX-M}, bla_{SHV} and bla_{TEM}, respectively. Co-occurrence of *bla* genes was also noticed; co-occurrence of bla_{CTX-M} with each of bla_{SHV}, bla_{TEM} and with both bla_{SHV} + bla_{TEM} was noticed in 20.4% (10), 2% (1) and 34.6% (17) isolates, respectively. CTX-M groups were characterized by multiplex PCR which indicated that all the isolates carry gene for CTX-M-genogroup-1 (Figure 1).

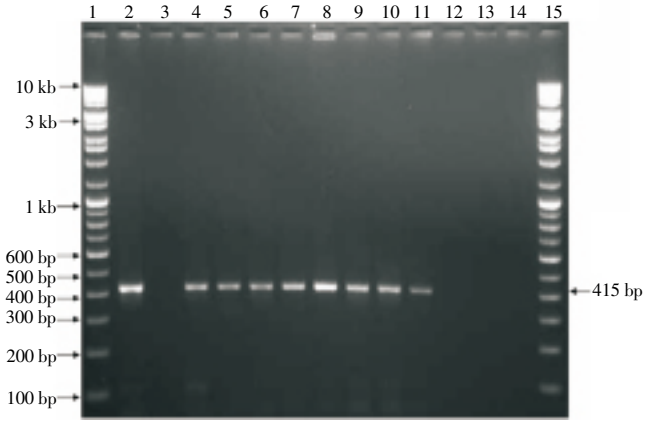

Figure 1. 2% Agarose gel is showing positive isolates of CTX-M type gene (Lane 4-11) of 415 bp with positive control strain D3 (*E. coli*) in lane 2 and negative control in lane 3, while lane 12-14 shows negative isolates, lane 1 and 15 are showing Genei High range DNA ruler.

3.4. Co-existence of bla_{ESBLs} with bla_{ampC}

Out of 49 isolates (collected during 2010) harboring bla_{ESBLs} 57.1% (28/49) showed co-existence for bla_{ampC} (Figure 2). In the collection of 76 isolates of 2009, 70 isolates were harboring bla_{ESBLs}. Of these 70 isolates harboring bla_{ESBLs}, 81.4% (57/70) showed co-existence of bla_{ampC} with bla_{ESBLs}.

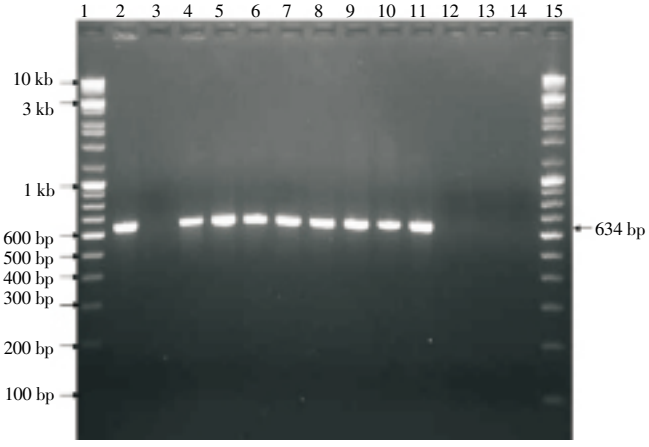

Figure 2. 2% Agarose gel is showing positive isolates of ampC type gene (Lane 4-11) of 634 bp with positive control strain D1 (*Citrobacter* spp.) in lane 2 and negative control in lane 3, while lane 12-14 shows negative isolates, lane 1 and 15 are showing Genei High range DNA ruler.

3.5. Comparative analysis of collections of year 2009 and year 2010

On comparison of genotypic results of two year's collections (2009 and 2010), we found nearly similar occurrence for bla_{ESBLs}; 94.2% (70/76) and 92.1% (49/52) in respective years, 2009 and 2010. In 2010, 82.6% (43/52), 59.6% (31/52), and 42.3% (22/52) isolates were found positive for bla_{CTX-M}, bla_{SHV}, and bla_{TEM}, respectively, while in 2009 these *bla* genes were present in 88.1% (67/76), 43.4% (33/76), 43.4% (33/76) isolates, respectively. On analyses of various combinations like bla_{CTX-M}, bla_{CTX-M}+bla_{SHV}, bla_{CTX-M}+bla_{TEM}, bla_{CTX-M}+bla_{SHV}+bla_{TEM} and bla_{SHV}+bla_{TEM} in 2010, we found these occurrences in 30.6% (15/49), 20.4% (10/49), 2% (1/49), 34.6% (17/49) and 4% (2/49) isolates, respectively, while in 2009 these combinations were present in 31.4% (22/70), 18.5% (13/70), 20% (14/70), and 25.7% (18/70), respectively; however the combination of bla_{SHV}+bla_{TEM} was absent in this collection of year 2009. By PCR, in all isolates harboring bla_{ESBLs}, bla_{ampC} was detected in 81.4% (57/70) and 57.1% (28/49) isolates from collections of year 2009 and 2010, respectively. In 2010, co-existence of bla_{ampC} was noticed in 46.6% (7/15), 50% (5/10), 100% (1/1), 76.4% (13/17), and 100% (2/2) isolates having different combinations of bla_{CTX-M}, bla_{CTX-M}+bla_{SHV}, bla_{CTX-M}+bla_{TEM}, bla_{CTX-M}+bla_{SHV}+bla_{TEM} and bla_{SHV}+bla_{TEM}, respectively. While in 2009, the co-existence of bla_{ampC} with the combinations of bla_{CTX-M}, bla_{CTX-M}+bla_{SHV}, bla_{CTX-M}+bla_{TEM}, bla_{CTX-M}+bla_{SHV}+bla_{TEM} was found in 86.3%(19/22), 92.3%(12/13), 85.7%(12/14), 72.2%(13/18) of isolates, respectively. In 2009 out of two isolates harbouring only bla_{SHV}, one was found positive for co-existence of bla_{ampC}.

4. Discussion

Nowadays, CTX-M type β-lactamases had become the principal cause of resistance to third-generation cephalosporins in *Enterobacteriaceae* worldwide[15]. In the present study, a collection of 52 isolates was characterized for ESBLs, which showed 94.2% (49/52) isolates carrying bla_{ESBLs}. In the previous collection of 2009 we also found comparatively similar percentage of isolates (92.1%) for the presence of bla_{ESBLs}. A previous report from south India in 2006[16] showed 35.89% prevalence for bla_{CTX-M} gene while in present study we observed a noteworthy increase 82.6% (43/52), which is inflexible in comparison to some recent reports from Ireland (89%), China (89%), Spain (86%), France (83%) and UK (85.2%)[17-21]. Since the first description of $bla_{CTX-M-15}$ in India[22], it is now considered a dominant type with some exceptions worldwide[15,23]. On

reviewing the literature of Indian studies we found only CTX-M group-1 as the dominant genogroup of CTX-M[13,24-26].

CTX-M-type extended spectrum β-lactamases (ESBLs) and AmpC-type β-lactamases have been found as two major contributors in recent years[1,2,27,28]. Reports of multiple β-lactamases in a single pathogen are increasing for *Enterobacteriaceae*[3,5-8,13]. CMY-2 type was found to be associated with CTX-M-14 in a Parisian *E. coli* isolate[4]. In Taiwan, CTX-M and SHV-type ESBLs with CMY- and DHA-type AmpC enzymes are the most common β-lactamases that conferred resistance to extended-spectrum cephalosporins in clinical *K. pneumoniae* isolates[6,29]. The emergence of a multidrug resistant *K. pneumoniae* isolate, which produces VIM-4, CTX-M-15, TEM-1, CMY-4 have been reported from France[30]. Co-existence of CMY-8 and CTX-M-3 was reported in a 269 kb conjugative plasmid from *K. pneumoniae*[31]. A recent report for co-existence of CMY-6 and CTX-M-15 has recently been reported on the similar type of plasmid from India[9].

These studies provided us to find out the co-existence of multiple genes of β-lactamases of two major classes, class A and class C in our isolates. So, a collection of 52 isolates obtained during 2010 and a previously characterized subset of 76 isolates (2009) were characterized to determine the frequency of co-existance of different combinations of bla_{ESBLs} with bla_{ampC}. In the present collection of 2010, among the bla_{ESBLs} harboring isolates (n=49), we found that highest number of isolates *i.e.* 34.6% (17) were positive for combination of bla_{CTX-M}+ bla_{SHV}+ bla_{TEM} while least number (2%) was positive for combination of bla_{CTX-M}+ bla_{TEM}. Out of 49 ESBL positive isolates, co-existence of *bla* genes with bla_{ampC} was present in 57.1% (28/49) isolates of *Enterobacteriaceae*. We looked co-existence of ampC, and found 46.6% (7/15), 50% (5/10), 100% (1/1), 76.4% (13/17), and 100% (2/2) of isolates positive for co-existence in different combinations of bla_{CTX-M}, bla_{CTX-M}+bla_{SHV}, bla_{CTX-M}+bla_{TEM}, bla_{CTX-M}+bla_{SHV}+bla_{TEM} and bla_{SHV}+bla_{TEM}, respectively, while in 2009 collection co-existence of bla_{ampC} occurred in 86.3%(19/22), 92.3%(12/13), 85.7%(12/14), 72.2%(13/18) isolates in different combinations of bla_{CTX-M}, bla_{CTX-M}+bla_{SHV}, bla_{CTX-M}+bla_{TEM}, bla_{CTX-M}+bla_{SHV}+bla_{TEM}, respectively.

In the present collection we could not find any positivity for co-existence of bla_{ampC} in those isolates which harbor only single gene of SHV or TEM, because of small sample size, but in our previous collection of 2009 one isolate was found positive for co-existence of bla_{SHV} and bla_{ampC}. On comparison of both collections of year 2009 and 2010, in 2009 we observed 81.4% (57/70) isolates positive for co-existence of *bla* genes with bla_{ampC}, while in the collection of 2010, we found co-existence only in 57.1% (28/49) isolates, and that indicates that the implementation of antibiotic prescription policies in our institution is moving on a right track. However further large scale studies are needed to address the trend of occurrence of *bla* genes over a period of time.

Conflict of interest statement

We declare that we have no conflict of interest.

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