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The production of β-lactamase was found as the single most prevalent mechanism which was responsible for resistance to β-lactams among clinical isolates of the family Enterobacteriaceae (Sanders et al. 1992) and extended spectrum β-lactamases (ESBLs) which mediate resistance to oxyimino-cephalosporins but not to cephemycins were observed worldwide in majority of species of Enterobacteriaceae (Bradford 2001). 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). CTX-M is the most common type of ESBL and has been reported in Enterobacteriaceae isolates from various countries including Poland, Canada, France, UK, Russia, Cameroon, Japan and Bulgaria (Bonnet 2004; Novais et al. 2007; Coque et al. 2008, Shahid et al. 2011), and also in India (Ensor et al. 2006).

There are reports from various countries describing the concurrent occurrence of bla_{CTX-M}, bla_{SHV} and bla_{TEM} in Enterobacteriaceae (Al-Agamy et al. 2009; Kalantar & Mansouri 2010; Bali et al. 2010) however, similar reports are fragmentary from India. There are only few recent reports describing the co-occurrence of these genes especially in Klebsiella, Enterobacter and Citrobacter spp. (Grover et al. 2006; Muzahed et al. 2009; Goyal et al. 2009; Kingsley et al. 2008; Shahid et al. 2011). Co-existence of class A and class C type β-lactamases in Enterobacteriaceae is frequently reported from many countries (Saladin et al. 2002; Song et al. 2006;
Moland et al. 2007), however fragmentary reports are provided from India regarding co-occurrence of class A and class C type β-lactamases (Shahid et al. 2009). Hence, this study was designed to investigate in greater details for the presence of bla genes of Class A type ESBLs and Class C type β-lactamases in Indian Enterobacteriaceae. Moreover due to paucity of Indian literature on mobile genetic elements (ISEcp1, IS26, ORF513, CS, Sul-1) and their association with ESBLs, especially CTX-M, this fact prompted us to investigate systematically the presence of the said mobile genetic elements and their association with ESBLs in our collection of Indian Enterobacteriaceae.

In the present study maximum numbers of isolates were obtained from pus samples (53.8%), followed by urine (31.5%), and then drain (5.3%). We obtained the major numbers of isolates from clinical samples of Surgery (57), followed by Gynaecology (29), Orthopaedic (25), Medicine (12) and Paediatric (7). Furthermore, to study the ESBL production in of our collection we subdivided this part in the phenotypic and genotypic experiments for determination of CTX-M type β-lactamase. We found most of the isolates, resistant to any of the third generation cephalosporins which included 100% resistance to cefotaxime, followed by the forth-generation cephalosporins, 78.4% resistance to cefpirome and 63% to ceftinime. Resistance to cefotaxime was the main marker of our bacterial isolates indicating the presence of bla_{CTX-M} genes. Along with rising resistance to cephalosporins, the second highest rates of resistance was noticed for fluoroquinolone (88.4% to ofloxacin and 83.8% to gatifloxacin) but the genes responsible for this resistance were not studied in this study. However, resistance rates to aminoglycosides in our collection were not comparatively so high (56.1% to gentamycin and 41.5% to amikacin) as compared to cephalosporins and
fluoroquinolones. Resistance rates to third- as well as fourth-generation cephalosporins in our collection indicate the presence of class A and class C type β-lactamases. On confirming ESBL-production by full for (DDST), (CDT) and (MTDET), higher numbers of ESBL producers were observed by CDT (89.2%) as compared to DDST (78.4%) and MTDET (80%). All isolates were found as ESBL-producers by any of the three phenotypic confirmatory tests. Genotypically by monoplex PCR, 86.1% (112) were found to harbour bla_{CTX-M} genes. Most probably, any other enzyme mimics the property of CTX-M type β-lactamases so we observed resistance to cefotaxime in higher number of isolates while bla_{CTX-M} was noticed in lesser number of isolates. On reviewing the Indian literature for the presence of different CTX-M type genogroups we found CTX-M genogroup-1 as the predominant genogroup (Ensor et al. 2006; Shahid et al. 2009). Similar to previous reports we also observed CTX-M-genogroup-1 as dominant genogroup, while in this collection we have also found five *E. coli* isolates harbouring one more genogroup in association with genogroup-1. Of which one isolate was found positive for genogroup-8 and four were found positive for genogroup-9. Of the four isolates that harboured CTX-M-genogroup-9 only one isolate provided the amolicon that corresponded with the exact amplicon size (205 bp) of genogroup-9 while other three isolates did not provided the amplicon that corresponding to the exact position for genogroup-9. On sequencing of the representative isolates, the amplicons of CTX-M-genogroup-1 were confirmed as CTX-M-15 type. The sequencing results of amplicon of genogroup-9 (that corresponded to the exact amplicon size) confirmed it to be CTX-M-9 like. Thus, we for the first time have observed the presence of CTX-M genogroup-9 in Indian Enterobacterial isolates. Moreover, it shows that the different
genogroups of CTX-M type of ESBLs are also rapidly emerging in Indian Enterobacteriaceae.

In the second part of this study all isolates were determined for co-occurrence of TEM and SHV type ESBLs by PCR. bla\text{CTX-M}, bla\text{SHV} and bla\text{TEM} alone were noticed in 29.7%, 2.4% and 3.3% of cefotaxime resistant isolates. Maximum number of isolates showed the combination of bla\text{CTX-M}+bla\text{SHV}+bla\text{TEM} (30.3%), followed by bla\text{CTX-M}+bla\text{SHV} (23.2%). The resistance pattern of the isolates for fourth generation cephalosporins showed the presence of class C type β-lactamases in the present collection. So in this part of study we also determined all bla\text{ESBL} positive isolates for co-existence of bla\text{ampC}. We noticed that 71.9% isolates showed co-existence of bla\text{ESBL}s and bla\text{ampC}. We noticed higher co-existence (81.2%) of these class C type β-lactamases in those isolates that harbours bla\text{CTX-M}+bla\text{TEM} and then in 76.9% and 76.4% isolates harbouring bla\text{CTX-M}+bla\text{SHV} and bla\text{CTX-M}+bla\text{TEM}+bla\text{SHV} genes, respectively. Least co-existence for bla\text{ampC} was noticed in isolates harbouring bla\text{CTX-M} genes only. This is showing that the probability for co-existence of class C type β-lactamases increases if any other type of ESBL gene also exists with bla\text{CTX-M} type.

On comparative evaluation of occurrence of Class A and Class C type β-lactamases in Enterobacteriaceae during 2009 and 2010, we noticed nearly similar occurrence of bla\text{ESBL}s in 92.1% and 94.4% isolates in the respective years, 2009 and 2010. Various combinations of bla\text{ESBL}s like bla\text{CTX-M}, bla\text{CTX-M}+bla\text{SHV}, bla\text{CTX-M}+bla\text{TEM}, and bla\text{CTX-M}+bla\text{SHV}+bla\text{TEM} were observed in 31.4%, 18.5%, 20%, and 25.7% isolates harbouring these genes, respectively, in 2009, while in 2010 these combinations were noticed in, 27.4%, 25.4%, 3.9%, and 31.3% isolates, respectively. We also observed that in 2010 the combination of bla\text{CTX-M}+bla\text{TEM} genes were observed in only 3.9%
isolates while in 2009, 20% isolates were found to harbour the same combination. In 2010, we have noticed 3.9% isolates harbouring combination of \( \text{bla}_{\text{SHV}} + \text{bla}_{\text{TEM}} \) while this combination was absent in 2009 collection. By PCR, in isolates which harboured \( \text{bla}_{\text{ESBLs}}, \) \( \text{bla}_{\text{ampC}} \) type \( \beta \)-lactamases were observed in 81.4% and 58.8% isolates in the collections of year 2009 and 2010, respectively. In 2009, co-existence of \( \text{bla}_{\text{ampC}} \) was noticed in 86.3%, 92.3%, 85.7%, 72.2% isolates which harboured different combinations of \( \text{bla}_{\text{CTX-M}}, \) \( \text{bla}_{\text{CTX-M}} + \text{bla}_{\text{SHV}}, \) \( \text{bla}_{\text{CTX-M}} + \text{bla}_{\text{TEM}}, \) \( \text{bla}_{\text{CTX-M}} + \text{bla}_{\text{SHV}} + \text{bla}_{\text{TEM}}, \) respectively, while in 2010 the co-existence of \( \text{bla}_{\text{ampC}} \) with these combinations was observed in 42.8%, 61.5%, 50%, and 81.2% isolates, respectively. In 2010 we observed that the isolates harbouring combination of \( \text{bla}_{\text{SHV}} + \text{bla}_{\text{TEM}} \) were found positive for co-existence of \( \text{bla}_{\text{ampC}} \). While in 2009, we also noticed that of the two isolates that harboured \( \text{bla}_{\text{SHV}} \) only, one was found positive for co-existence with \( \text{bla}_{\text{ampC}} \), while in 2010 none of the isolate harbouring \( \text{bla}_{\text{SHV}} \) or \( \text{bla}_{\text{TEM}} \) only showed this co-existence. This study reflects that the occurrence of various \( \text{bla} \) genes has reduced from 2009 to 2010. This is showing that the implementation of antibiotic prescription policies in our institution is moving on a right track.

In the third part we have studied all isolates for the presence of mobile genetic elements to understand the mobilization pattern of various \( \text{bla} \) genes among Indian \( \text{Enterobacteriaceae} \). These mobile genetic elements included integrons (\( \text{sul-1 type class 1 integrons and CS regions of integrons} \)) and insertion sequences (\( \text{ISEccp1, IS26, ISCR1} \)). We have noticed 89.2% isolates harbouring these mobile genetic elements. Of the total 112 isolates harbouring \( \text{bla}_{\text{CTX-M}} \) genes, 92.8% isolates were found associated with these mobile genetic elements. In all CTX-M positive isolates, 62.5%, 54.4%, 32.1%, 61.6%, and 60.7% isolates were found associated with \( \text{ISEccp1} \).
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ISCR1 (ORF513), IS26, sul-1 type class 1 integron and CS regions, respectively. This showed nearly equal number of CTX-M harbouring isolates was associated with ISEcp1, sul-1 and CS regions, followed by ORF513, but least number of the isolates were observed associated with IS26. 76.1% isolates showed the presence of Integrons which included sul-1 type class 1 integrons and CS variable regions of integrons. 59.2% isolates were noticed positive for the presence of sul-1 type class 1 integrons. We also observed that higher number of isolates harbouring bla<sub>CTX-M</sub>, bla<sub>TEM</sub> and bla<sub>SHV</sub>, were observed associated with sul-1 gene and suggests their mobilization by sul-1 type class 1 integrons. While least association with sul-1 type integron was noticed in isolates harbouring combination of bla<sub>CTX-M</sub>+bla<sub>TEM</sub>. 3.8% isolates were also found to possess sul-1 type class1 integrons but not associated with any bla gene noticed in this study; probably these integrons were mobilizing some other resistance genes such as aminoglycosides and flouroquinolones resistance genes. 55.3% isolates (harbouring all three types of bla genes i.e. CTX-M, TEM and SHV) were noticed positive for the presence of variable regions of CS integrons. We have also noticed isolates harbouring variable amplicon sizes of CS regions at ~1.6kb, ~900bp, ~800bp, ~500bp, ~450bp, ~350bp, and ~180bp, while some of the isolates were also harbouring multiple amplicons. 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 showed 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). We obserxed 17 isolates to harbour CS regions but not found positive for sul-1 genes, this is showing the presence of non-classic class 1 integrons as previously reported by Vinue et al. (2010), that the qacEA1–sul1 fragment of normal 3’CS region replaced by qacH–IS440–sul3.
ABSTRACT

On detection of insertion sequences like ISEcp1, ORF513 and IS26 we have noticed 83% isolates containing any of these insertion sequences to mobilize bla genes. Resistance genes of 56.9% isolates were mobilized by ISEcp1 type insertion sequences, while 53.07% and 33% isolates were mobilized by ORF513 and IS26, respectively. On analysing ISEcp1 type genetic element in the isolates harbouring various combinations of bla_{ESBLs}, maximum number (29.7%) of isolates harboured combination of bla_{CTX-M}+bla_{TEM}+bla_{SHV}. Those isolates that harbour bla_{TEM} and combination of bla_{TEM}+bla_{SHV} were not found to be associated with ISEcp1, while association of bla_{SHV} was noticed with ISEcp1. 53.07% isolates were detected for the presence of ORF513 (also known as ISCR1) associated with bla_{ESBLs}. It was found associated with all types of bla_{ESBL} v.i.z. bla_{CTX-M}, bla_{TEM}, and bla_{SHV}, and also in the isolates which harbour bla_{TEM} or bla_{SHV} alone. This is showing that ORF513 type insertion sequence can mobilize any type of bla_{ESBL}. In this study we have noticed only 33% isolates positive for IS26 type insertion sequences to mobilize associated bla genes. These isolates harboured variable size of amplicons i.e. 1.8kb, ~850bp, ~800bp, ~700bp, ~650bp, ~600bp, ~590bp, ~550bp, ~350bp, and ~180bp, some isolates also showed multiple amplicons of IS26 elements. In the present study we found that majority of the isolates showed amolicon of ~850bp. In our study, in isolates that harbour ISEcp1 like insertion sequences, 31.5% isolates were found flanking by the IS26 elements. In this study we found four isolates that harboured bla_{CTX-M} alone, and were associated with only IS26 elements. It represents that this gene can be mobilized only with the help of this mobile element. 79.3% isolates harbouring sul-1 type class 1 integrons were found associated with ORF513. This is showing higher occurrence of complex class 1 integrons in studied collection of Indian Enterobacteriaceae. On determination of various combinations of mobile
genetic elements in present collection, \( sul-1+CS+ORF513+ISEcp1 \) is the most common combination of mobile genetic elements followed by \( sul-1+CS+ISEcp1+ORF513+IS26 \). None of the studied isolates represents the \( ISEcp1+ORF513+IS26 \) combination of mobilizing elements. In this study, all isolates have shown a single plasmid of c.a.\(~23 \text{ kb}. On analysis of randomly selected plasmids for CTX-M genogroups by multiplex PCR, most of the isolates have shown occurrence of CTX-M-genogroup-1 on plasmids while the isolate found positive for genogroup-1 and genogroup-9 also showed the presence of these genogroups on plasmids. The isolate that harboured CTX-M genogroup-1 and genogroup-9 also showed the presence of \( sul-1+CS+ISEcp1+IS26 \) but was not found positive for ORF513.

On RAPD typing of our collection, we observed that out of 130 isolates, 51 isolates could be clustered into 18 clusters which included 15 clusters of \( E. \text{coli} \) and 3 clusters of \( \text{Klebsiella} \) spp. Most of the isolates that were clustered in different groups were from Surgery, and have also been found clonally related with the isolates collected from various other wards such as Orthopaedic, Medicine, Gynaecology and Paediatric. Only one isolate from Paediatric ward was found clonally related with isolates of Surgery and Gynaecology. Based on this study we reached to following conclusions:

Conclusions

- In the present study higher resistance rates were observed in our collection of Enterobacterial isolates. Among third-generation cephalosporins, 100% isolates were resistant to cefotaxime, followed by resistance to fourth-generation cephalosporins. In our collection fluoroquinolones also showed
higher resistance rates such as 88.4% to ofloxacin but comparatively lower resistance rates to aminoglycosides was noticed. Resistance rates and patterns to third- as well as fourth-generation cephalosporins denotes the presence of class A and class C type β-lactamases in this collection of North Indian Enterobacteriaceae.

- All cefotaxime resistant isolates were found as ESBL producers on the basis of any of the three detection methods such as double disk synergy test (DDST), combination disk test (CDT) and modified three-dimensional extract test (MTDET). In the present study we found CDT as the best detection method for identification of ESBL producers.

- In our Enterobacterial collection, on molecular basis we found 93% (121/130) occurrence for blaESBLs which included blaCTX-M 92.5% (112/121) as the predominant type of ESBL gene followed by blaTEM 54.5% (66/121) and blaSHV 45.4% (55/121). 62.8% isolates of this collection of Indian Enterobacteria showed co-occurrence of blaCTX-M with blaTEM or blaSHV or with both of these genes. Out of total blaESBLs harbouring isolates, 29.7% (36/121) were harbouring single gene of blaCTX-M while only 3.3% (4/121) and 2.4% (3/121) isolates harboured single gene of each blaTEM and blaSHV, respectively. This shows that in our collection higher occurrence of multiple genes has occurred due to the selection pressure developed by increasing usage of cephalosporins.

- In the present study, by multiplex PCR, we found CTX-M-genogroup-1 as the predominant genogroup, but for the first time we also observed CTX-M-
genogroup-9 in one E. coli isolate that was confirmed by sequencing. This is showing that other CTX-M types are also emerging in Indian Enterobacteria.

- Due to the presence of resistance to third- as well as fourth- generation cephalosporins in our isolates, of the ESBL positive isolates, 71.9% (87) also showed co-existence of bla\textsubscript{ampC} with bla\textsubscript{ESBLs}. Moreover, in 112 isolates that harboured bla\textsubscript{CTX-M} genes, 75% showed co-existence of bla\textsubscript{ampC}. This shows that in our collection of Indian Enterobacteriaceae class C type beta-lactamases is concurrently present with class A type beta-lactamases on the same plasmid.

- On comparative studies of class A and class C type \(\beta\)-lactamases in the Enterobacterial collections during 2009 and 2010, we found nearly similar occurrence of bla\textsubscript{ESBLs} in 92.1% and 94.4% isolates in the respective years, 2009 and 2010. On characterization of co-existence of class A and class C type \(\beta\)-lactamases, we found that co-existence of these classes decreased from 81.4% to 58.8% in the respective years, 2009 and 2010. This is showing improvement of the prescription policies of antibiotics as well as the implementation of strict infection control policies in our Institution.

- On determination of various mobile genetic elements (\textit{sul-1}, CS region, ISEcp1, IS26 and ORF513), we found 89.2% (116/130) isolates carrying these mobile genetic elements with various \textit{bla} genes and passively help in their mobilization. 92.8% isolates that harboured bla\textsubscript{CTX-M} genes were found to be associated with these mobile genetic elements. We noticed higher number of isolates harbouring combination of CTX-M+TEM+SHV that were associated with these mobile genetic elements for mobilization.
We also found 4.6% isolates which harboured only gene of bla<sub>CTX-M</sub> but not associated with any mobile genetic elements, and passively in these cases bla genes were mobilized through plasmids.

76.1% (99/130) isolates were found to harbour integrons (sul-1 and CS regions) and showed higher association of bla genes with sul-1 type class-1 integrons than CS region. This denotes higher occurrence of classic class-1 integrons in our collection as previously reported by Partridge et al. (2009) where it is being reported that presence of sul-1 gene in 3′CS region of class 1 integron is the characteristic of classic class 1 integron and absence of this normal 3′CS region denotes the presence of non-classic class 1 integron. We also found that 17 isolates that harboured CS regions but not associated with sul-1 gene which is showing the presence of non-classic class 1 integrons for mobilization of various bla<sub>ESBL</sub> in this Enterobacterial collection. We also found higher number of isolates harbouring combination of bla<sub>CTX-M</sub>+bla<sub>TEM</sub>+bla<sub>SHV</sub> associated with class 1 integrons.

We observed that all isolates that harboured SHV/TEM or combination of these genes were associated with sul-1 type integrons while none of the isolate was found associated with the CS region of integrons, only one isolate that harboured SHV gene was found associated with CS region and IS26 like insertion sequence. So we can hypothesized that in our Enterobacterial collection those isolates that harboured only SHV or TEM genes are mobilized more frequently by sul-1 type class 1 integrons.

On determination of various insertion sequences (ISEcp1, ORF513 and IS26) we found 83.8% (109/130) Enterobacteriaceae isolates that harboured
insertion sequences such as ISEcp1, ORF513 (ISCRI) and IS26. Like integrons we also found these insertion sequences in most of the isolates that harboured combination of $bla_{CTX-M} + bla_{TEM} + bla_{SHV}$. High frequency (56.9%) of isolates harbouring $bla_{ESBLs}$ was found associated with ISEcp1, while least (33%) were found with IS26.

- We also found that in the present collection the isolates that harboured TEM or TEM+SHV genes were not associated with ISEcp1, while the isolates harbouring only SHV-gene were associated with ISEcp1. This shows probably $bla_{TEM}$ and combination of $bla_{TEM} + bla_{SHV}$ is not mobilized by these mobile elements, while $bla_{SHV}$ gene is mobilized by this insertion sequence.

- In the present study we observed that IS26 is most commonly found in those isolates that harboured $bla_{SHV}$ genes (with other ESBLs) and this shows that the mobilization of SHV type of ESBLs is by IS26 elements. We also found various molecular sizes of IS26 elements inserted with $bla_{ESBLs}$, but amplicon size of ~850bp was the most frequently observed. We found four isolates that harboured only $bla_{CTX-M}$ genes associated with only IS26 elements, and thus suggests that this gene can even be mobilized only by the help of this insertion sequence.

- We also found CTX-M genogroup-9 like gene associated with ISEcp1 and IS26. while this gene was not associated with ORF513 (also known as ISCRI).

- Of the 69 isolates that harboured $sul-1$ type class-1 integrons, 73.9% (51) were found to harbour ORF513 which shows the higher occurrence of complex
class-1 type integrons with $bla_{\text{ESBLs}}$ including $bla_{\text{CTX-M}}$ in our North Indian collection of Enterobacteriaceae.

- We found all isolates to harbour ~23 kb plasmid, and on analysis for the presence of various CTX-M genogroups on plasmids, we found presence of CTX-M-genogroup-1 and -9 on the plasmid.

- By RAPD typing to determine the clonal relationship of our bacterial isolates, it can be concluded that probably the very same clone is 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 also found matching, so it can be concluded that still stringent sterilization conditions are not maintained in our hospital wards/operation theatres; however, variability among isolates is still maintained. Therefore, we suggest further work-out steps to minimize the spread of $bla$ genes in our hospital environment.
ABSTRACT

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