Discussion

The present study was undertaken to investigate the prevalence of quinolone resistant Enterobacteriaceae among hospital and community isolates from patients admitted to different wards, attended the outpatient departments of Silchar Medical College and Hospital and also those attended the private clinics of Silchar, Assam. The study was also extended to investigate the prevalence of quinolone resistance determinants, their molecular characterization, investigation of genetic environment and transmission dynamics. This study also analysed the mutation in the quinolone resistance determining region which attributes resistance to quinolone antibiotics.

Fluoroquinolones were first introduced into clinical use in 1985. The high clinical efficacy of orally available fluoroquinolones such as norfloxacin, ofloxacin and ciprofloxacin together with the initially very low incidence of resistance in *E. coli* and many other Gram-negative pathogens, in the treatment of urinary tract infections rapidly resulted in a widespread empirical use for this application. This high efficacy is due to the high affinity and inhibitory activity of the drugs to their target topoisomerases gyrase and topoisomerase IV which are A2B2 tetrameric enzymes sharing high structural and functional homology. Consequences of the irreversible enzyme inhibition are the arrest of replicative DNA metabolism and subsequent cell death due to secondary bactericidal mechanisms such as the introduction of DNA double strand breaks following the inhibition of DNA gyrase (Pohlhaus and Kreuzer. 2005; Heisig, 2009).

Quinolones have been prescribed widely to treat respiratory tract infections, including tuberculosis, urinary tract infections (UTIs), intraabdominal infections, skin infections, sexually transmitted diseases, and bone and joint infections. They have also been used for prophylaxis in neutropenic patients with cancers, in cirrhotic patients at risk for spontaneous bacterial peritonitis, and in urologic surgery (Freifeld et al., 2011; Bratzler et al., 2013). The national use of quinolones steadily increased from 1994 to 2000 in US intensive care units (ICUs), and this use was significantly associated with decreased overall susceptibility to ciprofloxacin in the same period (Neuhauser et al., 2003). The consumption of quinolones doubled during 2001-2012

in a Korean hospital with the increased ciprofloxacin resistance in clinical isolates of *Escherichia coli* in ICUs (Jun et al., 2013). While newer class quinolones that expand the spectrum of activity to include gram-positive bacteria and even anaerobes have been developed, quinolone resistance has nonetheless increased in many bacterial species, and no new quinolones with activity against gram-negative bacteria greater than that of ciprofloxacin have yet become available. The increase in quinolone re-sistance is now threatening the clinical utility for treatment of diverse infections (Lunn et al., 2010; Kandel et al., 2014).

Fluoroquinolones are broad-spectrum antibiotics that are used to treat several Gram-negative (GN) and Gram-positive (GP) bacterial infections. Since 1960, fluoroquinolones have become prevalent in the treatment of urinary, respiratory, gastrointestinal, urogenital, intra-abdominal, and skin infections. *E. coli* infections, especially in the urinary and gastrointestinal tracts, are frequently addressed with fluoroquinolones. The emergence of quinolone-resistance during treatment was first reported in *Staphylococcus aureus*, particularly along with the methicillin-resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa*. Fluoroquinolone resistance emerged rapidly and spread to Gram-positive (GP) and GN bacteria in hospitals, with minimal inhibitory concentration (MIC) values inhibiting 90% of pathogen growth specifically over a broad range from $\leq$ 0.015 up to $\geq$ 128 mg/L fluoroquinolones (Dalhoff. 2012). Drug-resistant subpopulations of pathogens became prevalent two decades ago and have remained almost unnoticed. Recent surveillance studies demonstrated that fluoroquinolone resistance rates have continued to increase, affecting patient management and necessitating the need for changes in the current treatment guidelines (Keddy et al., 2010).

In the present study, among 558 test isolates, 323 (57.89 %) were found to be quionolone resistant by disc diffusion method. Highest occurrence rate was found among *E. coli*, *K. pneumoniae* while in *Proteus vulgaris* the occurance rate was less. Among similar studies conducted in India, 76.71% of *K. pneumoniae* (Magesh et al.,2011) and 61% of *K. pneumoniae* (Tripathi et al.,2012) were quinolone resistant. Although current study showed a lesser frequency of quinolone resistance compared to other studies in India (Rath and Padhy. 2015), it was quite high in comparision with scenario of Europe, America and Africa (Gu et al., 2012). In France, there was continuous increase in fluoroquinolone resistant *E.coli* from 12.34% to 15.19%

(Gallini et al., 2010). However, in another similar study high prevalence of quinolone resistant *E.coli* (82.8%) were reported in Iran (Firoozeh et al.,2014). In Finland, quinolone resistance was observed in 27.78% non-typhoidal *Salmonella enterica* strains (Gunell et al., 2009). In a study carried out in China to investigate the cases of sporadic shigellosis, 12.2% of *S. flexneri* were resistant to fluoroquinolones (Pu et al., 2009). In another study from Tunisia, 58.08% of uropathogenic *E.coli* were reported as quinolone resistant (Tarchouna et al., 2015). Due to irrational use and over the counter availability, these drugs have often been misused and hence the higher incidence of quinolone resistance. The incidence of high resistance to fluoroquinolone is worrying and can compromise antimicrobial treatment.

In the current study, the incidence of quinolone resistance was 130/212 (61.32%) in community isolates and 79/212 (37.26%) among hospital isolates. Majority of community isolates were from urinary tract infection followed by upper respiratory infection, where most common community isolates were *E.coli* (n=97) and *K. pneumoniae* (n=23). In the Middle east (Iran), percentage of fluoroquinolone resistant isolates was significantly larger among hospital isolates (33%) than in community acquired infections isolates (4.1%) (Hashemi et al., 2013). The incidence of fluoroquinolone resistant isolates were significantly higher in hospital settings (38%) than in community settings (27%) in Australia (Fasugba et al., 2015). However in Brazil, the prevalence of quinolone resistant *E. coli* is 39% and *K. pneumoniae* is 15.8% in community settings (Holanda et al., 2015). In another study conducted in Meerut city on uropathogenic community isolates, 96.97% of *E.coli* and 79.31% of *K. pneumoniae* showed resistance against quinolone antibiotics (Prakash and Saxena. 2013). Moreover, the incidence of quinolone resistance in hospital isolates was quite high. Almost 90% of *E.coli* and *Klebsiella* spp. showed resistance to ciprofloxacin and norfloxacin (Vij et al., 2014).

From the findings of the study, the therapeutic option of quinolone need to be revised. Antimicrobial chemotherapy should be initiated only after analysis of resistance profile.

4.2 Phenotypic screening of quinolone resistance

Among different quinolone drugs, nalidixic acid showed highest resistance followed by lomefloxacin, ciprofloxacin, sparfloxacin and norfloxacin. In a similar study, resistance towards nalidixic acid and ciprofloxacin, reported in Ghana was 18.2% and 67% (Namboodiri et al., 2011), whereas in an another study 94% of resistance was found towards nalidixic acid, norfloxacin, ciprofloxacin in India (Pazhani et al., 2011). In an earlier study done in Turkey, uropathogenic *E.coli* strains displayed high rates of resistance to norfloxacin (80%) (Nazik et al., 2011). In another study conducted in Bangladesh, 96% of the *Shigella* spp. was found to be resistant to nalidixic acid and 44% to ciprofloxacin (Azmi et al., 2014).Reduced susceptibility to ciprofloxacin (resistance and intermediate susceptibility) was observed in 66 (71%) *Klebsiella pneumoniae* isolates investigated in the study conducted in Malaysia. Ciprofloxacin MIC values of the isolates ranged from 0.032 to ≥32 g/ml with MIC90 and MIC50 equal to ≥32 and 2 g/ml, respectively (Al-Marzooq et al., 2014).

In this study, high MIC was observed for quinolone antibiotics and majority of the isolates showed MIC values well above the break point ranging from 0.5 to > 256µg/ml which is an alarming situation and can lead to treatment failure. In a previous study conducted in Chennai India, MIC of quinolone resistant *Klebsiella pneumoniae* isolates ranged from >120 µg/ml -> 240 µg/ml (Magesh et al., 2011). High MICs (2000 µg/ml) were also observed for nalidixic acid and MICs ranged between 250 and 1000 µg/ml for norfloxacin and ciprofloxacin among enterotoxigenic *E.coli* strains from Ahmedabad, Gujarat (Pazhani et al., 2011). However in comparision to this study, in another study reported by Sangeeth and co-workers, MIC values of ciprofloxacin and levofloxacin were high (CIP- 16-64 µg/ml and LEV- 28-64 µg/ml) (Sangeeth et al, 2014) whereas in another study conducted in Dibrugarh, high MIC was observed for nalidixic acid against *Shigella flexneri* (Nath et al., 2013). However in comparison to this study, another study conducted in Spain reported MICs > 256 µg/ml for naldixic acid and >32 µg/ml for ciprofloxacin (Cano et al., 2009). Unlike the present findings, a study conducted in Milan reported basal MICs of *E. coli* strains ranging from 0.016 mg/L to 1 mg/L, from 0.004 mg/L to 0.5 mg/L and from 0.016 mg/L to 0.125 mg/L for levofloxacin, ciprofloxacin and

prulifloxacin, respectively. MICs of *Klebsiella* spp. ranged between 0.03 mg/L and 1 mg/L, 0.016 mg/L and 0.5 mg/ L, and 0.03 and 0.25 mg/L for levofloxacin, ciprofloxacin and prulifloxacin, respectively (Drago et al., 2010). In Central African Republic, all the CTX-M producing strains were resistant to nalidixic acid, norfloxacin and ciprofloxacin with MIC of nalidixic acid and norfloxacin 64 µg/ml and that of ciprofloxacin 32 µg/ml (Frank et al., 2011). Firoozeh and co-worker conducted a study in Iran where most of the quinolone resistant *Escherichia coli* showed MIC values of ciprofloxacin in the range 4- 512 µg/ml (Firoozeh et al., 2014).

4.3.1 Molecular characterization of quinolone resistance genes among clinical enterobacterial isolates.

This study could underscore presence of diverse quinolone resistance genes in this geographical region. The study identified *qnr*A, *qnr*B, *qnr*D, *qnr*S and *aac*(6')*Ib-cr* in clinical isolates of Enterobacteriaceae in the 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 the study. However previous study from Kolkata reported the presence of *qnr*A in 37%, and *qnr*B in 56 % of the *Klebsiella* isolates (Tripathi et al., 2012).

In the present study, *aac(6')-Ib-cr* (n=23,10.85%) was found to be the most predominant type of quinolone resistance in this geographical region. This fluoroquinolone modifying gene was first reported by Robiscek et al. (2006). In a previous study from India, *aac(6')-Ib-cr* and *qnr*B were identified from typhoidal Salmonellae indicating the trend of quinolone resistance in *Salmonella* (Geetha et al., 2014) which may lead to the worrisome condition as ciprofloxacin is a useful orally administered antibiotic for the treatment of enteric fever. However in another study conducted in Ahmedabad *aac(6')-Ib-cr* was predominantly found in ETEC (Pazhani et al., 2011). In a study conducted in Argentina, the most prevalent *qnr* genes were *aac(6')- Ib-cr* and *qnr*B among ESBL- producing Enterobacteriaceae (Cruz et al., 2013).

The gene *qnr*D (n=18) 8.49% was the second commonest among all quinolone resistant isolates. This *qnr* gene was earlier not reported from India and this appears

to be the first report of incidence of *qnr*D from India from both clinical and environmental isolates. A study conducted in China detected the presence of *qnr*D in multidrug resistant *K. pneumoniae* (Li et al., 2012) and another previous study from Tunisia also reported *qnr*D from *Morganella morganii* (Yaiche et al., 2014). A recent study from Latin America reported *qnr*D1 for the first time in *Morganella morganii* (Seija et al., 2015). The report of *qnr*D from this geographical part indicates the urgent need for further molecular investigation which can give clues to the origin and source of plasmid borne quinolone resistance determinants.

In this study *qnr*A (n=7) was also detected. This *qnr*A gene was first reported by Martinez and co-workers from *Klebsiella pneumoniae*. An analysis of the *K. pneumoniae* isolates obtained from tertiary care hospital in Chennai detected *qnrA*1, *qnrB1* and variant of *aac(6')-Ib-cr* gene in ceftazidime-resistant isolates (Magesh et al., 2011).

Furthermore, presence of *qnr*S (n=4) and *qnr*B (n=2) among enterobacteriaceae is also reported from the current investigation. In a study conducted in Brazil, isolates showed positive for the presence of *qnr*S1 and *qnr*B19 and *aac(6')-Ib-cr* in uropathogenic *E. coli* isolates which is the common cause of community acquired urinary tract infection (Paiva et al., 2012). In a study conducted in Bangladesh, *K. pneumoniae* was found to be harbour *qnr*B (Islam et al., 2013). Whereas a recent study conducted in Pakistan showed the prevalence of *qnr*B and *qnr*S in uropathogenic *E. coli* (Ali et al., 2016), Eftekar and Seyedpour have reported not only a high prevalence but also the co-carriage of *qnr*B, *qnr*S and *aac(6')-Ib-cr* genes in clinical isolates of *K.pneumoniae* from Tehran (Eftekhar and Seyedpour, 2015). Later from the same country, Ranjbar and co-workers reported the presence of *qnr*S in *Shigella* spp. (Ranjbar et al., 2016). Furthermore the coexistence of *qnr*S and *qnr*B was also reported in *Shigella* spp from India (Sethuvel et al., 2015).

Results of the present study are important in presenting the prevalence of PMQR determinants (specifically *aac(6')-Ib-cr* and *qnr*B) in enerobacterial clinical isolates and their association with multiple drug resistance. Considering the fact that these genes are often carried on mobile genetic elements and could easily be spread among the members of Enterobacteriaceae (Jacoby, 2005), such information is needed for

choosing a proper antibiotic therapy and may prevent the dissemination of these resistance determinants among important Gram-negative pathogens.

4.3.2 Molecular characterization of quinolone resistance genes among environmental enterobacterial isolates

Emergence of plasmid mediated quinolone resistance among enterobacterial isolates in the hospital and community environment poses to be a serious problem globally. As the prevalence of PMQR gene is a worldwide rather than a national or local problem, it is of much interest to identify the reason behind their spread in the different geographical locations which follow variable antibiotic policies, the selective antibiotic in hospital and community environment along with local antibiotic resistance burden and their genetic background would be a key factor to asses epidemiological relationship. It is also of importance how these genes are maintained in non-pathogenic organisms and whether environment act as a reservoir of the resistance genes. Therefore, the present was extended to determine the prevalence of PMQR genes among the environmental isolates.

In this study, *aac(6')-lb cr* (n= 25) was the most common among test isolates (n= 68), followed by *qnr*D (n=7), *qnr*A (n=5) and *qnr*S (n= 2). None of the isolates showed positive amplification for *qnr*C and *qnr*B genes. This appears to be first report of *qnr*D from environmental isolates. However, previously in a study from Central India, Ujjain, Madhya Pradesh, presence of *aac*(6')-*Ib-cr*, *qnr*A, *qnr*B was reported in *E. coli* isolated from hospital waste water (Diwan et al., 2012). In another study from Bangladesh, *qnr*B and *qnr*S were the predominant *qnr* genes among *E.coli* isolated from tap water (Talukdar et al., 2013).

Fluoroquinolone resistant enterobacteria isolated from water bodies, waste disposal and food sample, whether pathogenic or not, may come into contact with other microbes and transfer resistance genes. In this study, the most prevalent gut colonizer *E.coli* comprised 33.82% of total enterobacterial isolates in tested samples, which is an indicator of faecal contamination. This is in accordance with the study carried out by Chen et al. (2012) in China where the prevalence of *E. coli* was 36.4%. In another study carried out to assess the microbiological quality of ready to eat street vended food in porto region of Portugal (Campos et al., 2015) *E.coli* accounted for 55%.

The presence of *E. coli* in food sample indicates the lateral entry of enterobacterial isolates into food chain which may cause infection. Compared to this study, another report showed a different distribution in Mexico city (Amabile- Cuevas et al., 2010). Many studies were performed on the phenotypic detection of PMQR genes by using mostly nalidixic acid as an indicator (Mirarini et al., 2008). The study carried out by Cavaco et al. (2009) suggested that nalidixic acid alone did not confer the maximum effectiveness for the detection of these resistance determinants which is almost similar to our study where 39% of the isolates were simultaneously resistant to norfloxacin, ciprofloxacin and ofloxacin. The finding is in contrast to the observation made by Marti and Balcazar (2013) and Poirel et al. (2012) which stated that *qnr*S was the most commonly identified acquired *qnr* gene in the environment, because it is usually identified in waterborne organisms.

In this study, we reported the prevalence of plasmid mediated quinolone resistance genes in clinical and environmental enterobacterial isolates, while most *E.coli* isolates from sewage are likely of human origin; those from urban dust and lake water include intestinal micro-organisms from domestic animals. As it should be expected, the prevalence of FQ resistance is much higher among enterobacterial clinical isolates than it is among environmental ones. However, a remarkable high prevalence was found among strains isolated from the water bodies near hospital waste disposal; this prevalence is even higher than that reported for clinical isolates in other countries. This can be the result of an environmental selective pressure at play, favouring FQ-resistant strains, either directly or by co-selection. The nature of such pressure is unknown but could range from the presence of FQs themselves to the effect of environmental pollutants upon regulated bacterial responses that result in multiple-antibiotic resistance. This highlights the antibiotic resistance crisis and role of environment as reservoir of these genes. These also further underscore potential transmission dynamics and persistence in human environment interface. This warrants urgent review and analysis of residual antibiotics.

Hence, widespread use of fluoroquinolones has inevitably led to a sharp increase in the rate of resistance among different bacterial species in areas around the world.

4.4 Clonal dissemination of plasmid mediated quinolone resistant genes among enterobacterial isolates.

Plasmid appear to augment bacterial genetic diversity and acquiring genes as well as horizontal exchange among bacterial populations by conjugations or by mobilization (Francia et al., 2004). The genetic context and map of plasmid is key determinant for their mantainence and persistence within broad host range of Gram- negative bacteria (Helinski 2004). They contain gene 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 (Caratolli et al., 2005). The discovery of the plasmid-borne quinolone resistance determinants, named *qnr*, has substantially broadened our horizon on the molecular mechanisms of quinolone resistance. Several recent reports of Qnr or its homologues encoded by transferable plasmids in Gram negative bacteria isolated worldwide highlighted the significance of the emerging plasmid –mediated mechanism(s). This also alerts us to the potential rapid dissemination of quinolone resistance determinants.

In the present study, carriage of *qnr*A, *qnr*B, *qnr*D, *qnr*S and *aac*(6')-*Ib*-*cr* in transferable plasmid was observed in 82/98 (83.67%) clinical isolates and 27/36 (75%) environmental isolates. It was observed that in clinical isolates all the *qnr* and *aac*(6')-*Ib*-*cr* genes were horizontally transferable. However in case of environmental isolates, only *qnr*S and *aac*(6')-*Ib*-*cr* were horizontally transferable. Based on the transferability results it is clear from this study that *qnr* and *aac*(6')-*Ib*-*cr* genes are located on the plasmid. Unlike our findings, previous records showed that *qnr*D could not be transferred (Yaiche et al., 2014). In consistent with our findings, another study from India showed that *qnr* and *aac*(6')-*Ib*-*cr* genes are located on the transferable plasmid (Yugendran and Harish, 2016). Transferability of these resistance genes can result in community and nosocomial dissemination of resistant strains, which is a serious challenge towards treatment of infection.

While carrying out the transferability study, it was observed that the usage rate of the quinolone antibiotics selects the specific *qnr* genes in the environments or the organism harbouring these genes are adapted against specific antibiotic containing

environment. The assessement of horizontal transferability was done by selecting the transformants from the media containing ciprofloxacin, norfloxacin, ofloxacin and levofloxacin. Transformants carrying the *qnr* and *aac*(6')-*Ib-cr* genes showed biasness towards the specific quinolone antibiotics. Transformants carrying *qnr*A could be selected in the media containing norfloxacin, ciprofloxacin and levofloxacin whereas *qnr*B and *aac(6')-Ib-cr* were selected in norfloxacin and ciprofloxacin. The transformed *qnr*D could be selected in the media with norfloxacin and ofloxacin and *qnr*S was selected only in the presence of levofloxacin.

Ciprofloxacin which is a member of the large and widely used fluoroquinolone group of antimicrobial drugs, is considered as the empirical choice treatment of infections in adults. But the extensive abuse and self-medication of these drugs, viz norfloxacin and ciprofloxacin in last two decades has led to the development of resistance within pathogens associated with community acquired infection. The fact that abuse of antibiotics through over-the-counter (OTC) dispensing in India was supported by the study conducted in West Bengal (Hazra, 2014). Furthermore, the studies of Chaudhary et al. (2015) and Patel et al. (2013) provided evidence in support of self-medication that leads to development of resistance against fluoroquinolone and other group of antimicrobials.

This study established a scenario where it is observed that presence of *qnr*A. *qnr*B, *qnr*D and *aac(6')-Ib-cr* is associated with resistance against norfloxacin and ciprofloxacin. This study could give an insight into the usage pattern of different quinolone antibiotics and their role in carriage of specific quinolone resistance determinants within bacterial isolates. The predominance of different quinolone resistance determinants (*qnr*D, *aac(6')-Ib-cr*, *qnr*A, *qnr*B, *qnr*S) and their selection against corresponding quinolone groups well corroborates with the maximal usage of these in community acquired infection and their over the counter availability. All the *qnr*S (n=5) encoding plasmids were selected only in presence of levofloxacin and resistance against this drug can be predicted as phenotypic marker for carriage of *qnr*S. However, the usage of levofloxacin is quite low compared to other fluoroquinolones and possibly, the reason behind less occurrence of this particular gene in the present study.

This study also demands urgent need for review in fluoroquinolone therapy and their prophylactic use in both community and hospital acquired infections. More molecular epidemiological investigations must be carried out to correlate the maintenance and persistence of resistance gene within a host when a quinolone treatment is initiated. Thus, the study presents a scenario of plasmid selection by the pathogens due to quinolone stress and their role in horizontal expansion.

Antibiotic consumption is widely recognized as the main cause of emergence of resistance. Several studies have focused on the relationship between fluoroquinolone use and the emergence or dissemination of FQ resistant enterobacterial isolates, but none of the study has shown the relation between the presence of plasmid carrying *qnr* genes and their selection towards specific quinolone antibiotics (Mahamat et al., 2005). Antimicrobial use in the community is also believed to be an important determinant of resistance which influences the development of resistance scenario. As the fluoroquinolone antibiotics are readily available over the counter and often being misused, it is of interest to focus on the relationship between presence of *qnr* or *aac(6')-Ib-cr* genes and their specificity towards quinolone resistance. This study highlighted the fact that the presence *qnr* genes showed biasness towards the specific quinolone antibiotics and it may be used to develop a phenotypic marker which can detect the presence of specific *qnr* or *aac(6')-Ib-cr* genes. This study showed *qnr*S was mainly responsible for levofloxacin resistance and this was further supported by selection of *qnr*S gene only in the media supplemented with levofloxacin. Thus, occurance of few *qnr*S (n= 5) in clinical isolates well corroborates with very low level of levofloxacin resistance pattern.

Much remains to be understood about these genes, their basis of expression, but the incidence of their increasing resistance, their horizontal spread, and their co-selection with other resistance elements indicate that a more cautious approach to quinolone use and a reconsideration of clinical breakpoints are needed.

In this study it was noticed that there exist multiple sizes of plasmids which are transferable and encoding different *qnr* genes. Among them plasmids of~15 Kb,~18 Kb, ~20Kb and 25Kb were common in all transformants. Previously in Spain, in *Enterobacter* spp. a conjugative plasmid of 110Kb to 350Kb carrying *qnr*S was

found (Cano et al., 2009). In another study from Brazil, reported a *qnr*B carried in conjugative plasmid of 55Kb in *E. coli* and *K.pneumoniae.*(Mirarini et al., 2008) In a recent study from China, *qnr*D was detected on the ~2.7Kb or ~5.2Kb non-conjugative plasmid in tribe *Proteae* (Chen et al., 2016).

Plasmids have also a 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 known as bacterial conjugation. Conjugation involves direct cell to cell contact, mating pair information and 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 this study 76 (92.68%) isolates were found conjugatively transferable and *qnr* and *aac(6')-Ib-cr* genes were detected in these conjugative plasmids.

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 *qnr* and *aac(6')-Ib-cr* genes to new hosts and play a central role in the population dynamics of bacterial plasmids and maintenance within the host.

4.5.1 Genetic environment of *qnr* and *aac(6')-Ib-cr* genes

Integron acts as a tool for site specific recombination which is capable of integration and expression of gene enclosed in structures called mobile gene cassettes. Integrons were observed among pathogenic bacteria to be the 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., 1995). Integron are mainly divided into two major groups: resistant integrons (RI) and super integron (SI). These integrons are divided into three classes, the commonest one is class 1 followed by class 2 while class 3 is rare. Multidrug resistant integron (MDRI) mostly carry class 1 integrons and large array of different antibiotic resistance gene cassettes (Partridge, 2005).

In the present study, sequencing of the amplified product revealed that *qnr*D and *aac*(6')*Ib-cr* are located within the variable region of class 1 integron, whereas other quinolone determinants showed no association with the gene capture mechanism. Previous records showed that *qnr*A, *qnr*B, *qnr*S were present within class1 integron (Lui et al., 2015), however, in present study these genes were not associated with the gene capture mechanism.

4.5.2 Association of *qnr* and *aac*(6')*Ib-cr* genes with mobile element

Presence of plasmid mediated quinolone resistance determinants is significant, because resistance determinants among pathogenic Enterobacteriaceae have become a major health problem in recent decades. Infection with organisms harbouring *qnr* determinants result in poor clinical outcomes, overdue initiation of suitable antibacterial therapy, longer hospital stays and greater hospital operating cost.

Sequencing results showed that *qnr*A gene was linked with *tnISEcp1*; *tnIS26;* and *IS26* in the upstream region while in case of *aac*(6')*Ib-cr* the upstream region showed the presence of *tnIS26*. No association was observed in other quinolone resistance determinants associated with mobile genetic elements. In a previous study, it was found that IS26 is present in the upstream and downstream of *qnr*B19 (Schink et al., 2012). IS*CR1* has been reported to be associated with *qnr*A1that mobilize *qnr* genes in Enterobacteriaceae (Frank et al., 2011). However, in Spain it was reported that *aac*(6')*Ib-cr* gene is linked with transposase of Tn*1721* (Ruiz et al., 2012).While in another report from the same country, IS*CR1* was found to be associated with *qnr*S which help to mobilize this gene in Enterobacteriaceae (Cano et al., 2009).

4.6 Transferability of *qnr* and *aac*(6')*Ib-cr* genes

Bacterial genetic diversity, acquisition of resistance gene as well as horizontal gene transfer is facilitated by conjugation of plasmid (Francia et al., 2004).

Bacterial resistance may be transformed horizontally when bacteria acquire the drug resistance by transformation or by other modes of genetic exchange (Tenover, 2006).

In the present study, carriage of *qnr*A, *qnr*B, *qnr*D, *qnr*S and *aac*(6')*Ib-cr* genes in transferrable plasmids was observed. Transferability of these resistance determinants can result in nosocomial dissemination of resistant strains, which is a serious

challenge towards treatment of infection. Therefore current study emphasizes significance of reasonable choice of antimicrobial agents and powerful monitoring of bacterial resistance. Further study possibly can slow down the emergence of resistance and control the dissemination of resistant strains. In the current study plasmids of~ 25 Kb,~ 20 Kb ~ 18kb,~ 15kb which were transferable and harbouring different *qnr* and *aac*(6')*Ib-cr* genes, were found to be common in all the transformants. Previously in Spain, conjugative plasmids of 110kb and 350kb were found to be associated with *qnr*S (Cano et al., 2009).

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 76 isolates were found conjugatively transferable and *qnr* and *aac*(6')*Ib-cr* genes were encoded in these conjugative plasmids. Unlike the present finding, previous studies showed that *qnr*B was located on *C. freundii* chromosome (Liao et al., 2015). In agreement with the present finding, previous records from India showed that *qnr*B was plasmid borne in *V. fluvialis* (Singh et al., 2012). Another study from Spain showed that *qnr*S and *aac*(6')*Ib-cr* genes were encoded in conjugative plasmid in *E. coli* but the two *Klebsiella* strains with a positive chromosomal location of the *aac*(6')*Ib-cr* gene also harboured the *aac*(6')*Ib-cr* gene in plasmids.

Hence this study underscores that conjugative plasmids are major contributor of bacterial adaptability and diversity.

4.7 Plasmid Incompatibility typing of transformants

Incompatibility (Inc) refers to the ability of the two plasmids which 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" (Carattoli et al. 2005). 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, 2011). In the present study, plasmids of different incompatibility (Inc) groups were detected in all 82 transformants carrying single as well as multiple Inc group types.

In this study, *qnr*A was found within P, B/O, HI1, T, F_{rep}B, B, K/B, I1 Inc types of plasmids in *E.coli, Klebsiella pneumonia* and *Klebsiella oxytoca*. The gene *qnr*D was found within FIB and K/B Inc types of plasmids in Enterobacteriaceae whereas *qnr*S harbouring transformants carrying F_{rep}B and K/B plasmids were also observed. In *aac*(6')*Ib-cr* harbouring transformants, diverse Inc groups were also observed. Inc group HI2, FIIS, K/B, P, F_{rep}B and FIB were found in *E.coli, Klebsiella pneumoniae, Proteus vulgaris* and *Proteus mirabilis,* and *qnr*B harbouring transformants were found in K, F_{rep}B and I1 in *E. coli* and *Klebsiella pneumoniae.* These findings correlate with the previous findings, whereas *qnr*B was associated with IncF type plasmid (Kaplan et al., 2015). Thus, this study could establish the fact that *qnr* and *aac*(6')*Ib-cr* genes are located in plasmids of different Inc types which could predict multiple sources and origins of their acquisition and propagation. This finding is of epidemiological interest as diverse Inc types indicate diverse sources of acquisition in the same geographical area which could be helpful in formulation of hospital infection control policy and treatment failure options.

4.8 Mutation in the quinolone resistance determining region

Fluoroquinolones are broad- spectrum antibiotics which inhibit DNA gyrase activity. DNA gyrase is a type II DNA topoisomerase that catalyzes the negative supercoiling of DNA and the separation of interlocked replicated daughter chromosomes. These processes are important for DNA replication and transcription and for the segregation of replicated chromosomes. DNA gyrase is a tetrameric enzyme with two A subunits and two B subunits encoded by the genes *gyr*A and *gyr*B, respectively. Another tetrameric enzyme, topoisomerase IV composed of two A subunit and two B subunits are encoded by the genes *par*C and *par*E, respectively. The major role of this enzyme is to decatenate daughter replicons following DNA replications (Vila et al., 1996).

The main mechanism of quinolone resistance in bacteria is an alteration of the GyrA subunit of DNA gyrase. Mutation in *par*C, which encodes the ParC subunit of topoisomerase IV, seems to play a secondary role in a great variety of enterobacterial species (Brisse et al., 1999). Mutation in the *gyr*B and *par*E also have been associated with quinolone resistance in both clinical and laboratory isolates; however the frequency of *gyr*B and *par*E mutation in clinical isolates is

much lower in comparision with the frequency of *gyr*A and *par*C mutation (Vila et al., 1996). Mutation in *gyr*A and *par*C leading to quinolone resistance has been reported by many authors (Weigel et al., 1998; Ogbolu et al., 2012; Gharib et al., 2013; Bae et al., 2013; Al-Marzooq et al., 2014;).

Several mutations in *gyr*A and *par*C have been reported in previous studies. Al-Agamy et al. in 2012 reported a C to T transition at 248th position which resulted in the substitution of serine to leucine in codon 83 whereas another transition mutation from G to A at 259th position which resulted in the substitution of aspartic acid to asparigine in codon 87 of *gyr*A. A nucleotide A was exchanged to G at codon 80 of *par*C, which exchanged aminoacid from serine to isoleucine (Al- Agamy et al, 2012). In the present study, this particular point mutation was observed. In particular, Ser83 Leu and Asp87 Asn or Gly substitutions were considered as mutational hotspots in GyrA subunit as reported by other authors (Gharib et al,2013; Liu et al., 2012). Though in the present study substitutions were found in the same position but in this case, Serine83 was substituted by Leucine or Glycine and Aspartic acid87 by Asparagine or Leucine. Fu et al. (2013) also reported Serine83 Tyrosine and Aspartic acd87 Tyrosine along with Serine83 Leucine and Aspartic acid87 Asparigine. The expression of high level fluoroquinolone resistance in Enterobacteriaceae requires the presence of multiple mutations in *gyr*A and/ or *par*C genes (Mirarini et al, 2012). According to Minarini et al, *Klebsiella oxytoca* isolates with reduced susceptibility exhibited Thr83 Ile and Asp87 Tyr in *gyr*A. In this study, another different substituted amino acid was detected in *gyr*A, Asp87 Leu instead of glycine, alanine or tyrosine in *gyr*A, which to our knowledge has not been described so far. The mutations found in this study is not uniform as found in the study of Pazhani et al. (2008) and also was not Ser83 Phe; Ser83 Tyr as described by Ling et al (2003).

Some of the mutations have been reported by other authors (amino acid substitution at codon 57, 85, 91), in addition to the mutations at codon 57, 85, 91, we also identified some silent mutations in codon in 85, 91, 100 and 111 in GyrA of resistant strain. These mutations have been described before by Van Hees et al. (2011). Previous studies have shown that mutation in the *gyr*A leads to reduction in

susceptibility to FQs; however, high level FQ resistance requires an additional mutation in *par*C at either position 80 or 84 (Bagel et al. 1999; Heisig. 1996). This seems to be the first report of combination of rare substitution Tyr74 Leu, Pro76 Phe, His77 Arg and Cys82 Leu with common substitution Ser80 Ile and Glu84 Gly in ParC. Mutation outside the QRDR in codon 131(Leu131Phe) was also reported in this study. Some unique mutations were also reported in codon Asn60 Asp, Lys61 Gly, Tyr63 Iso, Lys64 Leu of GyrA.

4.9 Therapeutic option

The fluoroquinolones have been used to treat a great variety of infections, including gonococcal infections, osteomyelitis, enteric infections or respiratory tract infections, and as a prophylaxis in neutropenic patients, surgery or to prevent spontaneous bacterial peritonitis in cirrhotic patients. They are most commonly used oral antibiotics which are often misused due to self medication. This situation has led to the development of fluoroquinolone resistant strains and infections caused by these isolates have to be treated by other group of antibiotics.

In the current study, when fluoroquinolone resistant strains were subjected to susceptibility study against other groups of antibiotics, polymixinB came up with good activity followed by cefotaxime, ceftazidime and imipenem. In a study conducted by Bouchillon et al. ertapenem and imipenem showed good susceptibility to FQR *E. coli* isolates. Amikacin also came up with good activity against *C. freundii* which complies with the present study though the isolates are different (Bouchillon et al, 2012). In another study by Cheong et al. it was reported that quinolone resistant *E. coli* bacteremia was treated with third generation cephalosporin alone or third generation cephalosporins with aminoglycosides (Cheong et al. 2001). Recently, a study conducted by Nandihal have shown good susceptibility of nitrofurantoin against fluoroquinolone resistant *E. coli* and *Klebsiella* spp.(Nandihal, 2015).

This study could highlight the need for a diagnostic and epidemiological marker which can help in future treatment options. Also investigation should be undertaken to determine the molecular and genetic background of quinolone resistance.