5. Discussion

The present study, spanning about one and half years was undertaken to investigate the occurrence of Extended spectrum β-lactamase producing organisms among hospital and community isolates from patients admitted to different wards and those attended the outpatient departments of Silchar Medical College and Hospital, Silchar, Assam. The study was also subjected to find out the spectrum of ESBL production among individual Gram negative isolates and their further molecular characterization, investigation of genetic environment and transmission dynamics.

ESBLs are a major global problem in the clinical and community settings. The prevalence of ESBLs among *Enterobacteriaceae* and non fermenting gram negative rods is variable from country to country and even among two different institutions in the same country and it is also continuously changing over the time. However the difference in the prevalence of ESBL producer among the gram negative rods from study to study may be attributed to the sensitivity of the method used for detection, the type and source of the clinical specimens (specimen type and hospital ward) from which these strains were isolated, the antibiotic administration policy (consequently the antibiotic selective pressure) in the medical institute.

In the present study, among 663 test isolates, 494 (74.50%) were suspected to be ESBL producer by screen agar dilution method and on further confirmatory testing by combined disk diffusion method 360 (54.29%) were confirmed producing ESBLs. Highest prevalence rate was found in *K. oxytoca* (86.11%), *P. vulgaris* (64.70%), *P. aeruginosa* (62.01%) while in *E. coli* prevalence rate was 54.62%. Although current study showed a lesser frequency of ESBL production compared to other studies in India, it is quite high in comparison with the scenario of Europe, America, and Africa. Among the similar studies conducted earlier in india, 87% of *E. coli* and 88% are of *K. pneumoniae* were ESBL producers (Uma Devi et al., 2011). However, in another similar study high prevalence of ESBL production (57.5%) was reported in tertiary care hospital at Jhalawar, Rajasthan (Dalela et al., 2012). In Germany, there was slight but continuous

increase in the percentage of ESBL producing *Enterobacter* spp. from 0.8% to 6.4% and in *E. coli* isolates from 0.5% to 3.8% (Grobner et al., 2009). However, in Latin America surveillance trial test showed ESBLs in 36.7% *K. pneumoniae* isolates and 20.8% in *E. coli* isolates (Rossi et al., 2008). In another study from Africa the prevalence of ESBLs in all gram negative bacteria was 29% among them the prevalence of ESBL in *K. pneumonae* was 64% and in *E. coli* it was 24 % (Mshana et al., 2009) and in Egypt, 16% ESBL producers were reported, off them 19% of *E. coli* and 14% of *K. pneumoniae* were ESBL producers (Fam et al., 2011).

In current study the rate of ESBL producing organism was 66.66% in hospital isolates and 37.81% among community isolates. In community isolates, majority of them were from urinary tract infection (63/90) and from wound infections (23/90), while the most common community isolates were *E. coli* (n=50), *K. pneumoniae* (n=8), and *P. aeruginosa* (n=39) (Figure 19 and 20). No such Indian study from this part is known to us so far to find out the frequency of ESBL production among community isolates. In the Middle East (Lebanon) proportion of ESBL producing isolates was significantly larger among indoor patients (15.4%) than in outdoor patients (4.5%) (Khanfar et al., 2009). However in Kuwait, the level of ESBLs were lower in community isolates of *K. pneumoniae* (17%) and *E. coli* (12%) than in the corresponding hospital isolates 28% and 26% respectively (Al Benwan et al., 2010). Moreover the production of ESBL was more in hospital isolates (28%) as compared to community isolates (6%) as reported in India (Sarojamma et al., 2011).

In this study the proportion of ESBL producers among hospital and community isolates were 7:3 which is quite high as compared to other studies. However in similar study the frequency of ESBL producing organisms was found to be 5.3% and ESBL infection incidence densities were found to 3.4 per 1000 patient-days in New Delhi (Vijayakanthi et al., 2013).

The current study emphasizes the high prevalence rate of ESBL could be due to lack of proper infection control management and antibiotic usage policy in this region.

Antibiotic susceptibility profiling

Antibiotic susceptibility profiling varies from center to center depending on the types of antibiotic used in a particular center. In this study, high MIC was observed for expanded spectrum cephalosporins and monobactam against ESBL producing organisms and majority of isolates showed MIC values well above the break point ranging from <4 to256µg/ml. But for cabapenems, most of the isolates showed MIC value in the susceptible range i.e. <4 µg/ml. The ESBL producers showing MIC towards cephalosporins (13%) below break point is indication of false susceptibility and is quite alarming with high potential to lead to treatment failure.

In a previous study conducted in India, MIC values of cefotaxime, ceftazidime and cefepime were very high (CTX-512µg/ml; CAZ->512µg/ml, FEP- 256µg/ml) (Aubert et al., 2004) whereas in another study MICs of ESBL producing isolates ranged from 8 to > 512 µg/ml for both ceftazidime and cefotaxime (Ramesh et al., 2008). However in comparison to this study, in USA, MICs were 4 µg/mL (susceptible) for ceftazidime and ≤0.25µg/mL for cefotaxime. All other screen positive clinical isolates had an MIC for cefotaxime of ≥0.5 µg/mL (Hadziyannis et al., 2000). In Europe, most of the ESBLs producing isolates showed MICs for aztreonam, ceftazidime and ceftriaxone below the susceptibility range (Nijssen et al., 2004) and in another study MIC values of cefotaxime and ceftazidime was quite low (CTX-MIC_{50} 4µg/ml and MIC_{90} 32µg/ml; CAZ- MIC_{50} 32µg/ml and MIC_{90} 256µg/ml) (Grover et al., 2006). However in a study from China, MICs was found >128µg/ml for cefotaxime; 8µg/ml for ceftazidime; 64µg/ml for ceftriaxone; 8µg/ml for cefepime and aztreonam (Yin et al., 2009). Higher MICs of ceftazidime for *E. coli* (MIC of 8 versus 1.5 µg/ml) and decreased MICs of other expanded cephalosporins (MIC of cefotaxime of 1 versus 32 µg/ml) in France (Djamdjian et al., 2011). In Brazilian study, ESBL-producing isolates have the MIC_{50}/MIC_{90} values (µg/mL) of aztreonam, cefepime, ceftazidime, cefotaxime and imipinem were 16/256, 6/16, 32/256, 32/256 and 0.125/0.19 respectively (Chagas et al., 2011) however in Russia, cephalosporin resistance of *P. mirabilis* K-27 showing MIC of cefotaxime was 32µg/ml and ceftazidime was 0.25µg/ml (Fursova et al., 2013).

In the present study, we have observed that imipenem (89.16%) was highly susceptible, followed by meropenem (86.55%) (Table 20). Whereas an earlier multicentric Indian surveillance study reported, 100% carbapenem susceptibility among ESBL positive strains (Manoharan et al., 2011). However in Turkey susceptibility against meropenem was found to be 100% (Kizirgil et al., 2005). In Europe, US and Thailand, imipenem and meropenem showed 100% susceptibility against ESBL producers (Goossens et al., 2005; Phongpaichit et al., 2011). Whereas in another study 98% susceptibility was reported in Czech Republic (Dolejska et al., 2012) and in Chinese study, it has been reported that imipenem was most active β-lactam with a susceptibility rate of 99.2% (Qing et al., 2014).

In the current study, resistance was noticed towards carbapenems among *Enterobactericaeae* could be due to production of AmpC β-lactamases and metallo β-lactamases while in non fermenters it could be both due to porin loss or co-production of AmpC β-lactamases and metallo β-lactamases as well.

Among non β-lactam antibiotics tigecycline showed 84.44% susceptibility, followed by amikacin with 55%, gentamicin with 42.22% and co-trimoxazole with 23.33% towards enterobacteriaceae. However lowest susceptibility was found against ciprofloxacin 18.61%. In a similar study, susceptibility against gentamicin and amikacin was 67.3%, and 88.9 % (Shahid et al., 2009) whereas in other study 89.7% susceptibility was found against amikacin in India (Manoharan et al., 2011). The susceptibility was found high in ESBL producing *E. coli* in Thailand, 65% isolates susceptible against amikacin; 39% against gentamicin and 42% against ciprofloxacin were found to be susceptible (Phongpaichit et al., 2011). In another earlier study done in Czech Republic, susceptibility was found higher against amikacin (96%) while lower in case of co-trimoxazole (14%), and gentamicin (31%) (Dolejska et al., 2012)

Thus finding of the current study suggests that carbapenem drugs remains the option to treat infections caused by ESBL producing organisms and tigecycline could also be a viable alternative to treat infection caused by ESBL producers especially for enterobacteriaceae.

Molecular characterization of ESBL genes

This study could underscore presence of diverse ESBL types in a single centre study which is the first of such kind from this part of the world. We have identified *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-148}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{OXA-2}}$, *bla*$_{\text{PER-1}}$, *bla*$_{\text{VEB-1}}$ and *bla*$_{\text{GES-5}}$ ESBLs types in clinical isolates of gram negative rods in this study area. In some isolates absence of an amplicon could be due to the presence of some other type of gene which could not be targeted by the primers used in this study. However previously, in one North Indian study from Aligarh, Uttar Pradesh, presence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{ampC}}$ was reported in clinical isolates (Shahid et al., 2009).

In the present study, CTX-M-15 (n=131) ESBL gene is found to be the most predominant type in India, the very first report of *bla*$_{\text{CTX-M}}$ producing isolates was from New Delhi (Karim *et al.* 2001), all of which were found to be CTX-M-15. In other study from India, it found that CTX-M was predominantly found in *E. coli* while in *Klebsiella* spp. and TEM, SHV, CTX-M was co-produced together (Manoharan et al., 2011).

SHV-148 (n=90) was the second commonest among all ESBL producing isolates and is the first report from India. This ESBL gene type was first reported from USA (Castanheira et al., 2013). Detection of OXA-10 gene (n=24) in *Enterobacteriacae* and in non fermenters in the present study is the first report from this part of the world.

Further, presence of OXA-2 (n=18) enzyme in enterobacteriaceae and *P. aeruginosa* is also the first report from this part of the world. Previously this gene was identified in clinical isolates of *E. coli* only (Bhattacharjee et al., 2007). Presence of PER-1 (n=45), VEB-1 (n=22), and GES-5 (n=2) in gram negative rods is also the first report from India. Presence of VEB-1 in *P. aeruginosa* mediated through integron is first report from India as well. In this study, there were several isolates (n=126) harbouring multiple β-lactamase genes which is quite alarming in the context of treatment of indoor patients which are prone to acquire nosocomial infections particularly in surgical wards.

Clonal dissemination of *CTX-M-15* and *OXA-10* ESBL gene

Emergence of ESBL producer in the hospital and community environment poses to be a serious problem globally. As ESBL production is worldwide rather than national or local problem, it is of much interest to identify the reason behind their spread in the different countries which already follow the variable antibiotic policies and therefore different antibiotic selective pressure in addition to the other factors like geographical location which make up the strong motive to examine for the presence of an epidemiologic link in different countries.

In previous few years CTX-M-15 and OXA-10 ESBLs have very rapidly disseminated and are now frequently reported from all over Europe and many parts of Asia (Canton et al., 2008; Kwan et al., 2008; Feizabadi et al., 2010; Dolejskaa et al., 2012). At present 160 $bla_{\text{CTX-M}}$ genotypes are described (http://www.lahey.org/studies accessed on 30/09/2014). Epidemiological reports demonstrate that some enzymes are more frequently observed than others, that predominant type of enzymes varies with country. Clonal dissemination of diverse CTX-M-15 types which contributed to the high prevalence was determined in Asian countries (Lee et al., 2011).

In the present study, by DGGE, it was observed that similar banding pattern in all CTX-M-15 and OXA-10 amplicons of isolates both from community and hospital environment. The banding pattern was identical with the CTX-M-15 and OXA-10 type of gene. The single clone of gene type was present in different members of *Enterobactariacae* family as well as in non fermentors such as *P. aeruginosa* and *A. baumanii*. Thus there is dissemination of CTX-M-15 and OXA-10 gene in the hospital and community as well.

Finding of the current study also has shown the ability of CTX-M-15 and OXA-10 types ESBL genes to spread among different species of gram negative rods. In the hospital settings conditions of overcrowding and poor sanitation in wards/clinics and selective pressure created by over use of multiple antibiotics in the hospital has enabled the wide spread of this particular clone of these genes. The two wards that are distant to each other

excluding inter-wards spread. Thus, the most possible clarification for these types is the beginning of these genes from the community or transfer via regular medical staff personnel. Besides these, dissemination could take place among diverse bacterial species through transferable plasmid during co infection because plasmids are the most important vectors for the spreading of ESBL encoding resistance genes in the community and hospital environment.

Despite the lack of predominant epidemic clone, occurrence of few sporadic clonal pairs, circulating in the clinical setting which excludes or greatly minimizes the role of clonal expansion in the dissemination of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-10}}$ in the hospital environment. However, transfer could have also occurred in the environment. Thus it shows the potential role of wild types isolates harbouring CTX-M-15 and OXA-10 type enzymes, once they spread in the clinical setting, as a source of similar ESBL determinants for other pathogenic bacteria. From epidemiological point of view, finding suggests that CTX-M-15 type is most predominant type followed by OXA-10 type in this geographical part of the world.

DNA fingerprinting of ESBL producing isolates

Epidemiological typing is needed to monitor infectious agents in the surroundings and to follow their transmission. Several outbreaks are frequently recognized from laboratory tests results and a patient charts time consuming procedure. In well known outbreaks, molecular typing such as PFGE is typically performed to track the outbreak. However to improve the speed of typing, DNA sequence based approaches, such as multilocus sequence typing used but these methods are still too expensive for routine use in this hospital environment and have lower discriminatory power compared with PFGE. However, ERIC, REP and PFGE is found to be the less time consuming, less expensive and the easiest to be used routinely in the clinical settings.

In the present study, there were diverse ERIC and REP types among *E. coli, K. pneumoniae, K. oxytoca, Proteus mirabilis, Proteus vulgaris, P. aeruginosa, Pseudomonas* Spp., and *A. baumanii* species isolates as well as diverse clonal types were

also observed. This data is in agreement with previous Turkish, Italian and Swedish study (Sumer et al., 2014; Giuffre et al., 2013).

In the current study, ESBLs are recognized as an important cause of expanded spectrum cephalosporins resistance in gram negative bacteria. In this study, the typing of isolates has brought important insight in to the spread of ESBL genes in this geographical location. The high degree of diversity of ERIC, REP and PFGE types of isolates of all species suggests that the clonal dissemination of producer strains did not play the predominant role in the overall situation. Thus it indicates either horizontal transfer of the β-lactamase genes and/or their mobilization by mobile gene elements like transposons, integron and insertion sequence.

The current study suggests that molecular typing of ESBLs producing organisms is very useful for surveillance purposes and to monitor outbreaks as well as to track nosocomial spread of the gram negative rods in hospital environment.

Genetic environment of bla_{ESBLs}

Integrons act as a tool for site specific recombination which is capable of integrating and expressing genes enclosed in structures called mobile gene cassettes. Integrons were first observed from pathogenic bacteria, to be a major pool of antibiotic resistance genes (Boucher et al., 2007). Integrons were revealed in clinical environments, where they have been integrated in the small arrays of resistance gene cassettes and these resistance gene cassettes frequently encode the genes for antibiotic resistance determinants (Hall et al. 1999). Integron are mainly divided in two major groups: resistant integrons (RI) and super integrons (SI). These integrons are divided in to three classes, the commonest one is class 1 followed by class 2 while class 3 is rare. Multidrug resistance integron (MDRI) mostly carry class 1 integrons and large array of different antibiotic resistance gene cassettes (Partridge, 2011). These arrays are diverse and could encode resistance to β-lactams, aminoglycosides, sulfonamides, and trimethoprim (Partridge et al. 2005).

In the present study; a high occurrence of integrons among ESBL producing isolates (70.83%) was observed. Of these majority of isolates (60.05%) were harbouring class 1 integron while class 2 integron was found in 4.72% isolates. On performing the genetic linkage study of different plasmid mediated ESBL genes with integrons, variable sizes of PCR products were observed with *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{OXA-2}}$, *bla*$_{\text{PER-1}}$, *bla*$_{\text{VEB-1}}$ *and* *bla*$_{\text{GES-5}}$ gene which proves their diverse sources of acquisition within the same hospital. Majority of the isolates showed multiple bands and similar banding pattern of amplicons which indicates presence of additional integrons or multiple copies of ESBL genes within the gene cassette. In some of the ESBL producing isolates there was no amplification which suggests that ESBL gene might be located far away from the conserved regions of the integrons or the amplified products formed is too long to be visualized on the agarsoe gel. In current study, prevalence of class 1 Integrons was 52% in *E. coli* and 48% in *K. pneumoniae*. In a recent report from China, class 1 integrons was found in 51.1% *K. pneumoniae* isolates (Li et al., 2013) and in Iran, 44% ESBL positive *K. pneumoniae* found to be harbored class 1 integron, 6% were carried class 2 integrons (Qamsari et al., 2013).

In current study, presence of class 2 integron was observed in one *K. pneumoniae* isolates which is very rare whereas presence of class 2 integron in *E. coli* isolates was 5.6%. However in Iran it was found 6% in ESBL positive isolates (Qamsari et al., 2013). In the present study 5.55% isolates carried both class 1 and class 2 integron. However in Northwest Iran 10.7% multi drug resistant *K. pneumoniae* isolates was found to be carrying both class 1 and class 2 integrons (Rezaee et al., 2012). Findings of current study indicate that clinical strains of gram negative rods with multiple ESBL genotypes were captured and expressed within integron are broadly disseminated among diverse range of hosts. However, in the present study role of class 2 integron in cephalosporin resistance was unclear.

This study could demonstrate that integrons act as proficient platform for site specific integration of ESBL genes and also play an important role in the dissemination of antimicrobial resistance through horizontal gene transmission as well as its contribution

to the occurence of transferable expanded spectrum cephalosporin resistance. Besides this, integrons are of extreme diverse array associated with a variety of drug resistant phenotypes. Continuous monitoring of gene cassettes in integrons is warranted to improve the understanding and control of drug resistance in this hospital settings.

Genetic mapping of integron gene cassettes carrying ESBL genes

Gene cassettes associated with recombination site contains diverse sequence families, which all share common structure. The original name given to these sites was 59 base element (59 be). Gene cassettes which are captured by the integron; an *IntI* mediated site specific recombination between 59 be and *attI* sites (Leon and Roy 2003).

In the current study OXA-10 was located within class I integron with five different types of arrangement (Figure 44). In these gene cassette arrangements OXA-10 gene was arranged in following manner: *dfrA12-OXA-10-aadA2-qacE-sul1* (Type 1); *arr2-aacA4-dfrA1-OXA-10-adA1-qacE-sul1* (Type 2)*; dfrA1-OXA-10-aadA1-qacE-sul1* (Type 3)*; dfrA7-aadA5-OXA-10-aac(6')-1b-qacE-sul1* (Type 4)*;* and *dfrA17-aadA5-OXA-10-qacE-sul1* (Type 5). Whereas in previous study OXA-10 was found to be present followed by *aac(6')-Ib* gene encoding an aminoglycoside acetyl transferase, which is capable of modification of aminoglycosides and quinolone antibiotics (Porto et al 2010).

In the present study, $bla_{\text{VEB-1}}$ was located within variable regions of class 1 integron with four types of diverse arrangements (Figure 45). The gene cassette arrangement of VEB-1 was: *dfrA1-orfC-VEB-1-qacE-sul1* (Arrangement 1)*; dfrA12-orfF-VEB-1-aad2-qacE-sul1* (Arrangement 2); *VEB-1-aac1b-qacE-sul1* (Arrangement 3); and *dfrA17-VEB-1aadA5-qacE-sul1* (Arrangement 4). However in earlier studies in France, it was observed that VEB-1 is commonly inserted into the variable region of different class 1 integrons in which it is always associated with a downstream located *aadB* gene cassette encoding an aminoglycoside adenylyl transferase (Aubert et al., 2012).

In current study integron mediated *CTX-M-15* in *P. aeruginosa* (Figure 46); carried the following resistance genes in their gene cassettes: *dfrA17-aadA5-CTX-M-15-qacE-sul1* which is also the first report from this part of the world.

It has been also observed that *P. aeruginosa* isolates carrying bla_{GES-5} was found to be located within variable regions of class 1 integron within two different types of genetic arrangement (Figure 47): *attI1-GES-5-qacE-sul1* and *attI1-GES-5-59be-aacA4-qacE-sul1* and is first report from India. However in previously class 1 integron with bla_{GES-5}-*aacA4* gene cassettes was found and bla_{GES-5} was present in isolates has the unusual truncated *attC* whereas in downstream regions of *GES-5* flanked by *qacE* and *sul1* gene (Fonseca et al., 2007).

Present study demonstrated that existence of diverse types of genetic arrangements in ESBL harbouring isolates; advocates that multiple resistance gene cassettes were linked with significant genetic vehicle in ESBL producing clinical isolates and also acts as an integron mobilizing unit. This work also contributes to a better perceptive of acquired resistance of gene cassette evolution as well as dynamics of mobilization of these structures into integrons.

Association of ESBL genes with mobile genetic elements

Production of ESBLs is the most significant, because resistance determinant among pathogenic *Enterobacteriaceae* as well as non fermenting gram negative rods has become a major public health problem in India (Bradford 2001). Infection with ESBL producing organisms result in poor clinical outcomes, overdue initiation of suitable antibacterial therapy, longer hospital stays and greater hospital operating cost.

Insertion sequences (IS) are most abundant transposable elements which are capable of self determining transposition in microbial genomes. These elements encode an enzyme named transposase and are responsible for mobilization with detection of inverted repeat sequences and integration process. They cause genome rearrangements and enhance the spread of resistance and virulence determinants within species (Mahillon et al., 1999).

Most of these *ISs* have been involved in the mobilization of antibiotic resistance genes and latter mobilization mechanism has been observed with *Tn21* and *ISEcp1* (Poirel et al., 2005). In the present study most *CTX-M-15* genes were associated with *tnpISEcp1; tnpIS26;* and *IS26.* Investigation revealed that $bla_{\text{CTX-M-15}}$ gene has heterogeneous contexts. In most of the isolates CTX-M-15 genes were associated with *ISEcp1* and *IS26* as well as *tnpISEcp1* and *tnpIS26* which were able to mobilize adjacent DNA segments. It was also observed that *ISEcp1* and *IS26* being apparently the most relevant player in the capture and mobilization of $bla_{\text{CTX-M-15}}$. Primarily in France, it was found the presence of *ISEcp1* in the upstream of plasmid mediated CTX-M-15 and *IS26* was too in upstream of the *CTX-M-15* gene and disrupting *ISEcp1* (Eckert et al., 2006). While in another report from the same country, *ISEcp1B* has been reported to be associated with and to mobilize the emerging ESBL genes in *Enterobacteriaceae* (Lartigue et al., 2006). In current study it was also found that *IS26* underlining the most important role in the mobilization and expression of SHV-148 gene and possibly *IS26* is a characteristic feature in acquisition of $bla_{\text{SHV-148}}$ genes.

However CTX-M β-lactamases are considered a paradigm in the evolution of a resistance mechanism. This has been mainly produced by the involvement of genetic mobilization units such as *ISEcp1* and later integration in hierarchical structures associated with complex genetic structures as well as complex class1 integrons and transposons. The capture of these $bla_{\text{CTX-M}}$ genes from the environment by highly mobilizable structures could have been a random event (Canton et al., 2012). In recent report chromosome encoded intrinsic cefotaximases in *Kluyvera* spp. are proposed to be the progenitors of CTX-Ms, while *ISEcp1*, *ISCR1* and plasmid are closely associated with their mobilization and dissemination (Zhao et al., 2013).

In current study *IS26* has been found to insert within *ISEcp1* although the insertion of *IS26* differs from strain to strain. However the function of *IS26* insertion into *tnpA* of *ISEcp1* is to inhibit further *ISEcp1* mediated $bla_{\text{CTX-M-15}}$ mobilization, effectively trapping $bla_{\text{CTX-M-15}}$ onto plasmid and ensuring that it is maintained in the gene pool.

Present study established the association of *tnpISEcp1; tnpIS26;* and *IS26* with CTX-M-15 gene and implies the role of these insertion sequences in plasmid integration, either through transposition or homologous recombination. And the presence of $bla_{\text{CTX-M-15}}$ in association with mobile elements points to an evolutionary process which likely promotes the maintenance of this ESBL gene in hospital environment. Present study confirmed that the predominant role of *ISEcp1* and *IS26* in the mobilization of $bla_{\text{CTX-M-15}}$ in this geographical location. Presence of insertion sequence *IS26* upstream of *SHV-148* advocates its significant role in the mobilization of $bla_{\text{SHV-148}}$ and also emphasizes emergence of this drug resistant determinant from this part of the world, which may be tracked in order to implement proper infection control policy.

Genetic arrangements of $bla_{\text{CTX-M-15}}$ genes with mobile genetic elements

In the current study, the neighboring DNA of $bla_{\text{CTX-M-15}}$ genes has been determined. $bla_{\text{CTX-M-15}}$ was found in ten diverse genetic array in *Escherichia coli* isolates (Figure 48) which is the first single centre study from India. In array type 1- *tnpA-IS26-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 2- *tnpA-IS26-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 3- *tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 4- *tnpA-IS26-tnpA-tnpR-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 5- *tnpA-IS26-tnpA-ISEcp1-CTX-M-15-ORF477*; in type 6- *tnpA-IS26-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 7- *tnpA-IS26-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 8- *IS26-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477*; in type 9- *ISEcp1-tnpA-CTX-M-15-ORF477*; and in type 10- *IS26-tnpA-ISEcp1-tnpA-CTX-M-15-ORF477* was present. Interestingly, findings are very different from earlier studies which were also on the record (Rodriguez et al., 2014; Seiffert et al., 2013; Lartigue et al., 2004).

In France similar genetic arrangements like; *ISEcp1-CTX-M-15-ORF477; tnpIS26-CTX-M-15-ORF477;* and *TEM-1-tnpR-tnpA-tnpISEcp1-CTX-M-15-ORF477* were also reported (Eckert et al., 2006). *ISEcp1* as mobilizing unit of *CTX-M* in *E. coli* is documented earlier and was well known in upstream of $bla_{\text{CTX-M}}$ genes in which 86.9% of the isolates (93/107) recovered from health and sick pets in China (Sun et al., 2010). However in United Kingdom genetic arrangements of *ISEcp1-CTX-M-15-ORF477*; *IS26-*

ISEcp1-CTX-M-15-ORF477; and *tnpIS26-CTX-M-15-ORF477* was found (Dhanji et al., 2011). In Japan, *ISEcp1-bla*$_{CTXM}$*-orf477* like gene cassette arrangements was found where it was found that *orf477* encodes a protein of 158 amino acids with unknown function and the *orf477* and *orf477*-like elements were found downstream of plasmid harbored *bla*$_{\text{CTX-M-3}}$ like genes *bla*$_{\text{CTX-M-1, -3, -15, -22, -32, -53, -55, -66}}$ (Zhao et al., 2013).

So, current study established that multiple genetic elements, especially IS*Ecp1* and *IS26*, are involved in the mobilization of *bla*$_{\text{CTX-M}}$ genes from the chromosomes to plasmids. Of the genetic platforms linked with *CTX-M-15, ISEcp1* is one of the most important elements. *ISEcp1* can mobilize in the downstream located with *bla*$_{\text{CTX-M-15}}$ gene and may be provide a promoter for its expression.

Present study established that mobile genetic elements and integrons are the efficient tools for mobilization and expression of β-lactamase genes. Regular monitoring of these mobile genetic element associated resistant determinant may help in executing the right antibiotics policy to reduce the irrational utilization of expanded spectrum cephalosporins and so as to decrease the treatment failure and antibiotic pressure in the clinical setting.

Transferability of *bla*$_{\textbf{ESBLs}}$

Plasmids emerge to augment bacterial genetic diversity and acquiring genes as well as horizontal exchange among bacterial populations by conjugation or by mobilization (Francia et al., 2004). The organization of plasmids was basically built on the genetic analysis of plasmid and approaches for broad host range replication in gram negative bacteria (Helinski 2004.). They contain genes essential for initiation and control of replication and accessory genes that may be useful to their bacterial host such as antimicrobial resistance or virulence genes (Carattoli et al., 2005). Bacterial resistance may develop horizontally when bacteria acquire the drug resistance genes from some other drug resistant pathogenic bacterial strains by transformation or by other modes of genetic exchange (Tenover 2006).

In the present study, carriage of *CTX-M-15*, *SHV-148*, *OXA-10*, *PER-1*, *OXA-2*, and *VEB-1* in transferrable plasmid was observed in 181 transformants. It was also observed that, $bla_{\text{OXA-10}}$ was horizontally transferable in *Enterobacteriacae* family. However unsuccessful transfer of $bla_{\text{OXA-10}}$ in *P. aeruginosa* could be due to their plasmid not being replicated within *E. coli* recipient. Unlike our finding, previous records showed that $bla_{\text{PER-1}}$ was chromosomally located in *P. aeruginosa* (Ranellou et al., 2012). In agreement with our finding *VEB-1* has been previously reported in *Providencia stuartii* (Mahrouki et al., 2014). Transferability of these resistance determinants can result in nosocomial dissemination of resistant strains, which is serious challenge towards treatment of infection. Therefore current study emphasizes that, it is significant that the reasonable choice of antimicrobial agents, powerful monitoring of bacterial resistance and further study possibly can postpone the emergence of resistance, and controls the dissemination of resistant strains. It was also noticed in the current study, that multiple sizes of plasmids which were transferable and harbouring different ESBL genes. Among them plasmids of ~15Kb, ~25Kb, ~35Kb, and ~40Kb was common in all the transformants. Previously in North India, in *E. coli* and *K. pneumoniae* isolates, a conjugative plasmid of 16.9Kb was found (Shahid et al., 2004). However, not much report is available in this country. Thus it could be observed that multiple co-existing plasmids were harbouring particular types of ESBL which reflected diverse pathway of horizontal mobility and their acquisition in a host in this hospital environment.

So, the present study established that plasmids are responsible for the transfer of the bla_{ESBL} genes to new hosts and host range are closely associated with the high prevalence and widespread of the ESBL genes in gram negative rods. If resistance plasmids spread easily and can be maintained in resident bacterial flora in the absence of selective pressure and there is little hope that antibiotic control strategies alone will eliminate, or even reduce the prevalence of plasmids carrying ESBLs.

Plasmids also governing role in the horizontal transfer of genetic information between bacteria and can transfer DNA between genera, phyla and even major domains by a mechanism that is known as bacterial conjugation. Conjugation involves direct cell to cell

contact, mating pair formation and DNA exchange mediated by conjugative pili. The genes responsible for the transfer of most proficient plasmids have been isolated from natural environments which have not been identified (Thomas 2000; Van Elsas and Bailey 2002). In the present study 160 isolates were found conjugatively transferable and ESBL genes and integron were encoded in these conjugative plasmids. However in previous studies acquired CTX-M genes were found in clinical isolates of *Enterobacteriacae* are generally carried by conjugative plasmids (Zhao and Hu 2013; Carattoli et al., 2011).

Hence this study advocates that conjugative plasmids are key players in the team of mobile genetic elements that fuel bacterial adaptability and diversity. Conjugative plasmids are responsible for the horizontal transfer of the bla_{ESBL} genes to new hosts and play a central role in the population dynamics of bacterial plasmids and maintenance within the host.

Study of Co-resistance among transformants and transconjugants

Antimicrobial resistance has been a barrier to successful infectious disease therapy for as long as antibiotics have been used. Single drug resistance phenotypes were not entirely surprising. By contrast, multidrug resistance was not predictable, because the co appearance of multiple mutations conferring resistance was considered to be ahead of the evolutionary potential of a given bacterial population (Cambray et al., 2010).

There are numerous reports which have emphasized the need for less and better use of antibiotics, improved infection control and the development of new agents. However, decreases in antibacterial use do not always lead to reduced resistance, possibly because bacteria are now well adapted to the carriage of resistance. It is probably inexperienced to anticipate reaching a grand “control” over resistance and attempts should center on management rather than elimination, with the objective of slowing the development of new resistance while continuing to develop new agents at a sufficient rate to keep ahead of the bacteria (Andrea et al., 2013; Seiffert et al., 2013). In current study, 38.68-39.14% transformants and transconjugants were sensitive against gentamicin; 37.27-44.2%

against ciprofloxacin and 32.3-38.68% against amikacin. MIC_{50} and MIC_{90} of all transformants and transconjugants was above the break point level against all tested cephalosporins i.e. 16-256<µg/ml while in case of carbapenem drug MIC_{50} and MIC_{90} was below the MIC range i.e. 2-8µg/ml. The present study could depict the transferability of other co-existing resistance mechanism against different groups of non β-lactam antibiotics, when the transformants are screened on media containing cephalosporin. This showed that the resistance (R) plasmid carried multiple antibiotic resistant determinants which would have the potential to expand as pan-resistant phenotypic traits of bacteria in this hospital setting.

As resistant plasmids are the major source of ESBLs in this study and it is also noted that the ESBL producing isolates of this medical centre constituted multiple phenotypes, thus it can be concluded that transferable elements conferring antimicrobials other than β-lactams travel on or alongside the ESBL containing plasmid, probably in the same gene cassette, yielding multidrug resistant bacteria. Current study suggests that there is urgent need to slow down the persistent of expanded spectrum cephalosporinase resistant determinants in hospital environment so as to minimize the causes of treatment failure in clinical settings.

Plasmid Incompatibility typing of transformants

Incompatibility (Inc) refers to the ability of the two plasmids which is coexist stably within the same cell and is controlled by *ori* regions. If two plasmids could be coexisting stably in cell, they belong to different Inc groups. Plasmids among the same replication control are designated as "incompatible" however the plasmids by diverse replication controls are known as "compatible" (Carattoli et al., 2009). The basis of two plasmids which belong to the same Inc group may not be disseminated in the same cell line. However Inc group detection has been commonly used to categorize plasmids in homogenous groups on the basis of their phylogenetic similarity and may be helpful to investigate their transmission in nature as well as linkage with host cell to determine the evolutionary origins of plasmids (Carattoli A. 2011). In the present study, it was

observed that different incompatibility (Inc) groups in all 181 transformants carrying single as well as multiple Inc group types (Table 52 to Table 60).

In the current study *bla*$_{\text{VEB-1}}$ was found within P Inc group in *P. aeruginosa* which is the first report from India (Maurya et al., 2014) but in *Enterobacteriace, bla*$_{\text{VEB-1}}$ was spread via diverse Inc type of plasmids such as FIA, FIB, P, FIC and FrepB Inc type. Previously, *VEB-1* was reported in IncA/C type in France, Canada, Thailand and Turkey (Poirel et al., 2007).

In this study, OXA-10 β-lactamase was found within P Inc type of plasmids in *Enterobactaeriacae* which is also first report from this part of the world. In *CTX-M-15* harbouring transformants, diverse Inc groups were also observed. Inc group I1, FIA, FIB, Y, P, FIC, FIIs, FrepB, K and B/o was found in *E. coli, K. pneumonae, K. oxytoca, P. mirabilis, P. vulgaris, P. aeruginosa, Pseudomonas* Spp., and in *A. baumanii* strains. However W and N type of Inc group was found only in *E. coli* isolates harbouring *bla*$_{\text{CTX-M-15}}$. Similar to *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-2}}$ was also within diverse Inc groups viz: FIA, FIB, FIC, FrepB, K and B/o. However Inc I1 and Y were only found in *E. coli* harbouring *bla*$_{\text{OXA-2.}}$ *bla*$_{\text{PER-1}}$, *bla*$_{\text{SHV-148}}$ also came out with same observation. Most common Inc type in the current study was Inc F, which was carrying resistant determinants and it seems that the high occurrence of the multiple Inc plasmid types strongly liked to constructive selection used by high antimicrobial practice which incrementing their frequency as compared to bacterial populations which are not preselected for antimicrobial resistance. Thus, this study could establish that a particular ESBL gene was located in different Inc type which could predict multiple source and origin of their acquisition and propagation. This finding is of epidemiological interest and helpful in formulation of hospital infection control policy.

Present study also established that possible use of the PCR based replicon typing has been a significant tool in tracing the diffusion of plasmids which are conferring antimicrobial resistance and it may also track the evolution and spread of emerging plasmids. This method could be applied in routine laboratory in the monitoring of transmission of plasmids within strains from diverse settings. The study data suggests

that acquisition of multiple resistance genes in plasmids may perhaps present a new tool in evolution of bacteria and employing adaptive strategies for exploring as well as colonizing novel hosts and environments in this centre.

Plasmid stability analysis

Treatment of infectious diseases caused by resistant bacteria is the great warning faced by clinicians in the 21st century. The successful expansions of resistance plasmids are often recognized by selective pressure which ensuing the extensive use of antibiotics in clinical practice (Davies and Davies. 2010). Lack of antibiotic pressure and inactivation of the antibiotic resistance gene also had no effect on plasmid persistence, conjugation rate as well as bacterial host biology (Cottell et al., 2012). In the present study it was observed that *bla*$_{\text{CTX-M-15,}}$ *bla*$_{\text{OXA-10,}}$ *bla*$_{\text{VEB-1,}}$ and *bla*$_{\text{GES-5}}$ was found highly stable after 115 serial passages however *bla*$_{\text{PER-1}}$ stable after 110, *bla*$_{\text{SHV-148}}$ stable after 91 and *bla*$_{\text{OXA-2}}$ stable after 89 serial passages (Table 61). Previously fitness cost of CTX-M-15 type extended-spectrum β-lactamase was also described in Hungry (Toth et al., 2014).

Acquisition of multiple antibiotic resistances should impose a cost on bacteria and incidence as well as rates of rise and dissemination of antibiotic resistance in bacterial populations are mainly projected to be directly related to the volume of antibiotic use and it may inversely related to the cost that resistance imposes on the fitness of bacteria. In current study, the isolates harbouring multiple ESBL genes *OXA-10+VEB-1+SHV-148*; *OXA-10+OXA-2+SHV-148+CTX-M-15+PER-1; OXA-2+PER-1; OXA-10+OXA-2+SHV-148*; *OXA-10+CTX-M-15+VEB-1*; *CTX-M-15+SHV-148+VEB-1*; and *GES-5+SHV-148+CTX-M-15* was found stable after 115 serial passages (Table 62). However previously, expanded spectrum cephalosporins resistance *E. coli* was found in community, outpatient clinics and hospital settings in Switzerland (Seiffert et al., 2013) and fitness cost of tigecycline resistance in *E. coli* were also described in Sweden (Linkevicius et al., 2013).

The present study observed plasmids have developed to enforce little impact on host strains and resolution of antibiotic resistance genes may be expected in the absence of antibiotic selective pressure in spite of antibiotic stewardship. The study data advises that

potential treatment managements and antibiotic stewardship policies should be judge the persistent nature of antibiotic resistance genes.

PCR mapping of integron carrying multiple ESBL genes

Antimicrobial resistance poses to be a serious problem in clinical medicine and worldwide which may results in increased morbidity, mortality as well as cost in treating the infections which they cause. Infection with resistant organisms is a challenging public health issue with rising difficulty in many bacterial pathogens mainly concern for hospital acquired nosocomial infections. Situation becomes more complicated when the resistant determinants are linked with mobile element and present within integron gene cassette that facilitate their horizontal transfer. However, it is unknown why an organism harbors multiple ESBL genes when their hydrolysis spectrum is same and how they are arranged within the host genome.

In the present study, by PCR mapping of integron in *E. coli* and *P. aeruginosa* isolates found that multiple ESBL genes were arranged within class 1 integron in six diverse types of arrangements (Figure 53) and is first report from this part of the world. In *P. aeruginosa* and *E. coli* isolates; four and two different arrangements were observed. In *P. aeruginosa* arrangements were: *OXA-10-OXA-2-SHV-148* (Arrangement 1)*; CTX-M-15-OXA-10-VEB-1* (Arrangement 3)*; CTX-M-15-SHV-148-VEB-1* (Arrangement 4); and *GES-5-SHV-148-CTX-M-15* (Arrangement 5) while in *E. coli*: *OXA-10-VEB-1-SHV-148* (Arrangement 2) and *PER-1-OXA-2-SHV-148-CTX-M-15-OXA-10* (Arrangement 6). Among in *P. aeruginosa*, two isolates were recovered from surgery ward, one from medicine and another one from pediatrics ward of the hospital however among in *E. coli* isolates one isolate were recovered from surgery ward and other was from female burn unit. In the integron of *E. coli* isolates SHV-148 was found in reverse orientation (Figure 53: Arrangement 2) whereas in other arrangement all the genes were found in reverse orientation (Figure 53: Arrangement 6).

The current study make clear that multiple combination of ESBLs in gram negative bacilli in hospital settings survive against high antibiotic pressure and they can capture

and transferred their resistant genes through integron carrying antibiotics resistant gene cassettes. So, this study concludes that PCR mapping of integrons will be useful tool for study of the evolution of cephalosporin resistant plasmids and dissemination of antibiotic resistance genes as well as enable to formulate infection control programme in the hospital environment.

Transcription level of multiple ESBL genes:

Bacteria produce ESBL enzymes which enzymatically hydrolyze β-lactam antibiotics. If this is the function of ESBL genes, then what could be the possible role of multiple ESBL enzymes in a single organism, whether they are specific towards a particular antibiotics or expression varies under different inducing conditions? This study could well establish that possession of multiple ESBL genes definitely have protective role against specific antibiotic pressure. In contrast to previous claims, where it was mentioned that CTX-M type of β-lactamases has more substrate activity towards cefotaxime (Bradford 2001). In this study experiment showed transcription level was maximum when ceftriaxone stress was provided to the organism. Thus, this study is unique of its own as no such study has previously compared expression level of multiple co-existing ESBL genes under diverse inducing condition with oxyimino cephalosporins.