Chapter 1

Introduction

1 INTRODUCTION

1.1. Gastroenteritis

Gastroenteritis or infectious diarrhea is a common medical condition due to the inflammation of stomach and small intestine. It is generally caused by viral or bacterial or parasitic infections and is manifested by diarrhea, abdominal cramp and vomiting [Singh and Fleurat, 2010]. The increase in morbidity and mortality rate due to acute gastroenteritis in the past decades incurs worrisome situation in public health. The diarrheal diseases exist as the second major cause of death in children worldwide (Fig. 1.1) accounting for 1.6 to 2.5 million deaths annually and in developing countries every child encounters 3 episodes of diarrheal infections per year [Kosek et al. 2003; WHO, 2008; UNICEF and WHO, 2009]. India ranks among the top countries with 336,600 cases and a very high number of annual child deaths due to diarrhea [WHO, 2008; UNICEF and WHO, 2009]. Though there is a decline in the mortality rate of diarrhea in the past few years, still it persists as one of the major causes of morbidity and mortality in children [Kosek et al. 2003]. The gastroenteritis caused by protozoan parasites accounts for relatively small proportion of cases in developing countries and is uncommon in developed countries. The parasitic agents such as *Giardia intestinalis*, *Cryptosporidium parvum*, *Entamoeba histolytica* and *Cyclospora cayetanensis* most commonly cause acute diarrheal illness in children [World Gastroenterological Organization (WGO), 2008]. The virus-mediated gastroenteritis is caused by the agents such as rotavirus, enteric adenovirus, norovirus, sapovirus, and astrovirus. Rotavirus remains as the most common etiological agent of diarrhea in children worldwide accounting for around 0.5 million deaths per year, followed by norovirus [Ramani and Kang, 2009; Jain et al. 2014]. Bacterial gastroenteritis is caused by the bacterial genus such as *Vibrio*, *Salmonella*, *Shigella* and also by *Campylobacter jejuni*, *Yersinia enterocolitica*, enterohemorrhagic *Escherichia coli* (EHEC) and other diarrheagenic *E. coli*. In developing countries, gastroenteritis through bacteria is more prevalent whereas viruses are the predominant cause of acute diarrhea, especially during the winter season in industrialized countries [WGO, 2008]. The frequency of

occurrence of intestinal infections due to *Vibrio* and *Shigella* species is more in developing countries like India [Niyogi, 2005; Faruque and Nair, 2008; WGO, 2008]. Hence, the following sections would focus on the major diarrhea-causing pathogenic bacteria in India i.e. *Vibrio* spp. and *Shigella* spp. and the problems in their treatment failure.

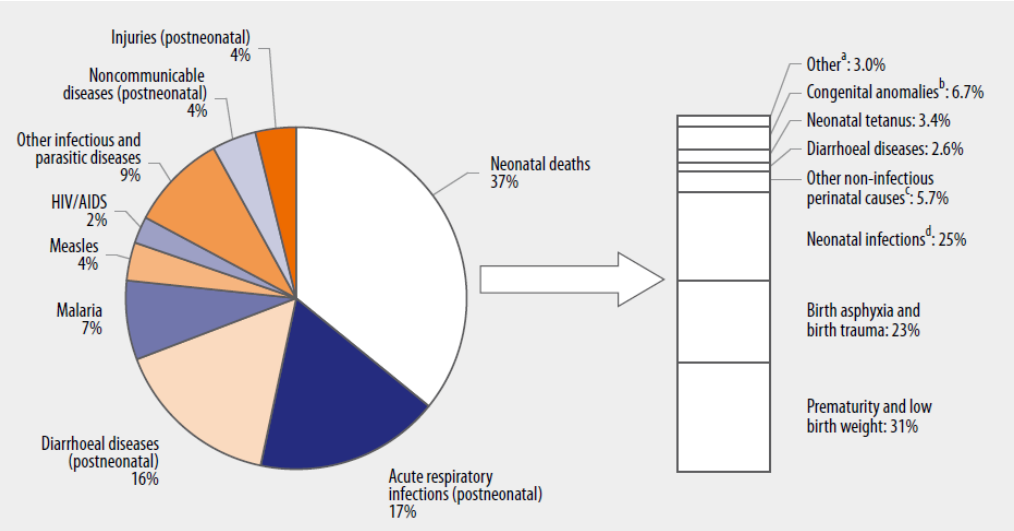

Figure 1.1. Distribution of cause of death among children aged under five years. Diarrheal diseases cause 16 % postneonatal and 2.6% neonatal deaths and thus exist as the second most common cause of child deaths worldwide. (Source: WHO, Global Burden of Disease estimates: 2004 update, 2008).

1.1.1. The enteric pathogens: *Vibrio* spp. and *Shigella* spp.

This section would describe briefly about *Vibrio* spp. and *Shigella* spp., the etiological bacterial agents of gastroenteritis in humans and their prevalence in the globe with a special focus on India.

1.1.1.1. *Vibrio* spp. and their outbreaks

The genus *Vibrio* consists of more than 100 species, but only about 13 of them were reported to cause diseases in humans [Ramamurthy et al. 2014]. Among different pathogenic vibrios, the focus here would be on three major diarrhea-causing vibrios, *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis*, as the incidences of occurrence of outbreaks of these three *Vibrio* spp. is more in India [Thekdi et al. 1982; Okuda et al. 1997; Srinivasan et al. 2006; Nair et al. 2007; Sen et al. 2007; Rajpara et al. 2009; Bhattacharjee et al. 2010;

Chowdhury et al. 2011, 2012; Kanungo et al. 2012; Sarkar et al. 2012; Singh et al. 2012].

The infection caused by the toxigenic *V. cholerae*, popularly known as cholera, is an ancient and heavily destructive disease that continues to pose a serious public health problem among developing world populations which have no access to adequate water and sanitation resources. The world has already witnessed seven pandemics in the past two hundred years (http://www.cdc.gov/cholera/index.html) (Fig. 1.2). The first six pandemics have been caused by the infection of *V. cholerae* O1 classical biotype that seems to be originated from Bangladesh. The seventh pandemic of cholera is due to *V. cholerae* O1 El Tor biotype and O139 serogroup [Mandal et al. 2011]. Globally, cholera outbreaks have increased continuously since 2005 and affected several continents (http://www.cdc.gov/cholera/index.html).

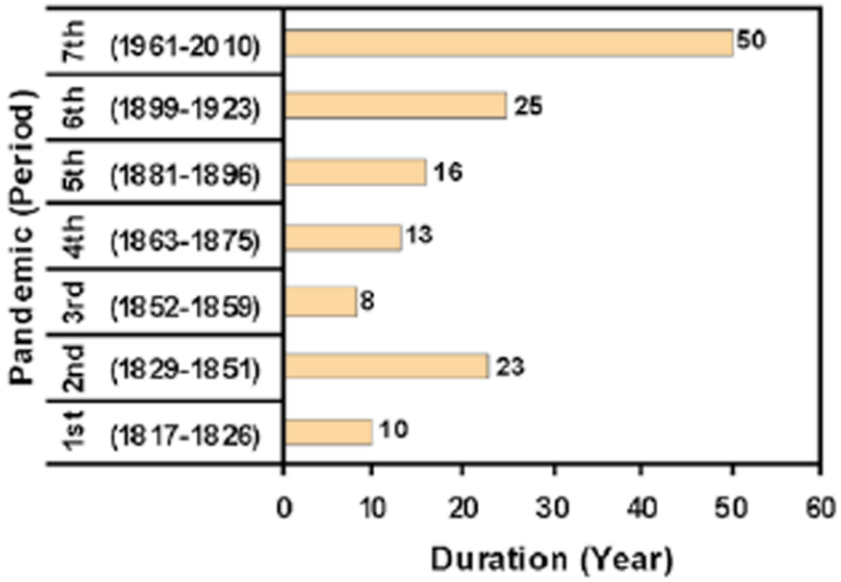

Figure 1.2. Duration and period of seven cholera pandemics [Source: Mandal et al. 2011].

African countries faced more cholera outbreaks than Asia and America. According to the weekly epidemiological record of WHO, in the year 2015, 55% of cases were reported from Africa, whereas, between 2001 and 2009, 93% to 98% of total cases worldwide were reported from that continent. In India, a total of 68 outbreaks were reported across 18 states and union

territories in the country between 1997 to 2006 and the unreported cases may exceed even more [Kanungo et al. 2010]. The Kolkata region of West Bengal is considered as the endemic region for cholera in India and it witnessed several outbreaks in the past two decades [Mandal et al. 2011]. The recent report showed that cholera occurs over a wider geographical area in the country, such as Maharashtra, West Bengal, Tamil Nadu, Andhra Pradesh, Delhi, Goa, Orissa and Madhya Pradesh [Kanungo et al. 2010; Sarkar et al. 2012].

V. parahaemolyticus, the causative agent of food-borne gastroenteritis is indigenous to estuarine, marine and coastal environments throughout the world. This species of *Vibrio* was first isolated in the year 1950 [Fujino et al. 1953] after which it has been recognized as the major cause of seafood-borne illness throughout the world. The species consist of more than 80 serotypes, based on the somatic (O) and capsular (K) antigens [Nair et al. 2007; Ceccaralli et al. 2013]. In India, the gastroenteritis caused by *V. parahaemolyticus* O3:K6 has increased at the beginning of 1996 in Kolkata. In the next few months, the gastroenteritis caused by the same serovar was reported in other neighboring countries such as Vietnam, Indonesia, Bangladesh, Japan, Korea and Thailand [Nair et al. 2007]. By the end of 2006, this serovar was isolated from Europe and the United States marking the beginning of the first pandemic of *V. parahaemolyticus* [Nair et al. 2007]. In Kolkata, the isolation rate of *V. parahaemolyticus* during 2001–2012 ranged from 0.5% to 4% of diarrheal cases. The overall isolation rate was 1.3%, which closely matched a report from Bangladesh [Pazhani et al. 2014]. In 2009, the isolation rate of *V. parahaemolyticus* increased to 4.2% compared to other years. The serovars predominantly isolated during 2009 were O1:K36, O1:K25 and O3:K6 [Pazhani et al. 2014].

V. fluvialis is one of the emerging foodborne pathogens all over the world which causes diarrhea in humans [Ramamurthy et al. 2014]. The organism was first isolated in 1975 from a hospitalized diarrhea patient in Bahrain and was categorized as group F *Vibrio* before it was named as *V. fluvialis* in 1981 [Lee et al. 1981]. *V. fluvialis* was found as the major causative agent of gastroenteritis in Tenri hospital, Japan, since 1979 [Aihara et al.1991]. In 1982

to 1988, about 10 cases of *V. fluvialis*-mediated gastroenteritis due to contaminated seafood were reported in Florida [Klontz and Desenclos, 1990]. *V. fluvialis* was the second major cause of diarrhea in Zhejiang province of China during 1991 [Jiang, 1991]. This pathogen was first reported in Brazil during 1990 [Magalhaes et al. 1990] and subsequently in 1991 [Libinzon et al. 1991]. During 1993, in Gulf-coast, 6% of Vibrio-mediated infections were caused by *V. fluvialis* [Levine and Griffin, 1993]. The prevalence of this species was 9.4% among the hospitalized patients in North Jakarta [Lesmana et al. 2002]. Bangladesh witnessed *V. fluvialis* outbreak after the 1998 flood [Tanabe et al. 1999]. In India, the first case of *V. fluvialis* was observed in Maharashtra during 1981 as a food-borne infection and it has been frequently reported from Kolkata since 1989 [Srinivasan et al. 2006; Rajpara et al. 2009; Chowdhury et al. 2011, 2012; Singh et al. 2012; Ramamurthy et al. 2014].

1.1.1.2. *Shigella* spp. and their outbreaks

Shigellosis is a global human health problem caused by four species of *Shigella* i.e. *S. dysenteriae*, *S. flexneri*, *S.boydii* and *S. sonnei*. Based on O-specific polysaccharide of the lipopolysaccharides on their outer membrane, each species of *Shigella* was subdivided into several serotypes [Niyogi, 2005]. It is reported to cause about 80 million cases of bloody diarrhea and 700,000 deaths per year [WGO, 2008]. Ninety-nine percent of infections caused by Shigella occur in developing countries, and the majority of cases (~70%), and deaths (~60%), occur among children less than five years of age [WGO, 2008]. *S. dysenteriae* type 1 causes more prolonged and severe illness and is frequently associated with life-threatening complications, with more attack rates and high fatality rates than other species of *Shigella*. The incidence of *Shigella* among children is more whereas *S. dysenteriae* type 1 infects all age groups [WGO, 2008]. In the developed countries, children in day-care centers, travellers to developing nations, migrant workers and homosexual men are prone to be affected most by these pathogens. *Shigella* is reported to be associated with 5% to 15% of cases of diarrhea and 30% to 50% of cases of dysentery. The majority of *Shigella* infections are due to endemic shigellosis which is responsible for approximately 10% of all diarrheal episodes among children younger than five years living in developing countries [Ferreccio et

al. 1991] and for 75% of diarrheal deaths [Bennish, 1991; Kotloff et al. 1999]. Epidemic and endemic diseases in developing countries are being majorly caused by *S. dysenteriae* and *S. flexneri* species, whereas, in developed countries, *S. sonnei* is a common cause of sporadic outbreaks. *S. sonnei*, transmitted by uncooked food or contaminated water is involved in over 75% of the cases annually in the United States [Niyogi, 2005]. In general, the illness caused by *S. sonnei* is less severe. The fourth species, *S. boydii*, was first found in India and since then the species is uncommonly encountered except in the Indian subcontinent [Niyogi, 2005].

During 2000, dysentery due to Shigella occurred in Sierra Leone and Lesotho and subsequent to that, Liberia and the Central African Republic faced shigellosis infection in 2003 (Global Alert and Response, WHO; http://www.who.int/csr/don/archive/disease/ shigellosis/en/). In 2004, shigellosis outbreak was reported from Sudan (Global Alert Response, WHO; http://www.who.int/csr/don/archive/disease/shigellosis/en/). In India, various shigellosis cases have been reported since the beginning of 1980 from different parts of the country such as Delhi, West Bengal, Bay of Bengal Islands, South and North Eastern India [Niyogi, 2005; Taneja, 2007].

1.2. Treatment complications in *Vibrio* and *Shigella* infections due to the problem of drug resistance

Oral rehydration therapy followed by administration of antibiotics is the standard acceptable form of treatment for *Vibrio* infections, whereas antibiotic administration comes as a primary line treatment in the case of *Shigella* infections [Niyogi, 2005; Faruque and Nair, 2008]. The introduction of antibiotics for treating diarrheal diseases in the twentieth century led to an incredible reduction in the death rate of humans caused due to these diseases. But the emergence of resistance to antibiotics in bacteria rendered them an ability to rise up against these magic bullets. This section explains the problem of drug resistance which causes the difficulties while treating the infections of *Vibrio* and *Shigella* species in humans.

Antibiotic resistance of a bacterium is its resistance to an antibiotic to which it was previously sensitive. Resistant microbes are able to withstand the effect of antibiotics so that standard treatments become ineffective and infections persist and may spread to other organisms. Bacteria resist antibiotic action mainly in three ways: by reducing drug accumulation by efflux action or porin mutations, by altering or protecting drug targets and by enzymatic modification/inactivation of drugs [Salyers and Whitt, 2012]. **Efflux pumps** play a major role in conferring resistance to antibiotics by efficiently recognizing and throwing them out of the cells (Fig. 1.3A). Efflux pumps confer only low level resistance to the bacteria towards drugs but their over expression or co-operativity with other mechanisms could result in moderate to high level resistance [Bhardwaj and Mohanty 2012]. **Porins** present in the cell membrane of bacteria are the passages which facilitate the entry and exit of antibiotics and other small organic molecules (Fig. 1.3A). Decrease in the expression of porins results in reduced uptake of antibiotics. **Mutations at the antibiotic target sites** are the main mode of resistance to most of the antibiotics. Mutations occurring as a result of replication errors reduce the affinity of the antibiotics to their targets resulting in the resistant phenotype (Fig. 1.3A). There are few **enzymes that either degrade or chemically modify** the antibiotics (Fig. 1.3A) so that they cannot exert their action and few **proteins are also known to protect the target** from antibiotics [Bhardwaj and Vinothkumar, 2014].

Though antibiotic resistance is ancient, the indiscriminate use of antibiotics created an immense selective pressure on the bacteria which ultimately facilitated the fast evolution of resistant bugs. This process was mediated through the pivotal role of various factors like efflux pumps /porins, mutations in the antibiotic target sites, and acquisition of resistance genes by horizontal gene transfer (HGT). The process of HGT enables bacteria to exchange genetic material within themselve without the requirement of cell division (Fig. 1.3B). Different kinds of mobile genetic elements (MGEs) are transferred between bacteria through this process leading to the adaptation and evolution of bacteria/bacterial communities in tune with the changing environments. HGT is mediated by the processes of transformation,

transduction or conjugation and different types of MGEs such as integrons, transposons and plasmids could move through these processes of HGT [Bhardwaj and Vinothkumar, 2014].

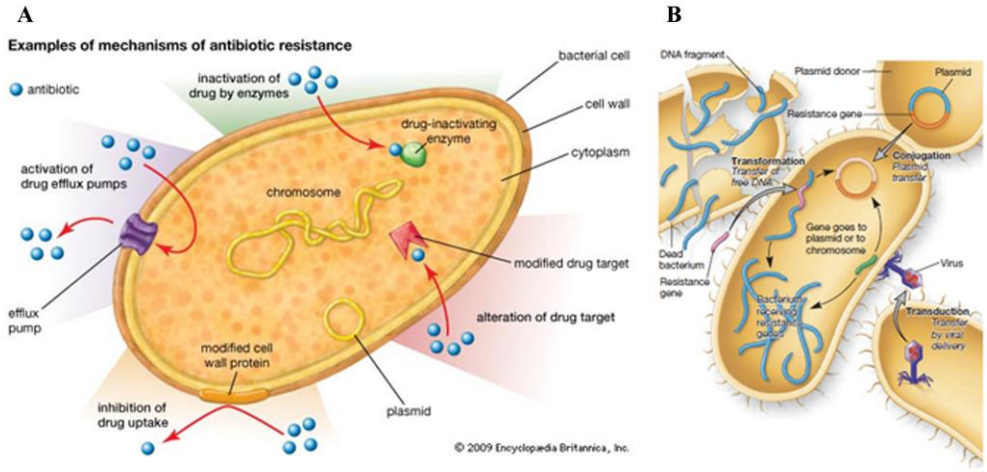

Figure 1.3. Factors responsible for antibiotic resistance in bacteria. (A) Various mechanisms exhibited by bacteria to combat antibiotics (source: Encyclopedia Britannica, Inc. 2009). (B) Different processes of horizontal gene transfer responsible for acquired resistance traits of bacteria (source: https://www.studyblue.com /notes/note /n/medical-microbiology/deck/5418270).

Bacteria exposed to multiple antibiotic environments were endowed with resistance mechanisms for multiple drugs which eventually exacerbated the treatment complication. The selective pressures in health care and community settings spawned multidrug resistance (MDR) as evidenced by the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Staphylococcus aureus* (VRSA), extended-spectrum beta-lactamase (ESBL)-producing bacteria and quinolone resistant bacteria. The incidence of drug resistance in the clinical isolates of bacteria is increasing at an alarming rate and treating the infectious diseases caused by these bacteria has become a great challenge to mankind. In 2009, WHO called antibiotic resistance as one of the three greatest threats to human health, and in 2011, the focus of World Health Day was ‘Combating Antibiotic Resistance’ [Chan, 2011]. A recent report from WHO revealed the pervasiveness of antibiotic resistance across many infectious agents, especially in seven different bacteria responsible for

common serious diseases such as bloodstream infections (sepsis), diarrhea, pneumonia, urinary tract infections and gonorrhea [WHO, 2014].

As mentioned above, the diarrheal diseases caused by multidrug resistant bacteria (like *Vibrios*, *Shigella* spp., etc.) are a major health problem in the developing countries. The increased drug resistance among bacteria towards various natural and semi-synthetic antibiotics led to the introduction of synthetic drugs such as quinolones due to their broad spectrum of activity and possibilities of the absence of resistance mechanisms in bacteria to these synthetic drugs. Nowadays, quinolone group of antibiotics are widely used clinically to treat these diseases. Soon, the bacteria also acquired resistance to quinolones further complicating the treatment of diarrheal diseases.

1.3. Quinolone class of antibiotics

The emergence of drug resistance concomitant with the introduction of antibiotics has made humans to continuously seek for new antibiotics which were expected to overcome the evolving pathogens. This paved the way for development of various synthetic and semi-synthetic antibiotics with improved efficacy and extended target coverage. Among different synthetic antibiotics like sulfonamides, quinolones and oxazolidinones, quinolones gained popularity because of their wider application, broader spectrum of activity and drug safety [Sarvanos and Duff, 1992; Brown, 1996; Walker, 1999]. In addition, it was hypothesized that resistance would not be mounted against the synthetic compounds which are not seen by bacteria in natural ecosystems. In this section, the focus would be on quinolone class of antibiotics, their targets in bacterial cell and the bacterial resistance machinery against these widely used synthetic antibiotics.

Quinolones are a class of antibiotics which specifically kill bacteria by inhibiting the synthesis of nucleic acids. The parent compound of quinolones, nalidixic acid, was derived from the antimalarial drug chloroquine in 1962 [Andriole, 2005; Bolon, 2011]. Nalidixic acid is composed of naphthyridine ring having ethyl and methyl group attached to its N1 and C7 position

respectively along with keto and carboxyl group attached to its C4 and C3 positions respectively (Fig. 1.4). Though it had narrow spectrum antibacterial activity, it was widely used for urinary tract infections and diarrhea until the introduction of broad spectrum fluoroquinolones [Drilca and Zhao, 1997; Van Bambeke et al. 2005]. Fluoroquinolones were derived from nalidixic acid by the introduction of a fluorine atom at C6 position of naphthyridine ring and also replacing the N8 by a carbon atom (Fig. 1.4) [Ball, 2000].

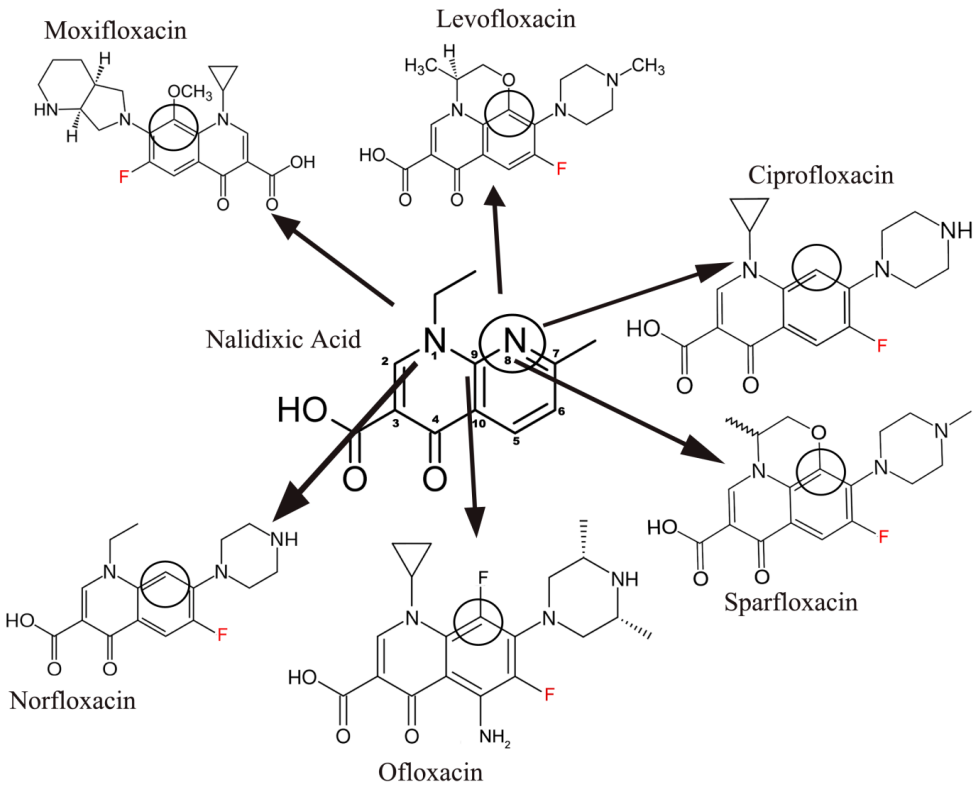

Figure 1.4. Structures of nalidixic acid and some of the fluoroquinolones. Derivation of fluoroquinolones from parent quinolone (nalidixic acid) by the addition of a fluorine atom (shown in red) at C6 position of naphthyridine ring and by replacing N8 by a carbon atom (shown inside circles).

All the available quinolones are grouped into four generations based on their spectrum of activity [O'Donnell, 2004; Bolon, 2011]. The first generation quinolones like nalidixic acid and cinoxacin were active against aerobic Gram-negative bacteria but showed little activity against aerobic Gram-positive bacteria or anaerobes. The second generation quinolones include

fluroquinolones such as norfloxacin, ciprofloxacin, ofloxacin, lomefloxacin and levofloxacin which showed broader Gram-negative spectrum and moderately increased Gram-positive spectrum. Piperazine moiety at C7 position of quinolone nucleus is the general feature of second generation quinolones with increased activity against *Pseudomonas aeruginosa*. Sparfloxacin, gatifloxacin and grepafloxacin fall under third generation quinolones that showed greater potency against Gram-positive organisms. A superior coverage against pneumococci and anaerobes were achieved by fourth generation quinolones like trovofloxacin, moxifloxacin, gemifloxacin and garenoxin [O'Donnell, 2004; Andriole, 2005; Bolon, 2011]. Although many of the quinolone derivatives were removed from the market because of some safety concerns, remaining drugs which were proved as safe, gained widespread use due to their favorable pharmacokinetics and broad antimicrobial spectra [Bolon, 2011].

1.3.1. Targets of quinolones in bacterial cell and their mode of activity

Quinolones target two enzymes, DNA gyrase and topoisomerase IV, which are essential for the vital activities of the bacterial cell such as DNA replication, transcription, recombination and repair. DNA gyrase is a special type II topoisomerase which is present in prokaryotes only and it introduces negative supercoiling rather than removing it [Watson et al. 2004]. During replication, the two strands of DNA are separated by helicase action that causes a reduction in linking number which in turn results in positive supercoiling in front of the replication fork. DNA gyrase removes the positive supercoiling ahead of the replication fork by introducing negative supercoiling and thereby renders an uninterrupted movement of the replication fork. The topoisomerase IV enzyme is known for unlinking of daughter chromosomes after replication (decatenation). DNA gyrase is a tetrameric protein having two A (GyrA) and two B (Gyr B) subunits which wrap DNA into a positive supercoil. The active site of the enzyme consists of tyrosine, which breaks the phosphodiester bond of a duplex DNA and forms phospho-tyrosine bond through its hydroxyl group. The other end of the DNA is also held by the enzyme to form a protein bridge. Another region of DNA is passed through the nick created by DNA gyrase and then the nick would be resealed [Drilca and Zhao, 1997; Watson et

al. 2004]. Both DNA gyrase and topoisomerase IV create double strand break and allow double strand passage and they require ATP for this action. The main difference in the action of these two enzymes is that gyrase wraps DNA around itself while topoisomerase IV does not which may lead to the functional differences [Drilca and Zhao, 1997].

It was evidenced that quinolones form a ternary complex with topoisomerase enzymes and DNA and cease the enzyme activity [Drilca and Zhao, 1997]. This complex is called as cleaved complex as it contains broken DNA. As discussed above, type II topoisomerase enzymes cause a double strand break and allow the passage of another duplex through the nick. Quinolones were found to interact with both the DNA and the enzyme in the cleaved complex. But the reversible binding of quinolones with the DNA-enzyme binary complex allows the enzyme to generate the double strand break only and not the passage of another duplex [Shen et al. 1989; Kampranis and Maxwell, 1998]. The drugs, with the help of aromatic rings stack against the DNA bases (-1 and + 1 base pairs) at the site of cleavage and thus cause a misalignment of DNA at both the sides of the break which eventually prevents the religation of the cleaved DNA. Similarly, helix-4 of the GyrA or ParC which harbors quinolone resistance determining region (QRDR) of the enzymes seems to interact with drug [Berger et al. 1996; Morais Cabral et al. 1997; Laponogov et al. 2009; Bax et al. 2010; Laponogov et al. 2010; Wohlkonig et al. 2010].

Though binding of quinolones to the enzyme-DNA complex leads to inhibition of nucleic acid synthesis, it does not cause lethality to the cells. The binding of quinolones just causes a bacteriostatic action as the formation of cleaved complex is reversible. The lethality to the cell is caused as a result of chromosome fragmentation and cell death induced by reactive oxygen species (ROS) [Drlica et al. 2009]. The chromosome fragmentation occurs in two ways, one is protein synthesis-dependent as it involves proteases or nucleases in releasing DNA breaks from the cleaved complex and the other is protein synthesis-independent where quinolones facilitate the dissociation of gyrase subunits and release the double strand break [Ikeda et al.1982; Ikeda et al.1984; Malik et al. 2006] (Fig. 1.5). The former pathway can be inhibited by chloroamphenicol and so the first generation quinolones like nalidixic acid fail

to kill the cells in the presence of chloroamphenicol whereas second generation quinolones like ciprofloxacin are not influenced by it [Drlica and Zhao,1997; Drlica et al. 2009; Collin et al. 2011] (Fig. 1.5). The chromosome fragmentation eventually triggers the accumulation of highly toxic ROS which amplifies the lethal action of the drug ultimately causing the cell death [Dwyer et al. 2007; Kohanski et al. 2007; Kohanski et al. 2010].

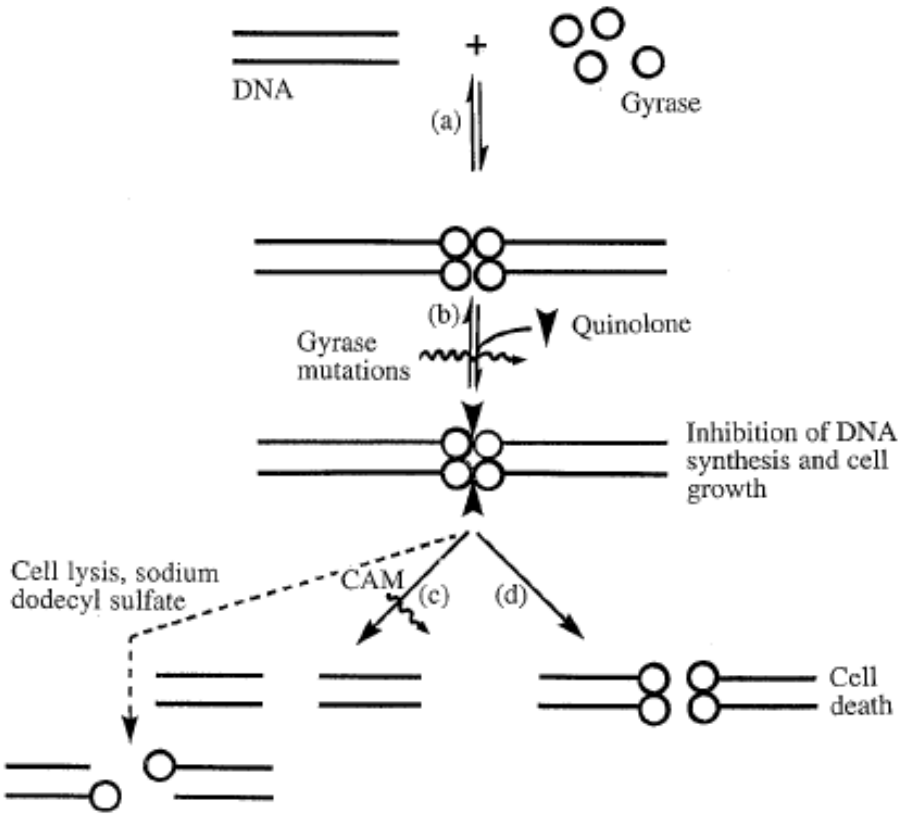

Figure 1.5. Intracellular action of quinolones. (a) DNA gyrase interacts with DNA to form a cleaved complex. (b) Quinolones trap cleaved complex. Trapping of complex blocks DNA synthesis and cell growth. Gyrase mutations prevent trapping by the quinolones. (c) lethal double-strand DNA breaks are released by a putative trapped complex removal system. Chloramphenicol (CAM) or rifampin treatment of cells blocks this reaction. (d) fluoroquinolones at high concentrations stimulate gyrase subunit dissociation, which releases lethal double-strand breaks. The dotted line indicates release of staggered double-strand DNA breaks when cell lysates are treated with ionic detergents such as sodium dodecyl sulfate. [Source: Drilca and Zhao, 1997].

1.3.2. Quinolone resistance mechanisms

Development of resistance towards quinolones began to emerge right with the introduction of first generation quinolone, the nalidixic acid in 1962. The increased use of this drug led to the increased development of resistance [Jacoby, 2005]. Earlier it was believed that target mutations (mutations in topoisomerase enzymes) and efflux pumps were the possible mechanisms of quinolone resistance. As there was no natural producer of quinolones, the quinolone resistance genes harbored in plasmids or other MGEs which facilitate horizontal gene transfer were not expected. Later that belief was disproved due to the discovery of various MGEs-borne molecular factors like target-protecting proteins, quinolone-modifying enzymes and efflux pumps [Jacoby, 2005; Hernandez et al. 2011]. As the bacterial pathogens are gaining resistance to quinolones, treating the infections caused by them has become difficult nowadays. Quinolone resistance could be mediated by either chromosome-borne or plasmid-borne genetic elements. The chromosome-borne genetic factors involve, (i) mutations in the DNA gyrase (*gyrA* and *gyrB*) and (or) topoisomerase IV (*parC* and *parE*) genes, (ii) chromosomal efflux pumps genes and (iii) chromosomal quinolone resistance (*qnr)* genes. The plasmid-mediated quinolone resistance (PMQR) has also been described with three mechanisms, (i) a quinolone-resistance/topoisomerase-protection mechanisms encoded by the *qnr* genes, (ii) a ciprofloxacin-modifying enzyme encoded by *aac(6')-Ib-cr* gene (Robicsek et al. 2006); and (iii) plasmid borne-efflux pumps [Perichon et al. 2007; Yamane et al. 2007]. These PMQRs are transferable traits and therefore, play a major role in dissemination of quinolone resistance. In the following sections, all these mechanisms are discussed in detail.

1.3.2.1. Mutations in the topoisomerase genes (chromosome-borne)

Mutations in the genes of topoisomerase enzymes are the main cause of quinolone resistance. Such spontaneous mutations occur as a result of replication error in a bacterium, at rates as high as 1 in 10^6 to 1 in 10^9 [Sanders et al. 2001]. A mutant bacterium having an alteration in topoisomerase enzymes could withstand antibiotic stress and evolve as a resistant bug.

Mutations tend to cluster in a region called the QRDRs of subunits of DNA gyrase or topoisomerase IV, which results in reduced drug affinity of those enzymes [Yoshida et al. 1990; Jacoby, 2005; Van Bambeke et al. 2005; Kakinuma et al. 2012]. Mutations in *gyrA*, *gyrB*, *parC* and *parE* genes have variable effects on MICs in different species of bacteria. In Gram-negative bacteria, high-level quinolone resistance is mainly due to the mutations in the genes encoding gyrase subunits, *gyrA* and *gyrB* (mainly in *gyrA*), whereas mutations in genes encoding topoisomerase IV subunits *parC* and *parE* are prevalent in Gram-positive bacteria [Hernandez et al. 2011]. Mutations conferring resistance typically occurs in a stepwise manner. Generally, the initial mutation occurs in *gyrA* (in the case of Gram-negative bacteria) or *parC* (in the case of Gram-positive bacteria) genes [Drlica and Zhao, 1997; Van Bambeke et al. 2005]. The first mutation helps to select the bacteria in quinolone stress by reducing the susceptibility of topoisomerase to the drug and allows accumulation of more mutations in the subunits of same or the other target enzymes. The accumulation of multiple mutations in the drug targets facilitates the development of high-level resistance to quinolones in bacteria [Drlica and Zhao, 1997; Dougherty et al. 2001; Van Bambeke et al. 2005; Jacoby, 2005]. The type of bacterial species and the kind of quinolone used determines the order in which mutations occur and the quantum of change in MIC [Bearden and Danziger, 2001]. Clinical failure of quinolones can occur as a result of many such bacterial mutations. Mutations at amino acid positions 83 and 87 of GyrA and positions 80 and 84 of ParC have been reported as a cause for reduced susceptibility of bacteria towards quinolones [Drlica and Zhao, 1997; Drilica et al. 2009; Chowdhury et al. 2011; Singh et al. 2012; Kutar et al. 2013; Fu et al. 2013]. The substitution of hydrophilic amino acid (serine) by hydrophobic residues (leucine or isoleucine), or substitution of acidic residue (aspartic acid) by basic amino acid (asparagine) or substitution of negatively charged amino acid (glutamic acid) by positively charged residue (lysine) are the well-documented substitutions which reduce the susceptibility of the target enzymes to quinolones. These residues are known to interact with the drug at quinolone binding pocket (QBP), the region where quinolones interact with both QRDR of enzyme and cleaved DNA [Heddle and Maxwell, 2002]. The alteration in the residues of QRDR of

topoisomerase enzymes causes a conformational change in QBP, which eventually prevents the binding of the drug in the pocket [Heddle and Maxwell, 2002].

Generally, these mutations occur as a replication error prior to the antibiotic exposure and at the time of antibiotic pressure, it helps the cells to resist the drug beyond the concentration required to kill wild type cells. The wild type cells fail to form colony at or above minimum inhibitory concentration (MIC). The selective enrichment of resistant mutants occurs only above MIC and the concentration where the colony's recovery of mutants ceases, is called mutant prevention concentration (MPC). Additional mutation is required for the bacteria to withstand the concentration beyond MPC, which is a rare phenomenon. So the drug concentration range, which favors the selection of mutants, between MIC and MPC is called Mutant selection window [Drlica and Zhao, 2007]. As described above, the resistance conferring mutations occur in a stepwise manner and at each step of acquisition of mutations, the values of MICs and MPCs increase [Eliopoulos et al. 1984; Li et al. 2002]. In other words, selection window increases at each step of mutation acquisition. Hence, the mutant selection window is important to optimize antimicrobial dose regimens and to avoid the emergence of resistant mutants [Heddle and Maxwell, 2002].

1.3.2.2. Efflux pumps (chromosome- and plasmid-borne)

The second resistance mechanism involves expression/overexpression of efflux pumps that transport quinolones and other antibiotics out of the cell. Efflux pumps are ubiquitous and are encoded either by chromosomal genes or by the genes associated with MGEs. These genes are responsible for intrinsic resistance under constitutive expression and cause low to moderate level of quinolone drug resistance under induced or activated conditions [Van Bambeke et al. 2005]. Mutations that occur in the regulatory elements of efflux pumps lead to overexpression of pumps which ultimately causes increased efflux activity [Cohen et al. 1989, 1993 Alekshun and Levy, 1997; Wang et al. 2001]. Quinolone-specific efflux pumps have been reported and characterized (e.g. SmrA, PmrA, NorA, NorM, PmpM, AcrB, VcrM, VcmA,

BmrA, MepA, VCH and VFH) from different bacterial species [Morita, 2000; Van Bambeke et al. 2005; Piddock et al. 2006; Mohanty et al. 2012]. The MATE (multidrug and toxic compounds extrusion) family efflux pumps like NorM, VCH, VFH are known to effectively efflux out hydrophilic quinolones such as norfloxacin, ciprofloxacin and ofloxacin and not the hydrophobic quinolones such as sparfloxacin, nalidixic acid and moxifloxacin [Morita, 2000; Dougherty et al. 2001; Mohanty et al. 2012]. Resistance due to efflux pumps causes only low to moderate level of resistance but they favor the emergence of resistance mutants by rendering the survival ability to the cells at suboptimal concentrations of antibiotics [Lomovskaya et al. 1999; Dougherty et al. 2001; Van Bambeke et al. 2005].

Two plasmid-mediated quinolone transporters (QepA and OqxAB) have been described [Hansen et al. 2004, 2005, 2007; Yamane et al. 2007; Cattoir et al. 2008; Zhao et al. 2010]. The presence of *qepA* in *Enterobacteriaceae* and *Vibrionaceae* was reported from different parts of the globe [Cattoir et al. 2008; Kim et al. 2009; Ma et al. 2009; Amin and Wareham, 2009]. QepA is a 511-amino acid protein belonging to MFS (major facilitator superfamily) transporters and shown to efflux out mainly norfloxacin, ciprofloxacin, nalidixic acid and also other compounds like erythromycin, acriflavine and ethidium bromide. The RND (resistance nodulation division) family pumps OqxAB confer resistance to Olaquindox (a quinoxaline derivative), nalidixic acid and ciprofloxacin [Hansen et al. 2004, 2005, 2007; Strahilevitz et al. 2009; Rodriguez-Martinez et al. 2011].

1.3.2.3. *qnr* genes (chromosome- and plasmid-borne)

Qnr proteins belong to pentapeptide repeat family and are capable of protecting DNA gyrase from quinolone action. These proteins are characterized by five semi-conserved tandem repeat motifs represented by general pentapeptide repeat formula (Ser, Thr, Ala or Val) (Asp or Asn) (Leu or Phe) (Ser, Thr or Arg) (Gly) [Vetting et al. 2006; Strahilevitz et al. 2009; Rodriguez-Martinez et al. 2011]. Qnr proteins consist of two domains of pentapeptide repeats separated by a single amino acid, usually glycine [Vetting et al. 2006; Strahilevitz et al. 2009]. Qnr acts by protecting DNA

gyrase and topoisomerase IV from quinolones by binding to the enzyme prior to the binding of DNA [Tran and Jacoby, 2002]. These proteins mimic the structure of DNA and they compete with DNA for enzyme binding. As Qnr occupies the DNA binding site of the enzyme, it prevents the binding of DNA to the enzyme and hence the number of enzyme-DNA complexes, the target of quinolone is reduced. As a result of this, the formation of cleaved complex is minimized and eventually cells are protected from the lethal action of quinolones [Tran et al. 2005a, 2005b]. Several *qnr* genes have been widely reported from *Enterobacteriaceae* and *Vibrionaceae* families and *Vibrionaceae* family was thought to be a possible reservoir for Qnr-like quinolone resistance determinants [Poirel et al. 2005a]. So far, five families of *qnr (qnrA, qnrB, qnrC, qnrD* and *qnrS*) have been reported in plasmids among the bacterial species [Strahilevitz et al. 2009; Rodriguez-Martinez et al. 2011]. Both *qnrA* and *qnrS* genes encode 218-amino acid proteins and exist as seven and nine alleles respectively. The *qnrC* gene encodes 221-amino acid protein whereas *qnrD* encodes 214-amino acid protein. The allelic forms of *qnrC* gene have not been reported so far and *qnrD* exists as two alleic forms. The 214-amino acid protein encoding *qnrB* genes are found to exist in eighty allelic forms (http://www.lahey.org/qnrStudies/) [Jacoby et al. 2008; Strahilevitz et al. 2009; Rodriguez-Martinez et al. 2011].

Genes for pentapeptide repeat proteins with sequence similarity to plasmid-borne Qnr proteins have been reported on the chromosomes of both Gram-positive and Gram-negative bacteria [Strahilevitz et al. 2009]. The chromosome-borne *qnr*-like genes were largely reported in *Vibrionaceae* family (*V. Vulnificus*, *V. fisheri* and *Photobacterium profundum*) [Poirel et al. 2005a; Saga et al. 2005; Kim et al. 2010]. *qnrVC1* isolated from *V. cholerae* O1 from a cholera epidemic in Brazil was found as a gene cassette in a chromosomal class 1 integron [Fonseca and Vincente, 2012]. The origin of these *qnr* genes is likely to be the chromosomes of aquatic environmental organisms. Accumulation of quinolones in the environment enriched the organisms having *qnr* genes that acted as reservoirs from where other pathogenic organisms acquired these genes [Strahilevitz et al. 2009; Rodriguez-Martinez et al. 2011]. A *qnrVC5* gene of chromosomal origin was

found to be associated with transferable plasmids from clinical isolates of *V. fluvialis* [Singh et al. 2012]. This study showed that though these genes were of chromosomal origin, they were circulating among the bacterial community through plasmids. Qnr proteins are found to have functional similarity with other well-studied pentapeptide repeat proteins like MfpA and McbG having amino acid identity 18.9% and 19.6% respectively with that of QnrA. The organisms producing the microcin B17, a topoisomerase poison, also produce McbG to protect its own DNA gyrase from the toxic effect of microcin B17 [Garrido et al. 1988; Heddle et al. 2001]. So it is evident that the pentapeptide repeat proteins are generally evolved for protecting topoisomerases from the naturally occurring toxins that inhibit those enzymes and also evolved to protect the enzymes from other topoisomerase-inhibiting agents like quinolones. Though higher-level *qnr*-mediated resistance has not been reported, they could help the isolates to attain clinical breakpoint of quinolone resistance in combination with the other mechanisms [Baranwal et al. 2002; Srinivasan et al. 2006; Chowdhury et al. 2011; Singh et al. 2012].

1.3.2.4. Ciprofloxacin modifying enzyme (AAC (6') -Ib-Cr)

AAC(6')-Ib-cr, a variant aminoglycoside acetyltransferase, capable of reducing ciprofloxacin activity in addition to modifying aminoglycosides, is carried on plasmids and more prevalent than Qnr proteins [Robicsek et al. 2006]. This enzyme has acquired the ability to inactivate quinolones (ciprofloxacin and norfloxacin) by N-acetylating the amino nitrogen on its piperazinyl group. Two amino acid changes (Trp102Arg and Asp179Tyr) render the ability to the enzyme to additionally inactivate quinolones apart from aminoglycosides. The effect on MIC by AAC (6')-Ib-cr is less than that conferred by Qnr protein and the drug spectrum covered by this enzyme is also small (ciprofloxacin and norfloxacin only) [Robicsek et al. 2006].

It is reasonably well documented that quinolone resistance in pathogenic bacteria through intrinsic and acquired traits causes a major health problem. The synergistic action of all these chromosome- and plasmid-borne factors help the pathogen acquire high-level resistance towards quinolones, as

described by many researchers [Baranwal et al. 2002; Srinivasan et al. 2006; Rushdy et al.2013; Zhu et al. 2013a].

1.3.3. Databases available for quinolone resistance genes

Since the discovery of first PMQR resistance gene *qnrA1* in 1998, a considerable number of *qnr* genes and other PMQR genes have been discovered and reported in the last decade. The *qnr* genes reported from several parts of the globe lacked a standard nomenclature or numbering system which created a chaos. In order to avoid the ambiguity and to systematically name the *qnr* genes of varying sequences, Jacoby et al. attempted to frame the criteria based on which these genes could be classified [Jacoby et al. 2008]. They have created a database where all the newly found *qnr* alleles can be verified and numbered accordingly (http://www.lahey.org/qnrStudies/). It has a collection of 80 alleles of QnrB as of 31st December, 2015. Similarly, in order to find the *qnr* genes in a fragmented nucleotide sequence of metagenomic data set, a tool/software was developed by Systems Biology and Bioinformatics group of University of Gothenburg, Sweden [Boulund et al. 2012] (http://bioinformatics.math.chalmers.se/qnr/index.html). They have developed this tool in order to understand the role of the environment as a reservoir of these kind of genes and to focus on their routes of transfer.

1.4. Prevalence of quinolone resistance in *Vibrio* and *Shigella* spp.

Since the introduction of quinolones, the resistance of the bacteria to these antibiotics has become common, widespread and generally non-clonal [Strahilevitz et al. 2009]. As these drugs were in use in India and other countries for a long time for treating different infectious diseases including cholera, the emergence of fluoroquinolone resistance was reported from early 2000 [Ghosh and Ramamurthy, 2011]. Worldwide surveillance studies suggested that resistance rates of fluoroquinolone increased faster in the past few years in almost all the bacterial species [Dalhoff, 2012]. The current

scenario increased the threats on the clinical utility of these drugs for the treatment of various infections [Kim and Hooper, 2014].

Vibrio species are known to resist quinolone action by exporting drugs through efflux pumps, chromosomal mutations and by acquiring the quinolone resistance gene-bearing plasmids or other MGEs [Sack et al. 2001; Ghosh and Ramamurthy, 2011; Kitaoka et al. 2011; Ramamurthy et al. 2014]. Mutation in GyrA followed by mutation in ParC is reported as the main mechanism of quinolone resistance in *Vibrios* [Ghosh and Ramamurthy, 2011]. *Vibrio* species utilize efflux pumps to throw the antibiotics and other foreign substances out of the cell. Vcam, one of the few ATP-binding cassette (ABC) superfamily efflux pumps in bacteria, was reported in *V. cholerae* to efflux out norfloxacin and ciprofloxacin along with other drugs such as tetracycline and doxorubicin [Kitaoka et al. 2011]. The MATE pump, NorM which effectively effluxes out hydrophilic fluoroquinolones was first reproted from *V. parahaemolyticus* [Morita, 1998]. *V. cholerae* non-O1/non-O139 is known to use an array of MATE efflux pump systems, namely VcmB, VcmD, VcmH, VcmN, VcmA and VcrM [Huda et al. 2003; Begum et al. 2005]. Two MATE-type efflux pumps, namely VFH and VFD in *V. fluvialis* were found to be responsible for fluoroquinolone resistance [Mohanty et al. 2012]. The PMQR determinants were frequently reported from *Vibrio* species. As mentioned in section 1.3.2.3, the *Vibrionaceae* family was found to be a possible reservoir for Qnr-like quinolone resistance determinants [Poirel et al. 2005a]. In *V. cholerae,* various alleles of *qnrVC* were found to contribute to quinolone resistance and those alleles were reported to be found in MGEs such as integrons, SXT and plasmids (Table 1.1) [Fonseca and Vincente, 2013]. *V. fluvialis* strains were also known to harbor *qnr* genes such as *qnrVC5* [Rajpara et al. 2009; Singh et al. 2012; Fonseca and Vincente, 2013] and *qnrA1* [Chowdhury et al. 2011]. The *qnr* homologue VPA0095 was reported from *V. parahaemolyticus* [Saga et al. 2005; Aedo et al. 2014] and *qnrVC5* and *qnrVC6* were found in the same species (Liu et al. 2013; Fonseca and Vincente, 2013). The *aac(6') Ib-cr* gene was reported from *V. fluvialis* [Rajpara et al. 2009; Chowdhury et al. 2011] and *V. parahaemolyticus* [Aedo et al. 2014].

Table 1.1. *qnrVC* alleles and their association with MGEs

Allele	MGEs	Organisms
qnrVC1	Class1 integron, SXT element	*V. cholerae*, *P. aeruginosa*
qnrVC2	Plasmid	*V. cholerae*
qnrVC3	Class1 integron	*V. cholerae*
qnrVC4	Class1 integron	*V. cholerae*, *E. coli*, *Aeromonas* spp., *Pseudomonas* spp.
qnrVC5	Class1 integron, Plasmids	*V. cholerae*, *V. parahaemolyticus*, *V. fluvialis*
qnrVC6	Class1 integron	*Acinetobacter baumannii*, *V. parahaemolyticus*
qnrVC7	Plasmid	*V. cholerae*

Quinolone resistance in *Shigella* species is also a serious global problem, especially in Asia and Africa compared to Europe and US [Gu et al. 2012]. Quinolone resistance in *Shigella* species is widely increasing in developing countries [Sack et al. 2001]. Mutations in DNA gyrase or topoisomerase IV subunits were found to be the major factor for quinolone resistance in *Shigella* [Sack et al. 2001]. The involvement of efflux systems that pump out fluoroquinolones in *Shigella* species has been demonstrated by various groups [Azmi et al. 2014; Kim et al. 2008; Ghosh et al. 1998]. In the presence of ciprofloxacin, the induced expression of efflux pump genes, namely *tolC*, *mdfA* and *ydhE* was documented [Kim et al. 2008]. PMQR has been reported frequently in *Shigella* spp. [Strahilevitz et al. 2009; Rodriguez-Martinez et al. 2011]. The *qnrS* was first reported in *S. flexneri* [Hata et al. 2005] and subsequently, the same gene was reported from different geographic locations in the same genus [Pu et al. 2009; Xiong et al. 2010; Ghosh et al. 2014]. The other *qnr* genes such as *qnrA* [Xiong et al. 2010] and *qnrB* [Bhattacharya et al. 2011] were also found in *Shigella* species. The *aac (6') Ib-cr* gene was frequently reported in *Shigella* species worldwide [Pu et al. 2009; Xiong et al. 2010; Bhattacharya et al. 2011; Yang et al. 2013; Ghosh et al. 2014]. The plasmid-mediated quinolone specific efflux pump QepA was also reported from this genus [Yang et al. 2013; Xia et al. 2013].

The systematic review of quinolone resistance determinants reported from *Vibrio* and *Shigella* species in the past twelve years is listed in Table 1.2.

Table 1.2. Prevalence of quinolone resistance determinants in *Vibrio* and *Shigella* spp.

S.No	Organism	Topoisomerase mutations	PMQR factors	Remarks	Reference
1	*V. cholerae*		*qnrVC7*		Po et al. 2015
2	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser60Asn), (Tyr65Phe), (Ser85Ile), (Ala128Ser), (Lys129Arg)		Topoisomerase genes of Haitian variant *V. cholerae* O1	Divya et al. 2014
3	*V. cholerae*		*qnr* cassette in the small chromosome of *V. cholerae* MS6		Okada et al. 2014
4	*V. parahaemolyticus*	GyrA and ParC	ND		Jiang et al. 2014
5	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Kumar et al. 2014
6	*V. parahaemolyticus*		VPA0095, *aac(6') Ib-cr*		Aedo et al. 2014
7	*V. parahaemolyticus*		*qnrVC6*		Liu et al. 2013
8	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Ismail et al. 2013
9	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu) in native isolates, GyrA (Asp87Asn) and ParE (Asp420Asn; Pro439Ser) are	ND	Combination of accumulated mutation had been documented	Zhou et al. 2013

		introduced to form mutants			
10	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Marin et al. 2013
11	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Kutar et al. 2013
12	*V. fluvialis*	GyrA (Ser83Ile)	*qnrVC5*		Singh et al. 2012
13	*V. splendidus*		*qnrVS*	Drug-induced expression of the gene	Okumura et al. 2011
14	*V. fluvialis*	GyrA (Ser83Ile) and ParC (Ser85Leu)	*aac(6') Ib-cr, qnrA1*		Chowdhury et al. 2011
15	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Ismail et al. 2011
16	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Quilici et al. 2010
17	*V. cholerae*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Sjoiund-Karlsson et al. 2011
18	*V. cholerae*	GyrA (Ser83Ile)	ND		Kitiyodom et al. 2010
19	*V. cholerae*		*qnrVC3*		Kim et al. 2010
20	*V. cholerae*	GyrA (Ser83Leu)	ND	New intrinsic mechanism of resistance through *vca0421* gene	Okuda et al. 2010
21	*V. vulnificus*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Roig et al. 2009
22	*V. cholerae*		*qnrVC1 & qnrVC2*		Fonseca et al. 2008

23	*V. anguillarium*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Rodkhum et al. 2008
24	*V. anguillarium*	GyrA (Ser83Ile) and ParC (Ser85Leu)	ND		Colquhoun et al. 2007
25	*V. splendidus*		*qnrS* like		Cattoir et al. 2007
26	*V. cholerae*		NorM	Identification of amino acids involved in norfloxacin resistance	Singh et al. 2006
27	*V. fluvialis*	GyrA (ser83Ile)	ND		Srinivasan et al. 2006
28	*Vibrio spp.*		Qnr like	*Vibrionaceae* family was believed to be the source of *qnr* genes	Poirel et al. 2005a
29	*V. parahaemolyticus*		VPA0095	Demonstration of transformation	Saga et al. 2005
30	*V. parahaemolyticus*		NorM	Identification of essential amino acids	Otsuka et al. 2005
31	*S. flexneri*		*aac (6')Ib-cr* and *qnrS*		Pu et al. 2015
32	*Shigella spp.*	GyrA (Ser83Leu; Asp87 Asn or Gly) and ParC (Ser80Ile)		Novel mutations in GyrA (Val196Ala) of clinical isolates and ParC (Phe93Val), (Asp101Glu), (Asp110Glu) of laboratory mutants	Taneja et al. 2015
33	*Shigella spp.*	GyrA (Ser83Leu; Asp87 Asn or Gly) and ParC (Ser80Ile)	*aac (6')Ib-cr* and *qnrS*		Pu et al. 2015

34	*Shigella spp.*		*aac (6')Ib-cr, qnrB* and *qnrS*		Ahmed and Shimamoto, 2015
35	*Shigella spp.*	GyrA (Ser83Leu)			Dutta et al. 2014
36	*S. flexneri*	GyrA (Ser83Leu; Asp87 Asn or Gly) and ParC (Ser80Ile)		Novel mutation GyrA (His211Tyr)	Azmi et al. 2014
37	*Shigella spp.*		*qnrS1* and *aac(6') Ib-cr*		Ghosh et al. 2014
38	*S. sonnei*	GyrA (Ser83Leu) (Asp 87Gly)	ND		Ruekit et al. 2014
39	*Shigella spp.*	GyrA (Ser83Leu; Asp87 Asn or Gly or Tyr); ParC (Ser80Ile) and ParE (Asp408Gly; Ser458Ala)	*aac (6')Ib-cr* and *qnrB*		Bhattacharya et al. 2014
40	*Gram –ve bacteria*		Many	*qepA* in *Shigella*	Xia et al. 2013
41	*Shigella spp*	GyrA (Ser83Leu; Asp87 Asn or Gly or Val) and ParC (Ser80Ile)	*qepA, aac(6') Ib-cr*	Multiple mutations in various combinations	Yang et al. 2013
42	*Shigella spp*		PMQR + ve		Zhu et al. 2013 (b)
43	*S. flexneri*	GyrA (Ser83Leu; Asp87 Tyr or Gly) and ParC (Ser80Ile)	ND	Ciprofloxacin-induced mutational frequency was studied	Pu et al. 2013
44	*S. flexneri*	GyrA (Ser83Leu; Asp87 Asn) and ParC (Ser80Ile)	ND		Jeon et al. 2012

45	*S. flexneri*	GyrA (Ser83Leu; Asp87Asn or Tyr) and ParC (Ser80Ile)	*aac (6') Ib-cr*, *qnrS* and *qnrB*		Liu et al. 2012
46	*Shigella spp.*		ND	Multiplex RT-PCR method to detect *gyrA* and *parC* mutation	Kim et al. 2012
47	*Shigella spp.*	GyrA (Ser83Leu; Asp87Asn or Gyl) and ParC (Ser80Ile)	ND		Zhang et al. 2011
48	*Shigella spp.*	GyrA (Ser83Leu) and ParC (Ser80Ile)	*qnrB*, *aac(6') Ib-cr*		Bhattacharya et al. 2011
49	*Shigella spp.*	GyrA (Ser83Leu; Asp87Asn or Gyl) and ParC (Ser80Ile)	*qnrB*, *qnrS* and *aac(6') Ib-cr*		Folster et al. 2011
50	*S. flexneri*	GyrA (Ser83Leu; Asp87Asn or Gyl or Val) and ParC (Ser80Ile)	*qnrA*, *qnrS* and *aac(6') Ib-cr*		Xiong et al. 2010
51	*S. flexneri*	GyrA (Ser83Leu; Asp87Asn or Gyl or Val) and ParC (Ser80Ile)	*qnrS* and *aac(6') Ib-cr*		Pu et al. 2009
52	*Shigella spp.*	GyrA (Ser83Leu; Asp87Gly) and ParC (Ser80Ile; Arg 91Gln)	ND	Multiple mutation in GyrA and ParC	Kim et al. 2008
53	*Shigella spp.*	GyrA (Ser83Leu; Asp87Asn or Gly) and ParC (Ser80Ile)	ND	Efflux activity	Pazhani et al. 2008
54	*Shigella spp.*	GyrA (Ser83Leu; Asp87Asn or Tyr) and	ND		Mensa et al. 2008

		ParC (Ser80Ile)			
55	*Shigella spp.*	GyrA (Ser83Ile) and ParC (Ser64Cys or Asp)	ND	Novel mutations in ParC	Hu et al. 2007
56	*S. dysenteriae*	GyrA (Ser83Leu; Asp87Asn or Gly) and ParC (Ser80Ile)	ND	Both single and double mutation was observed	Talukder et al. 2006
57	*S. dysenteriae*	GyrA (Ser83Leu; Asp87Asn or Gly) and ParC (Ser80Ile)	ND		Dutta et al. 2005
58	*S. flexneri*		*qnrS*	First discovery of *qnrS*	Hata et al. 2005
59	*S. dysenterie*	GyrA (Ser83Leu; Asp87Asn or Gly)	ND		Talukder et al. 2004

1.5. Need for this study

It is reasonably well documented that quinolone resistance in pathogenic bacteria through intrinsic and acquired traits causes a major health crisis and treatment failure. The synergistic action of all these chromosomal and plasmid-borne factors help the pathogen to confer higher-level resistance towards quinolones as described by many researchers [Baranwal et al. 2002; Srinivasan et al. 2006]. Though these factors have been characterized to a considerable extent in a number of pathogens, there is more information yet to be explored on the role they play in *Vibrio* spp. and *Shigella* spp. which are major diarrhea-causing pathogens in our country, India. There is a limited information regarding the molecular epidemiology of antibiotic resistance and the contribution of each quinolone resistance determinant towards the antibiotic action in these bacteria [Niyogi, 2005; Faruque and Nair, 2008]. Active surveillance of diseases and its etiological agent is an absolute requirement to keep pace with these continuously evolving pathogens. Therefore, the prevailing situation necessitates a thorough understanding of the mechanisms which are likely to be associated with quinolone resistance.

Through this work, the aim is to unravel/decipher the arsenal for the quinolone resistance determining genes in the clinical isolates of *Vibrio* spp. and *Shigella* spp. After mining all these genes, it is attempted to understand the synergy among these genetic factors which help the bacterium to attain particular quinolone drug resistance phenotype.

1.6. Aims and Objectives

The objectives of the proposed study would be as follows:

- Isolation, cloning and characterization of the quinolone-resistance-determining genes from the clinical isolates of *Vibrio* spp. and *Shigella* spp.
- Sequence analysis of these genes and submission to GenBank.

- Confirmation of the expression of the putative quinolone resistance genes in the native host by reverse transcription PCR.
- Cloning and expression of these genes in a heterologous host like *E. coli* to obtain recombinants with isolated resistance genes.
- Study the functionality of the recombinant quinolone-resistance-determining proteins by various assays like minimum inhibitory concentration (MIC) test and drug accumulation assay using fluorimeter.