DISCUSSION

CHAPTER 5

DISCUSSION

Extended Spectrum β-lactamases (ESBLs) are widespread all over the world, but the prevalence and phenotypic characteristic among clinical isolates may vary between geographical areas (Winokur et al. 2001; Navon-Venezia et al. 2003). This geographical difference may be due to the differences in the use of antibiotics and selection of organisms. TEM and SHV were found as the most prevalent type of ESBLs followed by CTX-M enzymes in various countries (Xiong et al. 2002; Mulvey et al. 2004). However, in India, CTX-M is reported as the most prevalent enzyme than any other ESBL (Karim et al. 2001; Ensor et al. 2006; Shahid 2010; Shahid et al. 2011b).

Some studies from India have reported 6.6-68% Enterobacteriaceae isolates as ESBL producers (Menon et al. 2006). From South India Subha et al. (2002) has reported 6.6% prevalence of ESBL production among Klebsiella pneumoniae isolates from Children. 68% of Gram-negative bacterial isolates from a tertiary care hospital from Delhi were reported as ESBL producers (Mathur et al. 2002). One study from north India, on ESBL production in uropathogens, has shown 26.6% ESBL producers comprising of Klebsiella, E. coli, Enterobacter, Proteus, and Citrobacter species (Khurana et al. 2002). 86.6% of Klebsiella spp., 73.4% of Enterobacter spp. and 63.6% of E. coli isolates from cases of neonatal septicaemia were reported as ESBL producers (Jain et al. 2003), while another study 48.3% urinary isolates were found as ESBL producers (Tankhiwale et al. 2004). In a report from Tamil Nadu, 40% and 41% isolates were found as ESBL producers among K. Pneumoniae and E. coli,
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respectively (Babypadmini et al. 2004). Whereas another study reported, 58.06% *E. coli* and 57.14% *Enterobacter* spp. isolates as ESBL producers (Ananthakrishnan et al. 2004). A previous study from our college hospital (JNMCH) reported 42% of isolates as ESBL producers among which 34.42% were *E. coli* isolates (Akram et al. 2007), but a subsequent study from the same place found 14.3% *E. coli* and 24.5% *K. Pneumoniae* isolates as ESBL producers (Shahid et al. 2008).

In the present study, 130 *Enterobacteriaceae* isolates (108 *E. coli* and 22 *Klebsiella* spp.) were selected based on the resistance to any of the third generation cephalosporin. The susceptibility pattern to various antibiotics noticed 100% (130) resistance to cefotaxime, while majority of the isolates 95.3% (124/130) were also found resistant to ceftazidime. Other antibiotics such as ceftriaxone, cefixime, cefoperazone and aztreonam were also showing significantly high rates of resistance, 93% (121/130), 90% (117/130), 82.3% (107/130) and 91.5% (119/130), respectively, while none of the isolate was found resistant to imipenem. A previous study by Shukla et al. 2004 has revealed 72% of Gram-negative bacteria resistant to all third generation cephalosporins. In this study resistance rates to fourth generation cephalosporins, cefepime and cefepirome, was noticed in 63% (82/130) and 78.4% (102/130) isolates, respectively. Concurrent high resistance rates to other antibiotics such as ofloxacin (88.4%) and gatifloxacin (83.8%) was noticed, however a comparatively low resistance to aminoglycosides like gentamycin (56.1%) and amikacin (41.5%) was noticed in our isolates. We found variation in the resistance rates and patterns in the isolates obtained from different wards. 100% resistance to ceftazidime was observed in the isolates obtained from Orthopaedic ward, while the isolates obtained from Medicine ward showed 100% resistance to
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defixime. The isolates obtained from paediatric ward showed 100% resistance to most of the antibiotics such as ceftazidime, cefoperazone, cefixime, ofloxacin, gatifloxacin and to aztreonam.

On analyzing the phenotypic detection of ESBL producers by double disk synergy test (DDST), combination disk test (CDT) and modified three dimensional extract test (MTDT) we found CDT as the most appropriate confirmatory test because this method showed 89.2% positivity in comparison to DDST and MDT that showed 78.4% and 80% positivity, respectively. In this study we used piperacillin/tazobactam combination instead of amoxycilav, and it gave us better results for detection of ESBL producers in comparison to amoxycilav (Amoxyclav was used in few representative isolates that showed negativity for ESBL producers). In MDT, we also used cefotaxime in place of ceftazidime, which again gave us much better result then ceftazidime and also helped confirming the presence of $\text{bla}_{\text{CTX-M}}$ genes in the Enterobacteriaceae isolates of this study.

In this study, genotypically, of the 130 cefotaxime resistant isolates, only 86.1% (112) were found positive for $\text{bla}_{\text{CTX-M}}$ genes that included higher number of $E.\ coli$ isolates (89.8%) then Klebsiella isolates (68.1%). We observed that of the all cefotaxime resistant isolates, 18 were not found to harbour $\text{bla}_{\text{CTX-M}}$ genes. May be any other enzyme mimics the activity of CTX-M type $\beta$-lactamases so we found these isolates resistant to cefotaxime, however $\text{bla}_{\text{CTX-M}}$ gene was absent. A previous study from south India in 2006 (Sekar et al. 2006), has reported 35.89% prevalence for $\text{bla}_{\text{CTX-M}}$ gene while in present study we observed a noteworthy increase 86.1%, which is inflexible in comparison to some recent reports from China (89%), Ireland (89%),
Spain (86%), France (83%) and UK (85.2%) (Yu et al. 2007; Morris et al. 2009; Blanco et al. 2009; Arpin et al. 2009; Xu et al. 2010).

Recently more than 113 different variants of CTX-M type β-lactamases (http://www.lahey.org/studies/webt.htm) belonging to six different groups has been reported (Rossolini et al. 2008; Shahid et al. 2011). Since the first description of \( \text{bla}_{\text{CTX-M-15}} \) in India (Karim et al. 2001), it is now considered a dominant type with some exceptions worldwide (Canton & Coque 2006; Hawkey & Jones 2009). On reviewing the literature of Indian studies we found only CTX-M group-1 as the dominant genogroup of CTX-M (Shahid 2010; Ensor et al. 2006; Kingsley & Verghese 2008; Shahid et al. 2011). So in this study we also determined the various CTX-M genogroups and we found CTX-M genogroup-1 as the most prevalent group, while only five isolates were found having multiple groups which included one for genogroup-8 and four for genogroup-9 along with genogroup-1. By monoplex PCR for these genogroups we found amplicons for genogroup-1 (415bp), genogroup-8 (666bp) and one isolate for genogroup-9 (205bp), whereas other three isolates gave positive results with genogroup-9-primers but the amplicon size did not correspond with genogroup-9 protocol, these genes amplified at molecular size of ~900bp. The sequencing of amplicons of genogroup-8 and genogroup-9, including those which did not correspond the exact amplicon size for genogroup-9 was determined. The single isolate with amplicon size 205bp was determined as CTX-M-9 like (which is for the first time isolated from India) but we obtained short sequence (~150 nucleotites) so the gene sequence was not accepted by gene bank. The representative isolates were also sequenced to determine the exact CTX-M genogroup-1 type (these isolates included those isolates harbouring genogroup-8 and genogroup-9) and we found
the presence of CTX-M-15 type. The occurrence of two other CTX-M-genogroups (-8 and -9) along with genogroup-1 in the present study is denoting the newly emerging mechanism of resistance in Indian Enterobacteriaceae.

Some global studies on occurrence of \textit{bla}_{CTX-M} and its co-occurrence with \textit{bla}_{ESBLs} reported 77.7% positivity for \textit{bla}_{CTX-M} gene among \textit{E. coli} ESBL-producing isolates from Germany (Mshana et al. 2009), while from New Zealand 97.6% ESBL-producing isolates were found to carry \textit{bla}_{CTX-M} ESBLs, of which 75.9% were CTX-M-15 type, 13.3% were CTX-M-14 type and only 1.2% each were reported to possess SHV and TEM types (Hefferman et al. 2009). In another study from Saudi Arabia all of the \textit{K. pneumoniae} isolates were found to carry CTX-M-15, SHV-1 and TEM-1 genes (Al-Agamy et al. 2009). In a recent study from Iran 63.8%, 51% and 23.4% of \textit{E. coli} isolates were found positive for \textit{bla}_{TEM}, \textit{bla}_{SHV} and \textit{bla}_{CTX-M}, respectively (Kalanter & Mansouri 2010) and from Turkey 73.43%, 21.87% and 17.18% of respective isolates of \textit{E. coli}, \textit{Acinetobacter} and \textit{Klebsiella} were found positive for \textit{bla}_{CTX-M}, \textit{bla}_{SHV} and \textit{bla}_{TEM}, respectively (Bali et al. 2010).

However, similar studies on concurrent occurrence of multiple \textit{bla} genes are fragmentary from India. In India, the prevalence rate of ESBLs varies in different institutions from 28-84% (Das et al. 2001). One institute from Chandigarh reported 12.6% prevalence rate for ESBL production in \textit{Enterobacteriaceae} (Datta et al. 2004). A study by Grover et al. 2006, reported 88.8% of the \textit{Klebsiella} isolates positive for \textit{bla}_{TEM} and/or \textit{bla}_{SHV}. Recently from south India 14% \textit{E. coli}, 15% \textit{Enterobacter} spp. and 45% \textit{Klebsiella} spp. isolates were reported to harbour \textit{bla}_{SHV} genes while 50% \textit{E. coli}, 28% \textit{Enterobacter} spp., 40% \textit{Klebsiella} spp. and 2% other Gram-negative bacilli were reported to possess \textit{bla}_{CTX-M} genes (Kingsley et al. 2008). Another study from
Eastern part of India reported co-occurrence of 85.4% $bla_{CTX-M}$ positive isolates with 32.9% $bla_{SHV}$ and 54.9% $bla_{TEM}$ either alone or in combination (Goyal et al. 2009).

In early 1990s publications on ESBLs reported $bla_{TEM}$ or $bla_{SHV}$ variants especially in *Klebsiella* but the situation has continuously been changing in the last few years and CTX-M β-lactamases are emerging as a predominant mechanism of resistance mainly among *E. coli* (Canton et al. 2008; Rossolini et al. 2008). So, in our study we also determined the other two most commonly occurring β-lactamases, $bla_{TEM}$ and $bla_{SHV}$, with $bla_{CTX-M}$ to find out the most prevalent ESBL in our collection of North-Indian *Enterobacteriaceae*. In the present study on determination of ESBLs like TEM and SHV, we found 93% (121/130) positivity for $bla_{ESBLs}$, including 92.5% (112/121), 45.4% (55/121) and 54.5% (66/121) isolates of $bla_{CTX-M}$, $bla_{TEM}$ and $bla_{SHV}$, respectively. While in a recent study by our group conducted on a collection of *Enterobacteriaceae* isolates from year 2007-2008, reported 28.8% $bla_{CTX-M}$, followed by 13.7% $bla_{SHV}$ and 10.9% $bla_{TEM}$ (Shahid et al. 2011b). The higher percentage of $bla_{CTX-M}$ is denoting that this gene is currently the most common type of ESBL then $bla_{TEM}$ and $bla_{SHV}$ in our North-Indian Tertiary Care hospital. In the present study we have noticed 9 isolates as ESBL-producers by phenotypic detection method but they were not found to harbour any of the screened $bla$ genes. Most probably, these isolates harbour any other type of $bla_{ESBL}$ that were not screened in the present study.

While on analysing the presence of various combinations of $bla$ genes ($bla_{CTX-M}$, $bla_{TEM}$ and $bla_{SHV}$), we found $bla_{CTX-M}^{+}bla_{TEM}^{+}bla_{SHV}^{+}$ as the most common combination in 28% (34/121) isolates followed by $bla_{CTX-M}^{+}bla_{SHV}^{+}$ in 21.4% (26/121), whereas only 13.2% (16/121) were having the combination of $bla_{CTX-M}$, $bla_{TEM}$ and $bla_{SHV}$. Least common combination was found in 1.6% (2/121) isolates having...
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$\text{bla}_{\text{SHV}}+\text{bla}_{\text{TEM}}$ type. But if we look for the presence of various genes alone then we found 29.7% (36/121) isolates harboured only $\text{bla}_{\text{CTX-M}}$ gene while 2.4% (3/121) and 3.3% (4/121) isolates were harbouring single gene of $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{SHV}}$, respectively, which again signifies that $\text{bla}_{\text{CTX-M}}$ is the most common gene than any other ESBLs. On analysis of these isolates for the presence of various $\text{bla}$ genes according to the wards from where they were collected, 100% (12/12) isolates from Medicine ward were found to harbour $\text{bla}_{\text{ESBLs}}$, while 96.5% (28/29), 96% (24/25), 91.2% (52/57), and 71.4% (5/7) collected from Gynaecology, Orthopaedic, Surgery and Paediatric wards, respectively, were found to harbour $\text{bla}_{\text{ESBLs}}$. All the isolates collected from medicine were $E. \ coli$ and found positive for various combinations of $\text{bla}_{\text{CTX-M}}$ and other $\text{bla}_{\text{ESBLs}}$ but none of the isolates was found to harbour single gene ($\text{bla}_{\text{TEM}}$ or $\text{bla}_{\text{SHV}}$) or the combination of both these genes.

CTX-M-type extended spectrum $\beta$-lactamases (ESBLs) and AmpC-type $\beta$-lactamases have been found as two major contributors for antibiotics resistance in recent years (Paterson & Bonomo 2005; Bonnet 2004; Philippon et al. 2002; Livermore & Woodford 2006). Reports of multiple $\beta$-lactamases in a single pathogen are increasing in Enterobacteriaceae (Hanson et al. 1999; Alvarez et al. 2004; Yan et al. 2004; Song et al. 2006; Moland et al. 2007; Shahid 2010). CMY-2 type was found to be associated with CTX-M-14 in a Parisian $E. \ coli$ isolate (Saladin et al. 2002). In Taiwan, CTX-M and SHV-type ESBLs with CMY- and DHA- type AmpC enzymes were reported as the most common $\beta$-lactamases that conferred resistance to extended-spectrum cephalosporins in clinical $K. \ pneumoniae$ isolates (Yan et al. 2004; Yan et al. 2006). The emergence of a multidrug resistant $K. \ pneumoniae$ isolate, which produces VIM-4, CTX-M-15, TEM-1, CMY-4 has been reported from
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France (Ktari et al. 2006). Co-existence of CMY-8 and CTX-M-3 was reported in a 269 kb conjugative plasmid from *K. pneumoniae* (Chen et al. 2007). 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 (Shahid et al. 2009). Recently *Enterobacteriaceae* isolates has been reported harbouring CTX-M-15 and CMY-6 in patients with bronchogenic carcinoma (Shahid et al. 2010). These studies prompted us to find out the co-existence of multiple genes responsible for production of β-lactamases especially of two major classes, class A and class C in our isolates. In this study 75% (84/112) isolates were showed co-existence of *bla*<sub>CTX-M</sub> and *bla*<sub>ampC</sub>. Simultaneously only 50% (36) isolates were showed co-existence of *bla*<sub>ampC</sub> with *bla*<sub>SHV</sub> (1) or combination of *bla*<sub>TEM</sub>+*bla*<sub>SHV</sub> (2), while none of the isolate has shown this co-existence with *bla*<sub>TEM</sub>. The higher occurrence of co-existence of *bla*<sub>CTX-M</sub> with *bla*<sub>ampC</sub> is denotes that these class A and class C type beta-lactamases may concurrently be present on the same plasmid. In various combinations of *bla*<sub>ESBLs</sub>, highest rate of co-existence of *bla*<sub>ampC</sub> i.e. 81.2% (13/16) was present with the combination of *bla*<sub>CTX-M</sub>+*bla*<sub>TEM</sub>, which was the least common combination of the *bla*<sub>ESBL</sub> genes in our *Enterobacteriaceae* isolates. While 69.4% (25/36), 76.4% (26/34), and 76.9% (20/26) co-existence of *bla*<sub>ampC</sub> was found with various combinations of *bla*<sub>CTX-M</sub>, *bla*<sub>CTX-M</sub>+*bla*<sub>TEM</sub>+*bla*<sub>SHV</sub>, and *bla*<sub>CTX-M</sub>+*bla*<sub>SHV</sub>, respectively, which shows the least co-existence with the single gene *bla*<sub>CTX-M</sub>. This revealed that if the *bla*<sub>ESBL</sub> genes possess *bla*<sub>CTX-M</sub> then the possibility for the presence of Class C type β-lactamases increases.

On comparative evaluation of occurrence of class A and class C type β-lactamases in *Enterobacteriaceae* during years 2009 and 2010, we found nearly similar occurrence of *bla*<sub>ESBLs</sub>: 92.1% and 94.4% in respective years, 2009 and 2010. On characterization
of various combinations like \( \text{bla}_{\text{CTX-M}}, \text{bla}_{\text{CTX-M+blashv}}, \text{bla}_{\text{CTX-M+blaTEM}}, \) and \( \text{bla}_{\text{CTX-M+blashv+blaTEM}} \) in 2009 we found 31.4% (22/70), 18.5% (13/70), 20% (14/70), and 25.7% (18/70) positive isolates, respectively, while in 2010 these combinations were present in, 27.4% (14/51), 25.4% (13/51), 3.9% (2/51), and 31.3% (16/51), respectively. We clearly observe here that in 2009 collection most of the isolates harboured single gene of \( \text{bla}_{\text{CTX-M}} \) while in 2010 collection the most common combination was \( \text{bla}_{\text{CTX-M+blashv+blaTEM}} \). In 2010, 3.9% (2/51) isolates were also found positive for combination of \( \text{blashv+blaTEM} \) while this combination was absent in 2009 collection. By PCR, in the isolates harboring \( \text{bla}_{\text{ESBL}}, \text{bla}_{\text{ampC}} \) was detected in 81.4% (57/70) and 58.8% (30/51) isolates from collections of year 2009 and 2010, respectively.

We also observed that in 2009, out of two isolates which harboured \( \text{blashv} \) gene only one isolate was found positive for co-existence of \( \text{bla}_{\text{ampC}} \), while in 2010 this co-existence was absent in all those isolates which harboured only single gene of \( \text{blashv} \) and \( \text{blaTEM} \) each (data submitted for publication). This reflects that the implementation of antibiotic prescription policies in our institution is moving on a right track.

Insertion sequences \( \text{IS26}, \text{ISEcp1} \) and \( \text{ISCR1} \) in association with class I integron structures, as well as phage related elements seem to have played a prominent role in acquisition of CTX-M genes (Arduino et al., 2002; Eckert et al., 2006; Oliver et al., 2005; Poirel et al., 2008). Studies analysing the presence of mobile genetic elements are also fragmentary from India (Shahid 2010; Bhattacharjee et al. 2010), therefore we looked our isolates for the presence of mobile genetic elements. Different genetic environments might be involved in the mobilization of \( \text{bla}_{\text{CTX-M}} \) and other ESBLs. On characterization of various mobile genetic elements (integrons and insertion
sequences) we found 89.2% (116/130) were harbouring these mobile elements i.e. sul-
l, CS-region, ISEcpI, IS26 and ORF513 (ISCR1). Higher percentage of mobile
genetic elements [28.4% (33/116)] was found to be associated with combination of
bla_{CTX-M}+bla_{TEM}+bla_{SHV}. 4.3% (5/116) isolates harboured mobile genetic
but were not found to harbour any bla gene, which shows that mobile genetic
elements in these isolates may be associated with any other type of resistance genes
(i.e. aminoglycoside or fluoroquinolones) which were not studied in this study. 4.6%
(6/130) isolates which harboured bla_{CTX-M} genes only, were not found associated with
any type of mobile genetic elements, so we hypothesize based on the current finding
that these bla genes were mobilized through plasmids.

Few studies have reported that classic class 1 integrons include in their 5’-conserved
segment (5’-CS) an integrase gene (intI1) and in their 3’-conserved segment (3’-CS)
the qacEΔ1+sul1 genes encoding resistance to quaternary ammonium compounds
(partially deleted) and sulphonamides, respectively (Partridge et al. 2009). However,
non-classic class 1 integrons lacking the 3’-CS region have also been reported (Liu et
al. 2009; Soufi et al. 2009; Chaunchuen et al. 2008; Sunde et al. 2008; Vinue et al.
2008; Antunes et al. 2007; Cocchi et al. 2007; Saenz et al. 2004). 76.1% (99/130)
isolates were found to harbour integrons (sul-1 and CS regions) and included higher
percentage of Klebsiella 77.2% (17/22) then E. coli 75.9% (82/108). Pitout et al.
(2005) has reported CTX-M types of genes associated with genetic structures such as
sul-1 type integrons, which might explain the multidrug resistant nature of organism
producing these enzymes. This structure is genetically linked to class-1 integrons
known to integrate antibiotic resistant gene cassettes responsible for resistance to β-
lactams, aminoglycosides, chloramphenical, sulphonamides, and to a lesser extent
rifampicin. Recently class-1 integrons were detected in 27% of *E. coli* isolates (Shaheen *et al.* 2010). The *sul*-1 gene, as part of the 3’-conserved segment (3’CS) of class 1 integrons were the most frequently detected integrons in *Enterobacteriaceae* (Carattoli 2001). In this study, *sul*-1 type class-1 integrons were found in 59.2% (77/130) *Enterobacteriaceae* isolates, while in a previous study from our laboratory, 32.5% *Citrobacter* spp. isolates harbouring *sul*-1 type class-1 integrons were reported (Shahid 2010). 61.6% (69/112) *Enterobacteriaceae* isolates which harboured *bla*<sub>CTX-M</sub> genes were associated with *sul*-1 type class-1 integrons in our collection. We noticed that the integrons were present in higher number of isolates [33.7% (26/77)] that harboured the combination of CTX-M+TEM+SHV genes. The occurrence of CS regions (specific regions for integrons) was noticed in 55.3% (72/130) whereas the *sul*-1 type class-1 integrons was observed in 59.2% (77/130). This is showing higher occurrence of classic class 1 integrons due to the presence of *sul*-1 gene in 3’CS region as previously reported by Partridge *et al.* (2009). CS regions and *sul*-1 genes were most commonly associated in those isolates which harboured the combination of *bla*<sub>CTX-M</sub>+*bla*<sub>TEM</sub>+*bla*<sub>SHV</sub>. We found that the isolates harbouring *bla*<sub>CTX-M</sub> genes were associated with nearly CS regions and *sul*-1 genes nearly in similar percentage 60.7% (68/112) and 61.6% (69/112), respectively. This is showing that in present study the *Enterobacteriaceae* isolates harbouring *bla*<sub>CTX-M</sub> genes were frequently mobilized by classic class 1 integrons. We also observed 17 isolates that harboured CS regions but not associated with *sul*-1 genes, this is showing the presence of non-classic class 1 integrons due to the absence of normal 3’CS region which contain the *sul*-1 gene, as recently reported by Vinue *et al.* (2010) that the qacED1–sul1 fragment of normal 3’CS region replaced by qacH–IS440–sul3. The *sul*-1 type class-1 integrons were found associated in the isolates that harboured either SHV/TEM alone or
combination of SHV+TEM ESBLs. But, the isolates that harboured TEM alone or SHV+TEM genes were noticed not to be associated with CS regions except one isolate that harboured SHV gene and was found positive for CS region. The occurrence of single bla\textsubscript{CTX-M} gene was higher 29.7% (36/121) than the combination of bla\textsubscript{CTX-M}+bla\textsubscript{TEM}+bla\textsubscript{SHV} [28% (34/121) of the all ESBL positive isolates], but in the present study we noticed that the association of integrons was higher (31.3%) in isolates with combination of bla\textsubscript{CTX-M}+bla\textsubscript{TEM}+bla\textsubscript{SHV} than the isolates (23.2%) which harboured only bla\textsubscript{CTX-M}. This shows here that the dissemination of more types of antibiotic resistance genes might be due to the higher occurrence of integrons as previously reported by Pitout et al. (2005). From different European hospitals commonly three sizes of inserted regions of DNA (800, 1000 and 1500bp) were reported among class 1 integrons in unrelated clinical isolates of Enterobacteriaceae (Martinez-Freijo et al. 1999). While in present study we found various bla genes associated with variable size of CS integrons such as ~1600bp, ~900bp, ~800bp, ~500bp, ~450bp, ~350bp, and ~180bp in 33.3% (24), 4.1% (3), 12.5% (9), 5.5% (4), 5.5% (4), 1.3% (1) and 1.3% (1) isolates, respectively. This shows that quite larger fraction of isolates harbours CS region of ~1600 bp. Most of our Enterobacterial isolates, which harboured CS regions of higher molecular weight, were found associated with sul-1 type class-1 integrons, while those which contain lower molecular weight amplicons of CS region (ranging from 180 to 450bp) were found devoid of sul-1 type class-1 integrons. We also found that one isolate showing two bands of CS amplicon, harboured sul-1 type class-1 integron but did not show presence of any bla\textsubscript{ESBL} (noticed in this study) or any other mobile genetic element (ISEcp1, ORF513, IS26). This class 1 integron may be responsible for mobilization of any other type of resistance genes such as aminoglycosides or fluoroquinolones. As
previously reported that \textit{qnrA}-like gene was located in \textit{sul-1} type class 1 integrons (Poirel \textit{et al.} 2005). Two other isolates harbouring \textit{sul-1+ORF513+IS26} and \textit{ORF513+ISEcp1+IS26} combination of mobile genetic elements and showing amplicons at 500bp and 900bp respectively, also found devoid of \textit{bla} gene screened here in this study. \textbf{Probably they may also be responsible for mobilization of resistance gene of other class of antibiotics.}

The association of insertion sequences with antibiotic resistance genes has been reported previously (Ford & Avison 2004; Miriagou \textit{et al.} 2005; Smet \textit{et al.} 2010). However, similar to the reports on mobile genetic elements, the reports on insertion sequences are also fragmentary from India (Karim \textit{et al.} 2001; Shahid 2010). On analyzing the insertion sequences we noticed that a total of 83.8\% (109/130) \textit{Enterobacteriaceae} isolates harboured insertion sequences such as \textit{ISEcp1}, \textit{ORF513 (ISCR1)} and \textit{IS26}; higher frequency of occurrence in \textit{E. coli} [87\% (94/108)] than \textit{Klebsiella} \textit{spp} [68.1\% (15/22)]. Similar to the results to integrons, these insertion sequences were also found highly associated in those isolates which harboured the combination of \textit{bla_{CTX-M}+bla_{TEM}+bla_{SHV}} [(27.7\%) (30/108) isolates], followed by the \textit{bla_{CTX-M}} (25.9\%). In this study we found 56.9\% (74/130), 53.07\% (69/130) and 33\% (43/130) \textit{Enterobacteriaceae} isolates harbouring \textit{ISEcp1}, \textit{ORF513 (ISCR1)} and \textit{IS26}, respectively. This shows the association of \textit{ISEcp1} is higher than \textit{ISCR1} and \textit{IS26} with \textit{bla_{ESBLs}}. Moreover, \textit{ISEcp1} elements and its remnants constitute an alternative promoter region (Karim \textit{et al.}, 2001) which leads to increased, clinically relevant expression of the \textit{bla_{CTX-M}} gene that is only weakly expressed in its natural reservoirs (Karim \textit{et al.}, 2001; Poirel \textit{et al.}, 2003). \textit{ISEcp1} is always found upstream of 5' end of \textit{bla_{CTX-M-15}} and is strongly implicated in the mobilization of this antibiotic resistance
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gene (Karim et al. 2001; Saladin et al. 2002). In the present study 94.5% (70/74) isolates harbouring ISEcp1 type insertion sequences were associated with various combinations of \textit{bla}_{\text{CTX-M}} with other ESBLs such as \textit{bla}_{\text{CTX-M}}+\textit{bla}_{\text{TEM}}, \textit{bla}_{\text{CTX-M}}+\textit{bla}_{\text{SHV}}, \textit{bla}_{\text{CTX-M}}+\textit{bla}_{\text{TEM}}+\textit{bla}_{\text{SHV}}. One isolate was also found to harbour ISEcp1 associated with \textit{bla}_{\text{SHV}}, while those isolates which harboured \textit{bla}_{\text{TEM}} and combination of \textit{bla}_{\text{TEM}}+\textit{bla}_{\text{SHV}} were not found to harbour ISEcp1. This shows probably \textit{bla}_{\text{TEM}} and combination of \textit{bla}_{\text{TEM}}+\textit{bla}_{\text{SHV}} is not mobilized by these mobile elements, while \textit{bla}_{\text{SHV}} gene is mobilized by this insertion sequence.

\textit{bla}_{\text{CTX-M}} genes belonging to the CTX-M-1, CTX-M-2 and CTX-M-9 clusters are associated with ISEcp1-like insertion sequences while ISCR1 elements are found upstream of the \textit{bla}_{\text{CTX-M-2}} and \textit{bla}_{\text{CTX-M-9}} genes (Eckert et al. 2006; Ryoo et al. 2005; Sabate et al. 2002). CTX-M-14 has been reported to be associated with ISEcp1, while an ISCR1 related CTX-M-14 gene is also reported from an \textit{E.coli} isolate (Bae et al. 2007). In the present study we found most of the isolates harbouring \textit{bla}_{\text{CTX-M}} genes associated with ISEcp1 and ORF513. Of these 69 ORF513 positive isolates, 27.5% (19/69), 15.9% (11/69), 15.9% (11/69), 28.9% (20/69), 2.8% (2/69), 1.4% (1/69), 1.4% (1/69), were associated with \textit{bla}_{\text{CTX-M}}, \textit{bla}_{\text{CTX-M}}+\textit{bla}_{\text{TEM}}, \textit{bla}_{\text{CTX-M}}+\textit{bla}_{\text{SHV}}, \textit{bla}_{\text{CTX-M}}+\textit{bla}_{\text{TEM}}-\textit{bla}_{\text{SHV}}, \textit{bla}_{\text{SHV}}, \textit{bla}_{\text{TEM}}, and \textit{bla}_{\text{TEM}}+\textit{bla}_{\text{SHV}}, respectively. This shows the higher association of ORF513 with \textit{bla}_{\text{ESBLs}} also in those isolates which harbour the \textit{bla}_{\text{TEM}} or \textit{bla}_{\text{SHV}} or combination of \textit{bla}_{\text{TEM}}+\textit{bla}_{\text{SHV}}. So we hypothesize that this is the most important insertion sequence required for mobilization of different types of \textit{bla}_{\text{ESBLs}} at least in our collection of \textit{Enterobacteriaceae}. We also found one isolate harbouring CTX-M genogroup-9 with genogroup-1 that was found associated with ISEcp1 but not with ORF513, while previous studies also showed presence of
DISCUSSION

ORF513 with bla\textsubscript{CTX-M-9} (Brigante \textit{et al.} 2005). On analysing the association of IS26 elements in these isolates with different combinations we found 27.9% (12/43) isolates which harbour bla\textsubscript{CTX-M}+bla\textsubscript{TEM}+bla\textsubscript{SHV} as the most common combination while 25.5% (11/43) isolates harbouring bla\textsubscript{CTX-M}+bla\textsubscript{SHV} as the second most common combination in which IS26 elements were found associated. Whereas only 20.9% (9/43) isolates were showing association of IS26 elements with bla\textsubscript{CTX-M} genes. Least number of isolates [9.3% (4/43)], which harbour bla\textsubscript{CTX-M}+bla\textsubscript{TEM}, were found associated with IS26 elements. In the present study we noticed that IS26 was most commonly found in those isolates that harboured bla\textsubscript{SHV} genes with other ESBLs and suggests us the mobilization of SHV type of ESBLs by IS26 elements. This was also suggested in a previous report that bla\textsubscript{SHV} originated from the chromosome of \textit{K. pneumoniae} and an IS26 element played a role in mobilization of bla\textsubscript{SHV} to plasmid (Ford & Avison 2004). Those isolate which harbour bla\textsubscript{TEM}+bla\textsubscript{SHV}, were not associated with IS26 elements and also with ISEcp1 but their association with ORF513 shows that this combination is mobilized by ISCR. In this study, variable sizes for the IS26 type insertion sequences were detected in: 6.9% (3/43), 39.5% (17/43), 4.6% (2/43), 11.6% (5/43), 2.3% (1/43), 9.3% (4/43), 2.3% (1/43), 2.3% (1/43), and 11.6% (5/43) isolates with amplicons of \textasciitilde1.8kb, \textasciitilde850bp, \textasciitilde800bp, \textasciitilde700bp, \textasciitilde650bp, \textasciitilde600bp, \textasciitilde590bp, \textasciitilde550bp, \textasciitilde350bp, and \textasciitilde180bp, respectively. This shows the presence of various molecular sizes of IS26 elements inserted with bla\textsubscript{ESBLs}, but amplicon size of \textasciitilde850bp was most frequently observed. This amplicon size has also been reported as the most common size in a previous study from our laboratory (Shahid \textit{et al.} 2010). Few isolates were also found to possess duplicate copies of IS26 elements in concordance to a previous report that insertion sequences connected to bla\textsubscript{CTX-M} as well as other genes are located between

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two IS26 elements (Literacka et al. 2009; Cullik et al. 2010). In this study some isolates also had multiple amplicons of IS26 (suggesting the presence of multiple IS26 copies) but these isolates could not be sequenced due to the presence of multiple amplicons in these isolates.

On analysing the presence of various combinations of mobile genetic elements in Enterobacteriaceae isolates we found most of the isolates harboured sul-1+CS+ORF513+ISEcp1 type combination of mobile genetic elements and the second most common combination was of sul-1+CS+ISEcp1+ORF513+IS26 type, while none of the isolate was found harbouring the combination of ISEcp1+ORF513+IS26 (Table 9). On analysing the occurrence of various combinations of mobile genetic elements with different combinations of \textit{MQRBSLU}, we found \textit{bla}\textsubscript{CTX-M}+\textit{bla}\textsubscript{TEM}+\textit{bla}\textsubscript{SHV} and \textit{bla}\textsubscript{CTX-M}+\textit{bla}\textsubscript{SHV} combinations of \textit{bla} genes associated with at least 16 different combinations of mobile genetic elements and \textit{bla}\textsubscript{CTX-M} was the second most common combination which was associated with 15 combinations of these genetic elements. The most common combination of \textit{bla} genes (\textit{bla}\textsubscript{CTX-M}+\textit{bla}\textsubscript{TEM}+\textit{bla}\textsubscript{SHV}) in this study was found associated with sul-1+CS+ISEcp1+ORF513+IS26 combination in majority of the isolates. In this study we found four isolates that harboured only \textit{bla}\textsubscript{CTX-M} genes and were associated with only IS26 elements, and thus suggests that this gene can even be mobilized only by the help of this insertion sequence. IS26 has been reported to be associated with \textit{bla}\textsubscript{CTX-M} genes including \textit{bla}\textsubscript{CTX-M-15} and more specifically inserted within ISEcp1, the insertion of IS26 differs from strain to strain (Eckert et al. 2006). It was observed in the present study that of the 76 isolates harbouring ISEcp1, 31.5% (24) were found flanking by the IS26 elements. This has already been reported previously that ISEcp1 is found disrupted by the insertion
sequences like IS26 (Munday et al 2004). A recent study also reported that the occurrence of IS26 together with ISEcp1 could play a critical role in the evolution of diverse multiresistant plasmids in clinical Enterobacteriaceae (Smet et al. 2010). In this study CTX-M genogroup-9 like gene was also found associated with ISEcp1 and IS26, while this gene was not associated with ORF513 (also known as ISCRI). It has been reported that ORF513 associated with class-1 integrons (known as a complex class-1 integrons) can mediate resistance to chloramphenicol, trimethoprim, aminoglycosides and tetracycline and may carry a range of β-lactamase genes as well as the qnrA gene (Toleman et al. 2006). The complex class-1 integrons contain the 5’CS and part of the 3’CS flanking one or more gene cassettes, this 3’ CS is a region known as the common region (CR) consisting of orf513 and a recombination crossover site followed by genes that do not resemble a gene cassette and flanking by another copy of the qacEAl/sul1 complex (Partridge & Hall 1995). More interestingly, in this study, out of 69 isolates that harboured sul-1 type class-1 integron, 73.9% (51) were found to harbour ORF513 which shows the higher occurrence of complex class-1 type integrons with blaESBLs, including blaCTX-M in our North Indian collection of Enterobacteriaceae.

On comparative analysis for the presence of various mobile genetic elements in the collections of 2009 and 2010, ISEcp1 was found as the dominant genetic element in 2009 while in 2010, CS region of integrons was the dominant element for mobilization of blaESBLs.

In this study we found that all isolates showed the presence of a single plasmid of ~23kb. On analysis of plasmids for the presence of CTX-M-genogroups in representative isolates, these plasmids were found to harbour CTX-M-genogroup-1
and genogroup-9 (205bp). This revealed that these genogroups are mobilized among the bacterial cells through plasmids.

In infections caused by the same bacterial clone (monoclonal outbreak) or by a few bacterial clones (Oligoclonal outbreaks), the pathogen usually migrate 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 (Paterson and Bonomo 2005). On genotyping by ERIC-PCR of Enterobacteriaceae isolates great diversity was noticed. A total of 51 isolates were found clustered in 18 clusters which included 15 clusters of 45 E. coli isolates and 3 clusters of 6 Klebsiella spp., while other 50 isolates were showing their own distinctive patterns. This RAPD-typing revealed diverse bacterial population and 18 dominant clones were identified. Analysis of RAPD profile of various isolates collected from different wards revealed most of the isolates clustered in different clusters were from Surgery (18), Orthopaedic (16), Gynaecology (12), while least number of isolates were from Medicine (4) and Paediatric (1). Isolates from Surgery were clustered in 11 clusters and found clonally related with Orthopaedic, Medicine, Gynaecology, and Paediatric wards. Isolates from Orthopaedic ward clustered in 9 clusters and clonally related with isolates of Surgery, Medicine and Gynaecology. Gynaecology isolates were clustered in 6 clusters and were clonally related with Orthopaedic and Surgery. Medicine isolates were clustered in 4 clusters and clonally related with isolates of Surgery and Orthopaedic wards, while the only 1 isolate from Paediatric ward was found clonally related with isolates of Surgery and Gynaecology. It can be concluded that probably the very same clone circulating in the gynaecology, surgery and orthopaedics wards as these wards are sharing the same building block in
our hospital and hence the chances of cross contamination are high. As some isolate from different wards were found matching, so it can be concluded that proper sterilization conditions are not maintained in our hospital wards/operation theatres; however, variability among isolates is still maintained.