CHAPTER 5

DISCUSSION

5. DISCUSSION:

The use of antibiotic is rightly considered to be the most significant health-related events. Since the time of their discovery antibiotics have been employed for a wide variety of purposes. However in recent decades therapeutic use of the agents are severely compromised due to emergence of multidrug resistant organism. The potential developments of resistance to the compounds were observed from the time when it was first employed. A wide range of physiochemical factors are found responsible behind this (Davies et al., 2010). The antibiotic resistance development has been rapidly observed over the past half century among Gram-negative bacilli. Several approaches to antibiotic prescribing have been employed like, introducing of newer, more potent antibiotics as well as combination therapy in clinical settings (Kapil, 2005). Gradually the inadequate use has emerged the resistance to an older agent and multi drug resistance (MDR) among gram negative bacilli became a growing threat for which no adequate therapeutic options would exist (Kapil, 2005).

Data suggest that the antibiotic use is relatively uncontrolled in these developing counties. The availability of antibiotics was very frequent and easily available in open market without a proper doctor's prescription (Kapil, 2005). The dissemination of antibiotic resistant pathogens in this region have been reported by different studies (D'Costa et al., 2006; Martinez, 2008; Thibault et al., 2012) and the genetic aspects of different phenomena associated with resistance development such as, gathering, heterogonous expression, horizontal gene transfer (HGT) and mutation (Davies et al., 2010) are also on record (Martinez, 2008). The contribution of HGT in rapid proliferation of antibiotic

resistance genes across the wide variety of bacteria are also reported previously (Aminov, 2011) and diversity of mobile genetic elements were described (Wozniak and Waldor, 2010; Bertels and Rainey, 2011) and showed the presence of other mechanism beyond horinzontal gene transfer. In the current study we tried investigate what are the possible mechanisms behind the rapid spread and acquisition of functional module which positively responsible for present scenario multidrug resistance. The origin of resistance genes could be due a natural process whereby the resistant genes are maintained in nature. The use of antibiotics should have manipulated the situation for microbial populations within clinical settings but genetic flexibility that allowed bacteria to survive and multiply under the antibiotic stress.

The mobile genetic element; integrons are present in approximately 10% of the bacterial genomes sequenced, and contribute to genomic plasticity and environmental adaptation of bacteria (Boucher et al., 2007). The previous observations support the view that integrons are not recent entities and that they have existed in bacterial populations for many decades (Rowe-Magnus et al., 2002). The introduction of antibiotics in clinical, veterinary and agricultural settings have most likely contributed to the selective adaptation of new resistance-encoding gene cassettes and increased the distribution and prevalence of integron-carrying bacteria with such cassettes (Kaplan et al., 1990; Kapil et al., 1994; Will et al., 1999). This work is mainly aimed to review the current status of mobile genetic elements with special reference to integrons associated with antibiotic resistance determinants. In fact, integrons can be considered one of the major genetic carriers of and vectors for dissemination of antibiotic resistance determinants in bacteria (Cambray et al., 2010). Integrons have been

classified according to their genomic context as either mobile integrons, whenever they are associated with transposons, or chromosomal integrons, when located in the bacterial chromosome (Cambray et al., 2010; Mazel, 2006). Mobile integrons are mainly found in Gram-negative bacteria, though a few studies have also reported their presence among Gram-positive species (Cambray et al., 2010; Domingues et al., 2012a). The genetic relatedness of the integrase gene of the mobile integrons is used to group them into distinct classes (Boucher et al., 2007; Stokes and Hall, 1989). Based on the nucleotide sequence of the integrase, five classes of mobile integrons have been described (Cambray et al., 2010). Class I integrons were described in 1989 (Stokes and Hall, 1989), and are reported as the most common and widespread, especially in clinical settings. This class mainly carries antimicrobial resistance genes and it is highly disseminated due to the close association with transposons, often embedded in conjugative plasmids (Cambray et al., 2010). Selection pressure from one antibiotic can simultaneously select to other antibiotic resistance genes and they are physically grouped together with the help of gene cassette in integron (Grape et al, 2004).

In India, the most important cause of hospital acquired infections isoccurred due to the gram negative bacteria. These bacteria have acquired resistance to multiple antibiotics those are not only in the hospital settings but also in the community acquired. Previously, the multi-centric study of (Kapil, 1994; Kapil et al., 2002; Thirunarayanan et al., 1993; Ramamurty et al., 2000; Dhawan et al., 2004) showed an increasing resistance to ampicillin, chloramphenicol, erythromycin and trimethoprim-sulphamethoxazole among clinical isolates from different parts of India

In our study, antibiotic susceptibility profiling was done against 268 isolates of Enterobacteriaceae. They were initially tested against five different groups of drugs those were commonly used therapeutically for a long time. As observed, the resistant rate was high towards co-trimoxazole (90.67%) followed by ampicillin (87.31%), gentamicin (81.34%), cefepime (58.20%) and ciprofloxacin (59.70%) (Table no. 15). Overall, bacterial resistance was observed mostly towards the antimetabolite group of drugs; co-trimoxazole and comparatively lesser against cefepime and ciprofloxacin.

The co-trimoxazole is a combination of drugs (trimethoprim and sulphomethoxazole) and commonly used for the treatment of Urinary Tract Infection (UTI). However, due to rapid spread of resistance towards this drug, there is a trend of declining consumption (Huovinen et al., 1985; Grape et al., 2004). In the 1970s, the trimethoprim (TMP) resistance in gram negative bacilli was observed only 10% (Huovinen et al., 1995) from outpatients but end of the 1980s it reached on 15-20% (Brumfitt et al., 1983; Gryson et al., 1990; Gruneberg RN, 1990). Resistance rates have been gradually increased at high level of 25-68 % in South America, Asia and Africa (Lee et al., 2001). This high resistance rate to trimethoprim as well as sulphamethoxazole and other antibiotics is one of the most important medical issues in India and this data is similar to that reported in other countries but clearly define the higher rate of resistance (Kunin et al., 1993; Grape et al., 2004; Javier et al., 2008; Lee et al., 2001).

The MIC against this particular group of drugs was higher and in agreement with disc diffusion result. This MIC of trimethoprim varied from 32 -240 µg/ml. This result might be produce due to the overproduction of Dihydrofolate reductase

(DHFR) in resistant strains. However, the sulphonamide is an agent which supposed to inhibit the growth of bacteria by interfering with the biosynthesis of folic acid (Brown, 1962). Resistance in most of the gram-negative species involved the acquisition of an additional Dihydrofolate synthetase (DHPS) genes on a plasmid or transposable element and produce an enzyme that is not inhibited by sulphonamide and that bypass inhibition of the chromosomally encoded enzyme (Huovinene et al., 1995). It is reported earlier that sulphonamide resistance genes can be horizontally transferred through integron, transposons and plasmids from commensal bacteria to a virulent one in human intestine (Guerra B, 2003 ; Soufi L, 2011). It is also hypothesised that prolonged use of co-trimoxazole therapy is responsible for selection of integron positive Enterobacteriaceae and in turn responsible for sulphonamide resistance (Vandeer Veen E.L, 2007). The consumption of sulphonamide in human has now become restricted but the sulphonamide resistance among bacterial population were still observed might be due to the acquisition and maintenance of sul gene.

It was known that the sulphonamide resistance determinant (sul1) is located within integron (Perreten et al, 2003) and also established that integrons were selected during use of trimethoprim/ sulfomethoxazole in the intestinal flora (Vandeerveen et al., 2007). Genetic locatization of sul genes on efficient mobile genetic structures probably contribute to the wide spread of sulfonamide resistance through the acquising of segments of modified sul gene by horizontal gene trasnfer (Rodstrom et al., 1992). This indicates that sulphonamide resistance is not originated from 3´CS region of Integron. In our study presence of other variants of sulfonamide resistance genes Viz; sul2 and sul3 were also responsible confering resistace. This study also underlines presence of three

sulphonamide resistance genes with harboring more than one type of sul gene and this presence is also established in other than integron. All the transformats were tested against sulfonamide resistance and MIC of sulfamethoxazole in the trasnformants was showed high (MIC>240). The higher MIC for transformants reflects the genetic environment and horinzontal transferability of resistant geens. However, in our study sul1 gene was found in integron- negative isolates as well. Thus, extra integron existence of sul1 gene also contributed phenotypic sulphonamide resistance, which too was evident by MIC study.Theses genes were probably selected during course of co-trimoxazole therapy which is very common in community-acquired infection in this region, and also maintained in the subsequent generation.Current study, probably the first study from India describing genotypic background of sulphonamide resistane. Further investigation is needed for assessment of their acquisition and expansion when co-trimoxazole pressure is withdrawn and their persistance through Class I integron within enteric pathogen.

The existence of integrons was confirmed in (73.13%) isolates, collected from members of Enterobacteriaceae showing resistant phenotype. Integrons are mainly consist of three essential elements, the *intI* integrase gene, which encodes the *IntI* protein responsible for the site-specific recombination of gene cassettes, the adjacent recombination site *attI* and the gene cassette promoter P_c (previously called P_{ANT}) (Hall and Collis, 1998), although the presence of the promoter has not been shown in all classes (Boucher et al., 2007). An additional promoter, P2, is present in some integrons (Collis and Hall, 1995) Class I integrons was the dominant class (55.6 %) whereas class II integron was observed in less number of isolates (5.6 %). Interestingly, class I integrons were

found higher in (62.64 %) of *E.coli* isolates which, probably the reason behind increased antibiotic resistance within this species. *Class II integron was detected in less isolates and this proportion was stated to have a limited distribution than reported here (Grape* et al.*, 2005, Glodstein* et al.*, 2001, Zhao* et al.*, 2003 and Marianne Sunde* et al.*, 2005).* The co-harbour of Class I and Class II positive were found in 13.22 % isolates. This percentage of Class I integron carriage in these antibiotic-resistant isolates was also compared with other studies reported worldwide and showed high percentage of among Enterobacteriaceae. According to Sue-Bee et al., 2013 48.3% of integron was found to be positive among Enterobacteriaceae within the hospital environment in Malaysia whereas, Lim et al., 2009, found Class I integron only in 41.2% isolates of ESBL *Klebsiella pneumonia* strains. However, Khosravi et al., 2011 demonstrated a high percentage of Class I integrons (60%) in imipenem-resistance isolatant from the same country. Other reports have also revealed the prevalence of Class I integrons in gram negative clinical isolates 54% in Taiwan (Sallen et al., 1995), 59% in France (Chang et al., 2000), and 49% in Australia (White et al., 2001) indicating that Class I integrns are wide-spread among gram-negative clinical isolates. As compared with other studies a significant increased level was found to be shown among MDR isolates of this particular region.

Multiple resistant phenotypes were observed among both Class I and Class II integron- containing isolates. A high correlation between the presence of integron carrying dihydrofolate reductase and phenotypic expression of resistance to trimethoprim was observed. It is probable that such resistance were encoded by non-integron elements also. Class I integrons have usually three distinct genetic regions: two highly conserved regions, the 5'-conserved segment

(5'-CS) and the 3'-conserved segment (3'-CS), flanking the central but variable region where the gene cassettes are located (Stokes and Hall, 1989); these integrons have been designated classic Class I integrons.The resistance gene cassette was successfully amplified and mapped with 5´-CS and 3´-CS primer pairs which bind in the conserved regions of this class (Levesque et al., 1995). It is noted that several other primer pairs have been developed and the PCR amplicon produced by these can have different sizes although they target the same arrays (Ajiboye et al., 2009; Sandvang et al., 1998; White et al., 2000). In the present study, some isolates failed to amplify with 5´-CS and 3´-CS primer, these phenomena could be happened due to alteration occurs in the primer target sites or too large to amplify which is reported in previous studies by Ndi and Barton 2011 and Yu et al., 2003. While all the integron negative isolates were found conferring high level of resistance in one or more antibiotics phenotypically. So it could be assumed that the resistance gene might be co-selected with the integrons involving other mechanisms. It is reported that in rare occasion 3´-CS of class I integron mobilized without any trace of a resistance gene cassette in its surrounding environment (Grape et al., 2005). In the present study sequence analysis identified four different *dfr* (*dfrA17*, *dfrA30*, *dfrA12*, *dfrA1*), four variants of *aad* (*aadA6*, *aadA5*, *aadA2*, *aadA1*) and one *aacA7* gene (confers resistance to aminoglycosides such as amikacin and netilmicin) in gene cassette. Five different class I integron arrangements in the present study was found to be responsible for resistance towards trimethoprim, spectinomycin, streptomycin. In one arrangement, two intact 3´-CS regions, indicates variability in their origin and selection. Association of integrons with *dfrA*17 and *dfrA*12 were the most prevalent in *Enterobactericeae* and frequently been observed to

be disseminated through horizontal gene transfer (Yu et al., 2004). Gene cassette such as *dfrA*1-a*adA*1, *dfrA*12-*orfF*-*aadA*2, *dfrA*17-*aadA*5 were first observed in the year of 1990 (White et al., 2001, Kumar et al., 2011). The cassette *dfrA1-aadA1 w*as previously mentioned, the *dfrA1* gene encoded a dihydrofolate reductase type I, conferring resistance to trimethoprim (Roberts et al., 2012; Van Hoek et al., 2011); and the *aadA1* gene encodes the enzyme aminoglycoside 3"-adenylyltransferase associated with resistance to spectinomycin and streptomycin (Ramirez and Tolmasky, 2010). The first report of this Class I integron array dates from 1990, in the plasmid pLMO229 of an Enterobacterium (Sundstrom and Skold, 1990). In the late of 1990s *dfr*A17 was found to be associated with Class I integrons (Yu et al, 2003). *dfrA17-aadA5:* the *dfrA17* gene encodes a dihydrofolate reductase type XVII, also conferring trimethoprim resistance (Roberts et al., 2012; Van Hoek et al., 2011); an aminoglycoside 3"-adenyltransferase, associated with spectinomycin and streptomycin resistance, is encoded by the *aadA5* gene (Ramirez and Tolmasky, 2010). This gene cassette array was reported for the first time in 2000, in the chromosome of a clinical *E. coli* isolate collected in Australia in 1998 (White et al., 2000). It is noted that the White et al. paper does not suggests the *aadA5* gene confer streptomycin resistance. However, our finding with different cassette arrays with resistant determinants could not be correlated with the antibiotic usage in our hospital setting, thus there must be some other factors which is response the for this persistence and vertical transfer in the hospital environment.

Furthermore the *aadA5* gene cassette was detected only with the *dfrA17* gene, indicating that the *dfrA17* and *aadA5* cassette genes were introduced to Class I integrons simultaneously (Je Chul Lee et al., 2001). The same

combinations of gene cassette in class1 integron have also been detected in other areas of the world (Yu et al., 2003) including Australia and Taiwan (Chang et al., 2000; White et al., 2000). According to a previous study it was observed that the prevalence of each particular combination of gene cassette differed according to the geographical area (Kor et al., 2013). In Taiwan, *dfrA12-orfF-aadA2* was the mostly detected in *E.coli* strains in 1993-1994, whereas, in Australia, *aadA1* was the most frequent gene cassette (Patridge et al., 2009). The differences in prevalence among different countries may have been caused by the different antibiotic therapy regimens used for bacterial infections in each country. However, this study showed the presence of integron mediated *dfrA*30 which was first identified in *Klebsiella pneumoniae* and reported by in 2011 (Kumar et al., 2011).

Integrons with identical gene cassette arrays are found in different species from diverse environments and geographic locations. Here, we assessed the occurrence and dissemination pattern of some of the most common gene cassette arrays found in mobile integrons. We mainly investigated the presence of trimethoprim and sulphonamide variants in clinical isolates. *dfr* variants such as *dfrA*17 and *dfrA*12 were the most prevalent genes and that *dfrA*1 and *dfrA*30. In a report from UK, *dfrA1* was the most frequent gene in urinary tract isolates (Lee et al., 2001). *dfrA*1 was initially identified as a component of Tn*7* and it was thought that the widespread of dissemination of the gene was caused by Tn*7* (Lee et al., 2001). This could be well explained by the fast the co-trimoxazole was the most common antibiotic used in the treatment of urinary tract infection in past decades (White et al., 2001).

Another, the most common gene cassettes was *aadA* family conferring resistance to streptomycin and stectinomycin. In fact, the *aadA* gene cassettes are the most often reported cassettes in bacteria isolates and seen to persist in environments regardless use of such antibiotics at present (Partridage et al., 2009; Moura et al., 2009; Guangming et al., 2013). *aad*A variants *aadA*1, *aadA*5 were the most prominent type. The *aadA1* gene encodes an aminoglycoside 3"-adenylyltransferase, associated with resistance to spectinomycin and streptomycin (Ramirez and Tolmasky, 2010). A Class I integron with this cassette will produce a PCR amplicon of 1009 bp. The *aadA1* gene has been associated with the *Tn*21 transposon for many years (Liebert et al., 1999); the first strain known to carry it was *Shigella flexneri*, isolated in Japan in the late 1950s (Nakaya et al., 1960). In 1989, when integrons were first described, this gene was found to be associated with Class I integron (Stokes and Hall, 1989). The first report of a Class I integron with this single cassette dates back to 1995, and refers to a clinical isolate of *Pseudomonas aeruginosa* isolated in Japan in 1965 (Kazama et al., 1995). Besides the classic Class I integron, the widely disseminated *aadA2* gene cassette is also present in complex Class I integrons, such as in the *Salmonella* resistance island SGI1 and some of the variants (Boyd et al., 2002; Boyd et al., 2008).

The *intI2* was first described as part of the transposons *Tn*7. It includes the *tns*transposition region and bounded by short segments, containing transposase-binding sites, called Tn*7*-L and Tn*7*-R, which are necessary for transcription (Peters and Craig, 2001). Most of the *intI2* contain an internal stop codon that makes them inactive. The natural suppression of the stop codon in *IntI2* or the action of other IntI in *trans* may allow occasional acquisition of new

cassettes (Hansson et al., 2002). The cassette array appears to end with a truncated cassette known as *orfX*/*ybeA* and the primers used to amplify the conserved region between *attI*2 to *orf*X (White et al., 2001). The variation of the gene cassette in class II integrons may reflect the horizontal gene transfer among Enterobacteriaceae. In this study four different types of class II intgeron gene cassette arrays were identified. The phenotypic resistance to tested antibiotics was observed in all isolates carrying the corresponding gene cassette. An integron of about 2.3 kb, present in Enterobacteriaceae harbour a common set of cassette arrays (*aadA1*, *dfrA*1 -*dfrA12* and *sat2)*. Presence of *dfr* cassettes, probably due to selection pressure and usage of trimethoprim antibiotic in hospital environment (Yu HS et al., 2004). Work done by Tajbakhsh et al., 2015, states that Class II integron doesn't carry determinants for nalidix acid, sulfamethoxazole-trimethoprim, nitrofurantion and imipenem and exhibited susceptibility towards these antibiotics. But in our study, sequencing indicates that class II integron carrying a single gene cassette with *dfrA12* gene, whereas rest of the isolates found to harbour *dfrA1*. The prevalence of aminoglycoside–resistant genes associated with class II integron has changed over the course of time, majority of isolates were carrying *aadA1* gene. The *aadA* gene confers the presence of streptomycin and spectinomycin and *sat2* confers the resistance to streptothricin which was occasionally used therapeutically, so far their prevalent within integron despite the fact that the integron encoded integrase is capable of excising them (white et al., 2001, Collis and Hall et al., 1992). Thus, even when antibiotics cease to be used therapeutically, genes encoding resistance to these antibiotics are not necessarily lost (white et al., 2001). Arrangement II showed cassette array where it associated with three additional genes with *intI2* (*dfrA1*-

sat2-aadA1-orfX-ybeA-ybfA-ybfB-ybgA) which may have occurred before it could spread in clinical samples in the antibiotic era and expanded by gene cassette system. From the previous studies it was also reported that, *ybeA* was most common array flanked by *intI2* (Partridge et al., 2009). The IV arrangement with 2.5 kb amplicon size carries a unique set of cassette array and based on the sequencing result we predict that this integron type might be formed by a complex recombination event (*dfrA1*, *linF* and *aadA1*). These gene cassettes have been reported to predominate in Enterobacteriaceae isolated from different European Hospital (Martinez et al., 1999). In this study it was observed that most of the gene cassettes encoding resistance in combination with aminoglycoside and trimethoprim whereas, other antibiotics resistance gene were present in a few isolates and this study were relevant with the other study reported before (Martinez et al., 1999; Heir et al., 2004). Except for IV arrangement all the other types of cassette array have been describe previously in members of Enterobacteriaceae. Other gene cassettes involved in antibiotic resistance included aacA7, sat2 and linF. These cassettes determine resistance to aminoglycosides such as amikacin and netilmicin, streptothricin and lincomycin respectively. Although in gram negative infection, lincomycin is not given. Presence of this resistance gene indicates possible lateral gene transfer from gram negative bacteria. This arrangement is also reported earlier from other parts of the world (Kor et al., 2013 and li et al., 2013, Mejia et al., 2008).

The *linF* gene confers the low level of resistance to both lincomycin and clindamycin have integrated into downstream of an integrase. In fact this group of antibiotics are not used to treat Enterobacteriaceae (Renee et al., 2006). The characterization of *linF* and their association with integron was first reported by

(Even Heir et al., 2004). Re-exposed to the antibiotic could demonstrate a form of genetic memory of the cassette region which are may be repositioned by the integrase, nearer to the promoter where they are expressed more efficiently (White et al., 2001). These cassettes have not received enough selective pressure to encourage their widespread dissemination but the potential of integron system will likely to make them more prevalent. This study also highlighted the extensive variety of drug resistance phenotypes as well as clones and clonal lineages among bacterial isolates in hospital settings. This diversity of structure were despite the fact that many of these class II integron had identical cassette array. However, this study has identified a set of gene cassette associated with *linF* gene. Accordingly continued surveillance of the epidemiology as well as further investigation of those class II integrns and their gene cassette is required to determine the impact and molecular mechanisms of acquisition of multiple drug resistance genes among Enterobacteriaceae of *intI2* gene upon the evolution of class II integron.

The increased prevalence of integrons is likely to be representing the outcome and selection of pharmaceutically produced antimicrobials. Integrons have therefore, together with other resistance-conferring mobile genetic elements, been seen as xenogenetic pollutants (Gillings, 2013). Integrons, similar to those found in clinical settings, have also been detected in remote communities with a history of minimal exposure to antibiotics (Pallecchi et al., 2007). This observation emphasized that antibiotic usage levels are not only the single factor for the existence of integrons in current bacterial populations.

The existence of cassettes is viewed as part of integron and resistance gene pool is supported by the microbial population, which reflects the variation

in cassette composition. Gene cassettes are simplest known genetic element but cannot move themselves, requiring the action in *trans* of an *IntI* (Recchia and Hall, 1995). Origin of gene cassette has not yet been achieved but their abundance indicates that organisms can create cassettes (*attC*) from single genes or a pairs of gene (Pertridge et al, 2009). The DNA sequence of *attC* site is diverged but the variation between closely related individuals was obtained by 59 base elements (be) PCR which amplify the open reading frame (ORF) of *attC* site which carried the antibiotic resistance genes (Stokes et al., 2001). Numerous combinations of gene cassettes have been reported (Pertridge et al, 2009) till date with a diverse number of new integron based cassette array and its unique nucleotide and amino acid sequence (Hall et al., 1991; Collis and Hall, 1992 and Stokes et al., 1997). So far more than 1050 gene cassette arrays with single or multiple nucleotide differences have been identified in Class I integrons (database last accessed on 11 February, **2015**). It is noted that the INTEGRALL database primary focuses on gene cassette compositions whereas early integron numbers also indicate the structure beyond the last cassette (Partridge et al., 2002). Thus the taxonomy of integrons has changed over time and may or may not consider both the gene cassettes and the characteristics of the flanking DNA (e.g. defective Tn*402* sequences). According to Partridge and colleagues (Partridge et al., 2009), the single gene cassettes *aadB*, *dfrA7*, *aadA1a*, *aadA2*, *blaP1* [now called $bla_{\text{CARB-2}}$ (www.lahey.org/Studies - accessed on 17 November 2014)], and the arrays *dfrA1-aadA1a*, *dfrA17-aadA5* and *dfrA12-aadA2* were the most common ones reported in Class I integrons surrounded by the 5'-CS and 3'-CS regions. Some commonly occurring gene cassette arrays were reported in this study (*dfrA1-aadA1*, *dfrA17-aadA2*, *dfrA17-aadA5* and *dfrA12-aadA2*) and

those were reported from Gram-negative bacterial species in previous studies also (Partridge et al., 2009). The dihydrofolate reductase and aminoglycoside adenyltransferase gene sequence were found in the majority of integron carrying isolates. Some of the *orf* region amplified the putative methylase transfarease gene (*met*) along with resistance gene cassettes. The gene cassette regions of few Class I and II integron positive isolates were found to be associated with more than one antibiotic resistance phenotype could not be amplified by conventional PCR. There might be one reason that the gene cassettes were mainly promoter less so to be expressed it required to be present relatively close to promoter and these cassette might be far from the common promoter region. Presence of potential putative gene along with other cassettes might be incorporated through the recombination mechanism in 3´ end of the cassette array. Presence of different arrangements of gene cassettes in birth class I and class II integron underscores their diverse source of origin and acquisition.

Further the typing of isolates was determined by ERIC–PCR revealing distinct patterns among studied strains. The primers are designed to target the copies of ERIC sequence to be amplified among different entrobacterial strains. ERIC sequences described as intergenic repetitive units of Enterobacteriaceae (Sharples and Lioyd 1990, Hulton, Higgins and Shrap 1991). It is known to be an imperfect palindrome sequence has been found only in intergenic regions within transcribed regions. The difference in copy number implies that orthologous intergenic regions may contain an ERIC sequence in one bacterial species but not in another. The fingerprint of done by using ERIC primers recognized that this species was unlikely to contain copies of ERIC sequence, they determined the terminal sequences of some of the fragments that had been

amplified: these fragments had similarity only to the primer and not beyond that. More recently, (Wei et al., 2004) determined the sequences of genomic fragments amplified using ERIC-PCR primers from unidentified microbial strains within human fecal samples. Our work was also comparable with the work stated by (Kang et al., 2005, Su et al., 2006, Niemann et al., 1999**).** Hak Sun et al.; 2003 used southern hybridization for detecting the integrons in *Escherichia coli* isolates by random labelling methods and observed most integrons were located in plasmids and could be transferred to other strains. Kor et al., 2013 used PFGE to determine the genotypic diversity of integron borne isolates among Enterobacteriaceae and reported cross transmission of integron carrying clones disseminating bacteria from patient to patient and their work was also supported by Nijssen et al., 2005 and Daikos et al., 2007. These epidemiological investigations include the molecular typing of strains those were clearly demonstrated a close genomic relationship exists among strains involved in outbreaks.

Horizontal gene transfer via plasmids represents a key mechanism by which resistance genes disseminate among different bacterial populations. Therefore monitoring the spread of plasmids is useful to follow the transmission of antimicrobial resistance genes from different environments. PCR based replicon typing was used in this study for plasmid identification, targeting the replicons of the major plasmid families occurring in Enterobacteriaceae. The predominant plasmid replicon types found in antibiotic resistant Enterobacteriaceae isolated from different clinical samples include incompatibility (Inc) groups, F, A/C, I/M, I1, HI2 and N. IncF plasmids likely contribute to the fitness of the bacterial isolates providing virulence and

antimicrobial resistance determinants but it also encoded several addiction systems that guarantee their maintenance and stability in the host cell independently. The positive selective pressure exerted by the antimicrobials. IncF plasmid families was found more prevalent in clinically relevant Enterobacteriaceae in this study and reported in previous studies also (Villa et al., 2010, Carattoli 2013). Woodford et al., 2009 also stated that, these plasmids showed multiple addiction systems which contribute to plasmid persistence during bacterial propagation. However, it was reported that plasmids IncF are limited by host range to the genera Enterobacteriaceae (Carattoli, 2009) and they are very stable in their bacterial host. Plasmids belonging to the IncI family are mostly responsible for the spread of bla_{CTX-M} gene in Europe, France, Belgium and Korea (Golebiewski et al., 2007). Our data showed the presence of 21 IncI positive Enterobacteriaceae which might be driven the dissemination of beta-lactamase mediated resistance in this region. The presence of IncI plasmid was also reported by Wu et al., 2010, which confers *sul1* gene containing integron. Overall, IncF seemed to be the most common replicon associated with *sul2*, which are considered having a narrow host range and being conjugative, seemed to be well adapted to Enterobacteriaceae (Boyd et al., 1996; Johnson et al., 2007). IncI was found to be most prevalent and associated with Class I integrons. However, isolates were resistant to ampicillin, streptomycinand trimethoprim. This indicates that resistance genes only account for small part of the plasmid gene sequence and more studies are needed to determine the contents and function of such plasmids. It was reported in a study that not all plasmid families occur at the same frequency in naturally occurring bacteria, some families are clearly more prevalent and diffused than others or show a

differential distribution in pathogens and commensal strains (Johnson et al., 2007). Abundant and common plasmids can be randomly targeted by mobile resistance determinants. It can be hypothesized that when a resistance gene targets a successful plasmid type, this can significantly contribute to the emergence and spread of that gene, being well adapted to different bacterial hosts. These plasmids can promote their spread in different bacterial species, being stably maintained very often independently by the harboured resistance genes. When a resistance determinant was acquired by one of these successful plasmids, the probability that it will spread and persist in different hosts and conditions increased. The prevalence of these rare plasmids can increase locally, thanks of the selective advantages offered by the acquired resistance gene when antimicrobials are used. In every case, plasmids represent the most difficult threat for the dissemination of antimicrobial resistance, since they efficiently contribute to the spread of emerging and relevant resistance determinants, being positively selected by antimicrobials, well adapted to the host, and difficult to be cured or counteracted by the current therapies.

The molecular background and gene cassette mediated antibiotic resistance in this region was observed in this study, where overuse and misuse of antibiotics is common in clinical practise. There might be various reasons which influence this current scenario like self medication, over the counter availability of antibiotics, aggressive marketing by pharmaceutical company, and lack of uniformity among physicians to follow antibiotic policy and most importantly absence of infection control management and awareness campaign. Although the findings represents in our study showed that strains contained integron responsible for multidrug resistance, we also found that gene cassettes within

integrons could not confer resistance to all the resistance phenotypes. These results implied that there may be other mechanisms responsible for bacterial resistance. We also observed the presence of more than one haplotypes of *Klebsiella pneumonia*, *Escherichia coli* and *Proteus mirabilis* carrying a particular integron. Most of the gene cassettes were carried by Class I, the transfer of the integron spread may be achived through the cross-transmission of intgeorn-carrying clones from one patient to the other an action known to be facilitated among Enterobacteriaceae in hospital settings which is also claimed earlier (Hall et al., 2002).

This study also drew an attention towards genetic vehicles responsible for horizontal gene transfer. Disease incompatibility types of plasmids observed in this study signifies their diverse source of origin, acquisition and mainteinance.