1. Introduction

1.1. Microorganisms in diarrhoeal diseases

Diarrhoeal diseases are the second leading cause of death after pneumonia, in children under five years of age [WHO, 2009]. Every day around 2195 and every year 801000 children die because of diarrhoea [Liu et al., 2012]. It is among the top 10 leading causes of overall death in the world (Figure 1.1). Diarrhoea is a symptom of gastrointestinal infection causing loose or watery stools at least three times per day or more frequently than normal for an individual. Though most episodes of diarrhoea are mild, severe cases can lead to significant fluid loss and dehydration, which may result in death or other major complications.

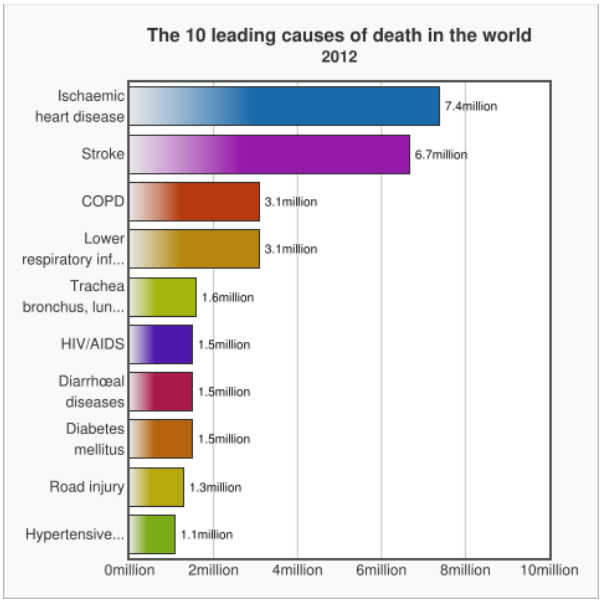

Figure 1.1. The 10 leading causes of death in the world in 2012. [Source: http://www.who.int/mediacentre/factsheets/fs310/en/]

The gastrointestinal infections are caused by a wide range of pathogens, including bacteria, viruses and protozoa. Rotavirus is one of the leading causes of acute diarrhoea. Bacterial pathogens like *Escherichia coli, Shigella, Campylobacter*, *Salmonella* and *Vibrio* are also majorly responsible for acute diarrhoea during epidemics. Diarrhoea can also be caused by protozoa such as *Cyclospora, Giardia, Entamoeba histolytica* and *Cryptosporidium* [WGO, 2008]. Diarrhoeal pathogens are

spread through fecal-oral transmissions. There are three main forms of acute diarrhoea; all forms are potentially life-threatening and require different treatments [http://www.who.int/mediacentre/factsheets/fs330/en/]. The first form, acute watery diarrhoea which includes cholera, is associated with significant fluid loss and rapid dehydration in an infected individual. It is generally caused by *V. cholerae* or *E. coli* or rotavirus. The second one is bloody diarrhoea, also referred to as dysentery, is marked by visible blood in the stools. It is associated with intestinal damage and fluid loss in an infected person. The most common cause of bloody diarrhoea is *Shigella.* The third form of diarrhoea is persistent diarrhoea, an episode of diarrhoea, with or without blood that lasts at least 14 days. Malnourished children and those with other illnesses such as AIDS are more likely to develop persistent diarrhoea.

In developing countries, diarrhoeal infections through bacteria are more prevalent whereas viruses are the predominant cause of acute diarrhoea, especially during the winter season in industrialized countries [WGO, 2008]. The frequency of occurrence of diarrhoeal infections due to *Vibrio* and *Shigella* species is more in developing countries like India, where the problem of poor sanitation and unsafe water supply exists [Bowen, 2016; Faruque and Nair, 2008; WGO, 2008; Niyogi, 2005]. Hence, the following sections would focus on *Vibrio* spp. and *Shigella* spp., their treatment and treatment failure due to drug resistance.

1.1.1. Diarrhoea due to *Vibrio* spp.

Vibrio cholerae, a Gram-negative pathogen, causes cholera, an acute dehydrating diarrhoea that occurs in endemic, epidemic and pandemic forms [Faruque et al., 1998]. *V. fluvialis* Gram-negative food-borne pathogen also causes clinically indistinguish from cholera [Ramamurthy et al., 2014; Faruque et al., 1998]. It is mostly caused by contaminated water and food. Profuse watery diarrhoea, vomiting, leg cramps, rapid loss of body fluids leading to dehydration and shock are the symptoms of cholera and cholera-like diarrhoea. The symptoms start two hours to five days after the infection. Pathogenic *V. cholerae* harbours key virulence factors that include cholera toxin and toxin co-regulated pilus [Nelson et al., 2009; Faruque et al., 1998]. Cholera toxin is an AB_5 type of toxin. Binding subunit B is a pentamer that binds to the monosialotetrahexosyl-gangliosides on absorptive epithelial cells and

activates endocytosis of the enzymatic subunit A, as a result of which it ADP-ribosylates a subunit of the G protein that controls adenylate cyclase activity [Nelson et al., 2009; Faruque et al., 1998]. This results in constitutive cAMP production, that leads to secretion of H_2O, Na^+, K^+, Cl^-, and HCO_3 into the lumen of the small intestine and rapid dehydration. There are other toxin/proteins in *Vibrio* spp. that play a role in enhancing the adhesion and intestinal colonization or help to modulate the infection in the small intestine. Some of these proteins are hemagglutinin, cytolysin, heat labile cytotoxin, hemolysin, mucinase and metalloprotease [Ramamurthy et al., 2014; Rajpara et al., 2013; Syngkon et al., 2010; Ghosh et al., 2006; Faruque et al., 1998].

V. cholerae infections are more prevalent in developing countries. In contrast, an infection caused by *V. parahaemolyticus* and other *Vibrios* is due to the contamination of seafood [Ramamurthy et al., 2014]. In the past two hundred years, the world has witnessed seven pandemics of cholera [http://www.cdc.gov/cholera/index.html] in which first six pandemics have been caused by *V. cholerae* O1 classical biotype [Mandal et al., 2011]. The seventh pandemic of cholera was caused due to *V. cholerae* O1 El Tor biotype and O139 serogroup. Cholera outbreaks have increased continuously since 2005 and affected several continents [http://www.cdc.gov/cholera/index.htm]. In 2015, 1,72,454 cases and 1,304 deaths due to cholera were reported worldwide to WHO in which 41%, 37% and 21% cases were reported from Africa, Asia and America respectively [WER, 2016]. In India, during 1997 to 2006, a total of 68 outbreaks of cholera were reported across 18 states and union territories [Kanungo et al., 2010].

V. fluvialis is one of the emerging pathogens all over the world and has epidemic-causing potential especially in coastal areas [Lu et al., 2014]. Moreover, *V. fluvialis* behaved more aggressively than *V. cholerae* O1 in an epidemic situation with a higher rate of infection in Sunderban, West Bengal, India [Bhattacharjee et al., 2010]. The bacteria was first isolated in 1975 from a patient with severe diarrhoea and categorized as group F *Vibrios* by Furniss et al and "EF-6 *Vibrios*" by the center for disease control [Seidler et al., 1980; Furniss et al., 1977]. Subsequently, it was named as *V. fluvialis* in 1981 [Lee et al., 1981]. The outbreaks of *V. fluvialis* infection were reported from Bangladesh, the United States and India [Chowdhury et al., 2012; Bhattacharjee et al., 2010; Huq et al., 1980; Levine and Griffine, 1993].

1.1.2. Diarrhoea due to *Shigella* spp.

Shigellosis is an intestinal infection caused by *Shigella* spp. that includes *Shigella dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* [Niyogi, 2005]. Shigellosis is caused by direct contact with the bacteria or through contaminated water or food. A small volume of inoculum of bacteria (as few as 200 bacteria) is sufficient to cause the infection. Patients infected with bacteria suffer from the frequent passage of small liquid stools that contain visible blood, with or without mucus, after one to four days of incubation period. Abdominal cramps, tenesmus, fever and anorexia are the common symptoms in shigellosis. Sometimes it causes some serious complications like hemolytic uremic syndrome, reactive arthritis, blood stream infections and seizures [CDC, 2013]. Young children, gay, bisexuals, HIV-infected persons and travellers have a high risk of shigellosis [CDC, 2013]. *Shigella* has the ability to invade and colonize the intestinal epithelium and cause its disruptions. *Shigella* can multiply within the intestinal epithelium cells and cause cell death. It also disseminates to infect and kill the adjacent cells, causing mucosal inflammation, ulceration and bleeding [Niyogi, 2005].

Shigellosis is estimated to cause 80-165 million cases of infections and 6,00,000 deaths worldwide [Bowen, 2016]. This disease is more prevalent in children under the age of five years and more common in developing countries. *Shigella* causes about 5,00,000 cases of dysentery in the United States annually while in Asia, around 125 million infections and 14,000 deaths are annually caused due to shigellosis [Scallan et al., 2011].

The geographical distribution of four species of *Shigella* is different and the reasons for this are still unclear [Keusch, 1998]. *S. dysenteriae* type 1 was historically responsible for large epidemics in Central America, Asia and Africa [Ries et al., 1994] but recently there are very few reports on the occurrence of this species [Livio et al., 2014]. *S. boydii* is reported occasionally whereas *S. flexneri* is most frequently isolated around the globe especially in resource-poor countries [Kotloff et al., 1999]. *S. flexneri* consists of 14 different serotypes that are distributed heterogeneously across different regions of the world, with predominant serotypes that include *S. flexneri* 2a, 3a, and 6 [Livio et al., 2014; Levine et al., 2007]. *S. sonnei* is also prevalent globally, most frequently detected in high-income regions and it has only

one serotype [Ram et al., 2008; Levine et al., 2007]. In general, the disease caused by *S. sonnei* is less severe when compared to shigellosis caused by other species [Acheson and Keusch, 1994].

1.2. Antibiotics for treatment of diarrhoea caused by *Vibrio* and *Shigella* spp., and antibiotic resistance

Oral rehydration therapy and in extreme cases, rapid intravenous replacement of lost fluids and ions are the first-line treatment for cholera. Antibiotics are used as a second-line treatment to shorten the duration of diarrhoea. They are often used in epidemic situations also as prophylactics. In severe cholera cases, doxycycline, tetracycline, ciprofloxacin, azithromycin, co-trimoxazole, chloramphenicol and erythromycin are recommended for the treatment [Saha et al., 2006; Kabir et al., 1996; Khan et al., 1996; Burans et al., 1989]. Ciprofloxacin, ampicillin, azithromycin, ceftriaxone, nalidixic acid, chloramphenicol, tetracycline and co-trimoxazole are used to treat the *Shigella* infections [Klontz and Singh, 2015].

The emergence of antibiotic resistance is becoming the most pressing problem in infectious agents including the *Vibrio spp*. and *Shigella spp*. Antibiotic resistance occurs due to misuse of antibiotics, excessive use of broad-spectrum antibiotics, sub-optimal dosing, use of antibiotics without a prescription and many other reasons [Bhardwaj et al., 2014]. Treatment failure, increased mortality rate, increased cost of treatment and emergence of untreatable infections are some of the consequences of antibiotic resistance.

1.2.1. Drug resistance in *Vibrio* spp.

Due to the emergence of drug-resistant strains, an increase in the case fatality rate from 1% to 5.3% was observed in Guinea-Bissau (Africa) during the 1996-97 epidemic of cholera [Dalsgaard et al., 2000]. In Bangladesh, most cases of cholera infections carried resistance for tetracycline, trimethoprim, sulfamethoxazole, and erythromycin [Sack et al., 2006]. Increased resistance to a number of antibiotics has been seen in *V. cholerae* during 1970 to 2010 from Kolkata, India [Ghosh and Ramamurthy, 2011]. In a study, from various regions of the world, *V. cholerae* O1 (between 1938 and 1993) showed resistance to 1 to 3 antimicrobials, whereas the

strains isolated from 1994 to 2005 carried 3 to 8 resistance markers including fluoroquinolones [Ghosh and Ramamurthy, 2011].

Antibiotic resistance was also reported in other clinical *Vibrios* [You et al., 2016; Xu et al., 2016a; Tra et al., 2016; Chowdhury et al., 2016; Shaw et al., 2014]. Many of the *Vibrios* including *V.fluvialis* were found resistant to ampicillin, carbenicillin, cefalotin, kanamycin and sulfadiazine, trimethoprim in Mediterranean fish farms [Laganà et al., 2011]. In China, the majority of the *V. fluvialis* isolates were resistant to azithromycin, β-lactams and sulfamethoxazole [Liang et al., 2013]. In India, there are many reports on drug resistance in *Vibrio fluvialis* isolates [Chowdhury et al., 2016; Chowdhury et al., 2013; Chowdhury et al., 2012; Singh et al., 2012; Rajpara et al., 2009; Srinivasan et al., 2006].

1.2.2. Drug resistance in *Shigella* spp.

Historically, shigellosis was treated with ampicillin, trimethoprim, sulfamethoxazole and ceftriaxone [WHO, 2014; CDC, 2013]. Unfortunately, these bacteria have become resistant to one or more antibiotics. The Center for Disease Control and Prevention has kept this organism in the category list of serious threat. They had also reported 27,000 cases of drug-resistant *Shigella* infections per year in the United States [CDC, 2013]. National Antimicrobial Resistance Monitoring System and Foodborne Disease Active Surveillance Network sites of United States revealed that 1118 isolates (2000 to 2010) were found resistant to ampicillin (74%), streptomycin (58%), co-trimoxazole (36%) and tetracycline (28%) [Shiferaw et al., 2012]. Additionally, these antibiotic resistance patterns differed by race, ethnicity, age, travel and species. For example, male individuals were more likely to be infected with multidrug-resistant *Shigella* than female individuals. Resistance to cephalosporins was markedly increased during 2010 to 2012 from Europe, America, Africa and Asia [Gu et al., 2015]. In a study of *Shigella* isolates from central Israel in 1998 to 2000, and comparison with the period 1991–1992, a significantly increased resistance to tetracycline (from 23% to 87%), high resistance to co-trimoxazole (94%) and ampicillin (85%) and developing resistance to quinolones (0.5–2%) was observed [Ashkenazi et al., 2003]. *Shigella* spp. were initially susceptible to co-trimoxazole but on the emergence of resistance to this antimicrobial, treatment recommendations were

shifted to quinolone group of antibiotics and azithromycin [WHO, 2014]. But eventually, these bacteria also developed the mechanisms for quinolone resistance and recently WHO reported the fluoroquinolone resistance in *Shigella* isolates from all over the globe [WHO, 2014]. Resistance to nalidixic acid and ciprofloxacin in Asia-Africa (during years 2007 to 2009) was progressively increased each year, reaching 64.5% and 29.1% respectively as compared to Europe and America [Gu et al., 2012]. There are many reports on azithromycin resistance in *Shigella* [Heiman et al., 2014; Hassing et al., 2014; Sj lund Karlsson et al., 2013; Howei et al., 2010].

This is a worrisome situation that *Vibrio* and *Shigella* are acquiring resistance to all the possible drugs which would complicate the treatment procedure.

1.3. Modes of action of antibiotics

Table 1.1. Classes of antibiotics, their modes of action, their activity and examples [Source: Salyers and Whitt, 2002]

Class	Modes of action	Spectrum activity	Examples
β-Lactams (Penicillin, cephalosporin, carbapenem, monobactams)	Inhibit transpeptidation step in peptidoglycan; bind penicillin-binding proteins	Gram-positive and/or Gram-negative bacteria	Penicillin, Ampicillin
Glycopeptides	Inhibit transglycosylation and transpeptidation steps in peptidoglycan synthesis by binding D-Ala-D-Ala	Most effective against Gram-positive bacteria	Vancomycin, Teicoplanin
Aminoglycosides	Bind 30S subunit of bacterial ribosome	Broadly bactericidal	Kanamycin, Gentamycin, Streptomycin
Tetracycline	Bind 30S subunit of bacterial ribosome; disrupt bacterial cell membrane	Broadly bacteriostatic; some protozoa	Tetracycline, Doxycycline
Macrolide/lincosamide	Bind 50S ribosomal subunit	Bacteriostatic for most; bactericidal for some Gram-positive bacteria	Erythromycin, Lincomycin, Clindamycin
Streptogramins	Bind 50S ribosomal subunit	Bacteriostatic for individually; bactericidal in combination	Synercid
Fluoroquinolones	Bind DNA gyrase	Broadly bactericidal	Ciprofloxacin, Norfloxacin
Rifampin	Binds β-subunit of bacterial RNA polymerase	Broadly antibacterial; effective against mycobacteria	Rifadin
Trimethoprim/ sulphonamides	Inhibit enzymes responsible for tetrahydrofolate production	Broadly antibacterial	Bactrim, Spectra
Metronidazole	Interferes with DNA replication	Antibacterial, antiprotozoal	Flagyl
Oxazolidinones	Bind 50S ribosomal subunit	Bacteriostatic; broad spectrum against Gram-positive bacteria	Zyvox

Antibiotics were used to treat the infections caused by bacteria and other microorganisms. Antibiotics kill or retard the bacteria by targeting the cellular processes in bacteria which differ greatly from that of their host counterparts [Salyers and Whitt, 2002]. The antimicrobial compounds that kill bacteria are called bactericidal while the others that merely retard the growth of bacteria are called bacteriostatic compounds. These drugs interfere with the vital housekeeping processes such as cell wall synthesis, protein, DNA and RNA synthesis or inhibiting the key enzymes of metabolic pathways [Willey et al., 2008]. Class of antibiotics, basic mechanisms of antibiotics and their action on bacteria are described in Table 1.1.

1.4. Mechanisms of Antibiotic Resistance

Development of antimicrobial resistance is a natural phenomenon that every organism undergoes for evolutionary fitness. Antibacterial resistance gets aggravated due to a wide variety of non-genetic factors and genetic factors.

1.4.1. Non-genetic factors governing MDR in bacteria

There are many other social/clinical/policy-related factors that lead to the emergence and dissemination of antibiotic resistant bacteria at a particular geographical location. These include the indiscriminate use of antibiotics, poor surveillance systems for various epidemics/pandemics, the absence of a comprehensive and coordinated response by government in case of spread of a serious infection, lack of preparedness in terms of efficient diagnostics, prevention and therapeutic tools [Sack et al., 2001]. In addition to this, the pharmaceutical companies have lost interest in the development of new antibiotics as this research is no longer lucrative. As anti-infective drugs are taken for shorter times till the infection persists, the companies refrain from investments on these pharmacologically active agents as compared to blockbuster drugs for lowering the cholesterol levels, for hypertension, diabetes etc which are taken for prolonged periods and mostly lifelong. Accordingly, the government funding in this area has also been diminishing. There has been a drastic cut down on antibiotic discovery programmes [Gould and Bal, 2013]. This amounts to the use of same old antibiotics in clinics and hospitals leading to the development of bacterial resistance against them.

1.4.2. Genetic factors/mechanisms governing MDR in bacteria

Antibacterials kill or retard the bacteria by targeting the cellular processes or structures in bacteria which differ greatly from that of their host counterparts. Bacteria evolve different mechanisms to counteract these antibacterial drugs and employ a large battery of genes to effect these mechanisms. These resistance mechanisms could be intrinsic/innate (chromosome-borne) or acquired (borne on mobile genetic elements [MGE]). Though innate resistance allows a bacterium to adapt to the changing environment, it gets restricted to that particular bacterium and its progeny by vertical gene transfer. In contrast to this, the acquired resistance leads to the resistance genes being disseminated quickly within different bacteria crossing the species and genera barriers. This process is carried out by mutations in the target genes and horizontal gene transfer (HGT) and could be mediated through different vehicles like plasmids, integrons, transposons and mechanisms of transduction, conjugation and transformation. The other mechanisms through which bacteria mediate resistance to antibiotics are: restricting the access of the antibiotics through porins and efflux pumps, enzymatically inactivating the antibiotics, modifying or protecting the antibiotic target and by hindering the activation of antibiotic [Willey et al., 2008; Salyers and Whitt, 2002]. Often, a bacterium counteracts an antibiotic by the synergistic action of more than one of the above-mentioned resistance mechanisms [Singh et al., 2012; Pazhani et al., 2011; Baranwal et al., 2002; Cavallo et al., 2002]. For example, the resistance of *P. aeruginosa* to ticarcillin has been attributed to overexpression of outer membrane protein OprM, production of β-lactamase and overexpression of AmpC cephalosporinase [Cavallo et al., 2002]. Similarly, resistance to tetracycline is often a result of synergy between the efflux pumps (encoded by *tetA* to *tetG*, *tetK*, *tetL*), oxidation of tetracycline and cytoplasmic proteins (encoded by *tetM, tetO*, *tetQ*) that confer protection to ribosomes from tetracycline [Salyers and Whitt, 2002]. In many cases, acquisition of a single gene/single mutation can offer protection against many different classes of antibiotics. For example, the efflux pumps with specificity for many different antibiotics can lead to resistance to all these antibiotics on overexpression or mutations. Similarly, the enzyme rRNA methylase (encoded by *ermA*, *ermB*, *ermF*, *ermG*) methylates an adenine on 23S rRNA that lies within a region that binds to three classes of

antibiotics; macrolides, lincosamides and streptogramins. Therefore, a single gene of this methylase confers resistance against these three structurally distinct classes of antibiotics [Salyers and Whitt, 2002].

It can be envisaged that the source of antibiotic resistance genes could be the bacteria that produce antibiotics. In such producers, these resistance genes provide protection to bacteria from the antibiotics produced by them. Another possible source of these resistance genes could be the organisms naturally resistant to some of the antibiotics. For example, *Lactobacillus* species do not use D-Ala-D-Ala dipeptide as part of their muramyl dipeptide and therefore, they are naturally refractory to the glycopeptide antibiotic vancomycin. Vancomycin prevents crosslinking of peptidoglycan by binding to the D-Ala-D-Ala of the muramyl peptide. Resistance to vancomycin arises due to the replacement of this peptide with D-Ala-D-lactate, a dipeptide that does not bind to vancomycin [Salyers and Whitt, 2002].

1.5. Mobile Genetic Elements

Bacteria possess an exquisite ability to adapt rapidly to the changing environments and this property is mediated by a large number of genetic elements that contribute to the genome plasticity. It was predicted that up to 32.6% of each bacterial genome is made up of non-native DNA that has been acquired by HGT [Sakharkar and Chow, 2008; Koonin et al., 2001]. MGEs which include integrons, integrative and conjugative elements (ICEs) and plasmids are the carriers of drug resistance genes (Figure 1.2).

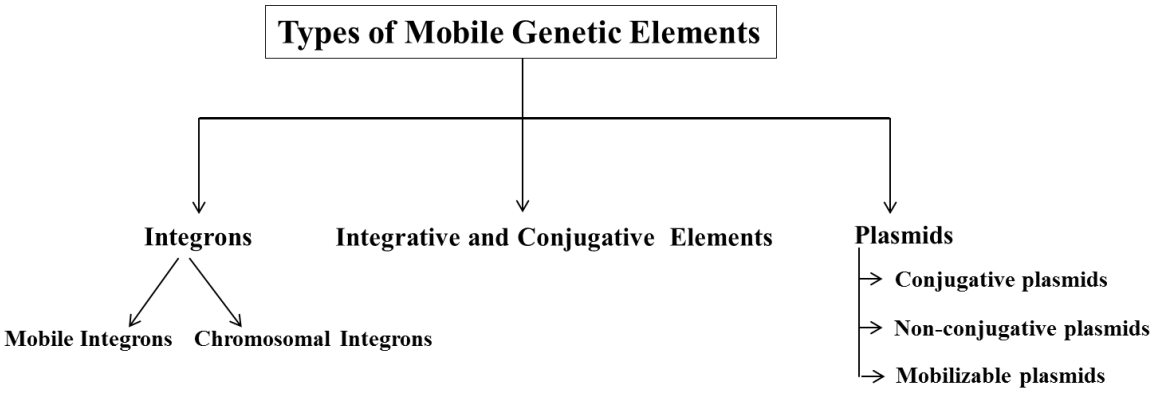

Figure 1.2. Schematic representations of various types of mobile genetic elements

Integron contains an integrase that enables integration, expression and exchange of the DNA segments (gene cassettes). ICEs are self-transmissible elements that contribute to the horizontal transfer of virulence genes, antibiotic resistance genes and other bacterial traits. Plasmids serve as vehicles for gene capture and its dissemination. These elements are described below in detail.

1.5.1. Integrons

Integron is a common tool for antibiotic resistance gene capture and dissemination. It is a platform to acquire open reading frames (ORFs) by site-specific recombination and convert them to functional forms by their expression [Cambray et al., 2010; Mazel, 2006]. Integrons were discovered in the 1980s [Stokes and Hall, 1989] and these mobile genetic elements harbouring antibiotic resistance gene cassettes are now known in clinical as well as agricultural and environmental samples [Partridge et al., 2009; Stokes et al., 2006]. Integrons have contributed to the emergence and dissemination of simultaneous resistance to streptomycin, tetracycline, chloramphenicol and sulphonamides in isolates of *S. dysenteriae* during last six to seven decades [Mitsuhashi et al., 1961].

1.5.1.1. Structure of Integrons

Most of the integrons have three key components: 1) an Integrase *intI* belonging to tyrosine recombinase family; 2) a promoter Pc that directs the expression of the captured genes and 3) a primary recombination site *attI*. Additionally, all gene cassettes that incorporate in the integron share some specific characteristics at 3' end of the gene which are imperfect repeats called *attC* (also called 59- base element) [Cambray et al., 2010; Mazel, 2006].

1.5.1.1a. Integrases

Integrases belong to site-specific recombinases known as tyrosine recombinases. Tyrosine recombinases include a wide variety of enzymes that use a tyrosine residue as the nucleophile in their strand exchange reactions [Nunes-Duby et al., 1998; Esposito and Scocca, 1997]. It is involved in integration and excision or inversion of gene cassettes. The catalytic domain of integrase contains conserved amino acids

Arg(R)-His(H)-Arg(R) and the nucleophilic tyrosine, Tyr (Y) [Demarre et al., 2007; Nunes-Duby et al., 1998]. Integron integrases pose additional domain which is distinct from other tyrosine recombinase family proteins [Messier and Roy, 2001]. The integrase mediates recombination through two sites i.e. the integrase-specific *attI* site and the gene cassette associated *attC* site [Mazel, 2006]. Integrase proteins consist of four subunits in which two subunits are active per cleavage round in site-specific recombination [Demarre et al., 2007] and remaining two subunits are involved in specific interactions with two nucleotide bases that protrude from the *attC* site of the gene cassettes.

1.5.1.1b. Promoter

Gene cassettes that get inserted in the integrons are generally promoterless but few reports revealed that some of the gene cassettes have their own promoter. For example, *cmlA* cassettes have both promoter and translational attenuation signals, *V. cholerae qnrVC* genes have their own internal promoter and the *qacE* cassettes have a weak promoter [da Fonseca and Vicente, 2012; Stokes and Hall, 1991; Guerineau et al., 1990]. The expression of the majority of promoterless cassettes is hence dependent on the proximity of an external promoter located either in the integrase gene or on *attI* site [Jove et al., 2010; Collis and Hall, 1995; Levesque et al., 1994].

1.5.1.1c. Primary Recombination site (*attI*) and 59-base element (*attC*)

Integrase recognizes and recombines two types of sites that have different structures, the *attI* type (non-palindromic) of site found in the integrase gene and *attC*/59-base elements (palindromic) found on the gene cassettes. The *attI* and *attC* sequences are complex attachment sites that include the crossover site and additional binding sites. Integrase monomers act as accessory factors at these additional sites [Collis and Hall, 2004; Gravel et al., 1998; Collis et al., 1998]. The *attI* sites are located at the end of the 5' conserved region (5'CS) of integrons and their sequences vary considerably. The *attC* sites share among themselves a common set of characteristics that enable them to be identified despite the diversity of their sequences and sizes [Gravel et al., 1998; Stokes et al., 1997]. They are characterized by a palindrome of variable length and sequence between the RYYYAAC (R= Purines; Y= Pyrimidine) inverse core site

and the GTTRRRY core site [Stokes et al., 1997]. The size of these recombination sites varies in length from 57 to 141 bp [Recchia and Hall, 1995]. Mostly gene cassettes are found integrated into the variable region of integrons or they can exist as covalently closed circular intermediates. The integrase recognizes *attC* and *attI* sites. In the event of integration, *attI*×*attC* recombination occurs and that allows expression of genes that are downstream of the promoter. In excision, *attC*×*attC* recombination occurs [Baharoglu et al., 2013; Cambray et al., 2010].

1.5.1.2. Types of Integrons

Two types of integrons are mobile integrons and chromosomal integrons.

1.5.1.2a. Mobile Integrons (MI)

Integrons associated with mobile DNA elements and primarily involved in the spread of antibiotic resistance genes correspond to the mobile integrons [Cambray et al., 2010]. The bacteria use these elements as natural genetic vehicles to transfer a wide array of genes including antibiotic resistance genes. More than 130 different antibiotic resistance cassettes have been identified in MIs [Partridge et al., 2009, Fluit and Schmitz, 2004]. These cassettes have resistance genes for the majority of antibiotic classes like β-lactams, aminoglycosides, chloramphenicol, trimethoprim, streptomycin, quinolones, rifampin, erythromycin and antiseptics of the quaternary ammonium compound family [Partridge et al., 2009; Mazel, 2006; Fluit and Schmitz, 2004].

Five classes of MIs have been well defined to date with ~40-58% identities based on integrase sequences [Cambray et al., 2010; Mazel, 2006]. Here, only three integron classes (class 1, 2 and 3) have been described. Class 1 integrons are the most widespread and well characterized. Class 1 integrons are embedded within larger transposon Tn 21 [Liebert et al., 1999]. Class 1 integrons consist of two conserved segments (CS) at 5′- (5’CS) and 3′- (3’CS) ends, separated by a variable region (VR) that usually comprises of one or more gene cassettes (Figure 1.3). The 5’CS region contains the integrase gene (*intI1*), the integration site (*attI1*) and a promoter region (Pc) that allows expression of any number of gene cassettes inserted at the *attI1* site in a suitable orientation. The 3’CS region usually comprises of *qacE*Δ1 encoding

resistance to quaternary ammonium compounds and *sul1* encoding resistance to sulphonamides [Recchia and Hall, 1995; Rouch et al., 1990]. Class 1 integrons are widely distributed in animal and human clinical strains of Gram–negative bacteria [Labbate et al., 2009; Goldstein et al., 2001] and also in some of the Gram–positive bacteria [Shi et al., 2006; Nandi et al., 2004; Nesvera et al., 1998; Martin et al., 1990].

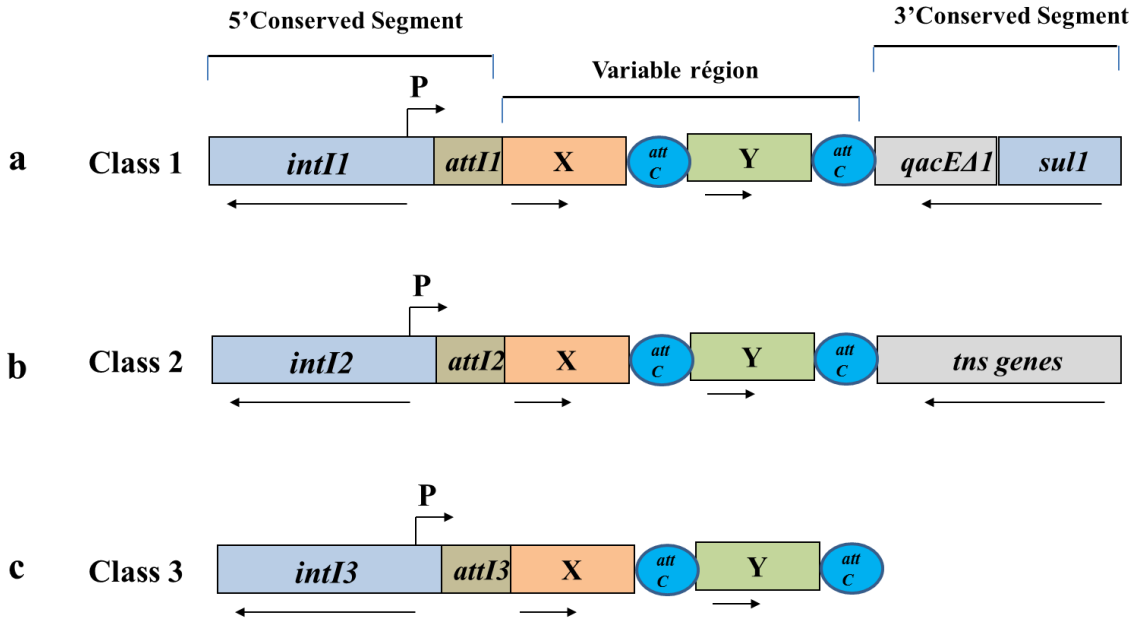

Figure 1.3. The structures of class 1, class 2 and class 3 integrons. Integrons consist of a gene *intI* encoding a site-specific recombinase called "integrase" belonging to tyrosine-recombinase family and a recombination site *attI* into which the exogenous gene cassettes (X and Y) harbouring the recombination site *attC* are inserted through site-specific recombination. In the 5'conserved sequences (5'CS), a promoter P located within *intI* drives transcription of the captured genes. *intI1, intI2* and *intI3*: integrases of class 1 (a), class 2 (b) and class 3(c) integron respectively. *attI1, attI2* and *attI3*: primary recombination sites of class 1, class 2 and class 3 integron respectively. *qacEΔ1* and *sul1* are conserved regions in 3'conserved sequences (3'CS) which contribute resistance to ethidium bromide and sulfonamides. *tns* genes: transposition genes; X and Y: gene cassettes [Source: Bhardwaj et al., 2014]

The atypical class 1 integron consists of 5'CS of class 1 integron, variable region and the IS1 element at 3' end, in place of 3'CS of *qacE*Δ1 and *sul1* [Pan et al., 2006]. The atypical class 1 integron was first found on pathogenicity island carrying *Shigella* Resistance Locus, on the chromosome of *S. flexneri* 2a strain YSH6000 and harbours two resistance determinants of chloramphenicol and tetracycline [Pan et al., 2006; Luck et al., 2001].

Class 2 integrons are found on Tn7 transposons and its derivatives. A class 2 integron consists of an integrase gene (*intI2*), a promoter and a primary attachment site (*attI2*) at 5' CS while transposition genes (*tns*) are carried at 3'CS (Figure 1.3).

Class 2 integrase is 325 amino acids long and 46% identical to class 1 integrase [Hansson et al., 2002]. The gene contains a nonsense mutation in codon 179 (ochre 179) and thereby it yields a truncated, non-functional protein which can be recovered by a single mutation. It has been proved experimentally that the mutation of the ochre 179 codon to glutamic acid encoding codon produces an integrase with full recombinase activity [Hansson et al., 2002; Collis et al., 2002a].

The class 2 integrons have been found in several species of Gram-negative bacteria isolated from human, animal and environmental sources [Uyaguari et al., 2013; Goldstein et al., 2001]. They have been shown to carry three resistance gene cassettes, *dfrA1, sat1* and *aadA1*, that confer resistance to trimethoprim, streptothricin and streptomycin/spectinomycin, respectively [Hansson et al., 2002]. In some instances, functional class 2 integrases have also been reported with genes other than above [Wei et al., 2014; Marquez et al., 2008; Barlow and Gobius, 2006]. A class 2 integron was associated with four non-antibiotic resistance gene cassettes while in another case, it carried *dfrA14* and a novel lipoprotein signal peptidase gene cassettes [Marquez et al., 2008; Barlow and Gobius, 2006]. There are few reports suggesting that the insertion elements such as IS911, IS630 and IS1 were present in class 2 integrons [Gassama Sow et al., 2008]. A class 2 integron lacking the *tns* (transposition) genes has been reported in *Acinetobacter baumannii* and *S. sonnei* [Gassama Sow et al., 2008; Ploy et al., 2000]. The class 2 integron from *A. baumanii* harbored the hybrid class2/class1 integron, which carried the 3' segment of class 1 integrons (*qacEΔ1*, *sul1* and ORF5) in place of transposition (*tns*) genes on class 2 integron.

Class 3 integron was first reported in *Serratia marcescens* strain in 1995 [Arakawa et al., 1995] and characterized later in 2002 [Collis et al., 2002b]. The configuration of the three potentially definitive features of these integrons, the *intI3* gene, the adjacent *attI3* recombination site and the P_c promoter that directs transcription of the cassettes was similar to that found in class 1 integron module (Figure 1.3). The *IntI3* integrase was active and able to recognize and recombine known types of *IntI*-specific recombination sites, the *attI3* site in the integron and different cassette-associated 59-base sites. Both integration of circularized cassettes into the *attI3* site and excision of integrated cassettes were catalysed by *IntI3* [Collis

et al., 2002b]. Class 3 integron with its genes encoding bla_{GES-1} and bla_{OXA}/*aac*(6')-*Ib* responsible for the β-lactam and aminoglycoside resistance have been reported [Correia et al., 2003; Arakawa et al., 1995].

1.5.1.2b. Chromosomal Integrons (CIs)

Chromosomal Integrons or "Super-Integrons" (SIs) are located on the chromosomes of a large number of bacteria [Drouin et al., 2002; Clark et al., 2000; Heidelberg et al., 2000; Mazel et al., 1998]. CIs are distinguished from conventional integrons by their size, placement of promoters, chromosomal location and the nature of gene cassettes they carry [Heidelberg et al., 2000]. CIs have been found in bacteria belonging to families such as *Vibrionaceae*, *Pseudoalteromonas*, *Xanthomonadaceae*, *Alteromonadaceae*, *Pseudomonadaceae* and *Spirochaetales* [Cambray et al., 2010; Mazel, 2006]. All of them share general characteristics like they are large (>20 gene cassettes and up to 200) and have homology between the *attC* sites of their endogenous cassettes.

The first SI was discovered in *V. cholerae* chromosome II in a clustered region spanning 126 kb. It harboured 214 ORFs out of which the functions for 179 cassettes were not assigned [Rowe-Magnus et al., 2002]. As a large number of gene cassettes are promoterless and are present in the Sis. The first few cassettes are expressed through the promoter of the integrase thereby gradually decreasing the expression in rest of the cassettes (Figure 1.4). Unexpressed cassettes in the array constitute a reservoir and provide a form of molecular memory. These cassettes are maintained in a large array of toxin-antitoxin systems [Cambray et al., 2010].

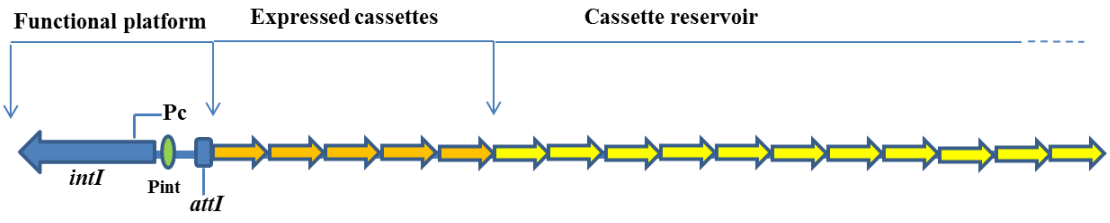

Figure 1.4. Schematic diagram of chromosomal integron. *intI*: integrase; *attI*: attachment sites; Pc and Pint: promoter

The evolutionary history of CI suggested that these elements helped in adaptation along with the change in environment, as well as were the main source of mobile integron's backbone and antibiotic resistance gene cassettes [Mazel, 2006]. It has been proposed that MDR integrons arose from SIs by the entrapment of *intI* genes and their related *attI* sites on MGEs such as transposons. The gene cassette reservoirs of SIs provide a source of gene cassettes that are recruited by multi-resistant integrons [Rowe-Magnus et al., 2002].

The difference between MIs and CIs is as follows [Rowe-Magnus et al., 2002]:

1. MIs contain <20 gene cassettes while SIs/CIs contains >20 gene cassettes.
2. In MIs, the cassettes typically code for antibiotic resistance genes whereas those of SIs are mainly unknown functions.
3. MIs are associated with mobile elements while the SIs coevolved with their host genomes which strongly suggest that SIs are sedentary.
4. The *attC* sites of the gene cassettes of MIs are highly variable in length and sequence while the *attC* sites of CI gene cassettes are closely related and species-specific.
5. Finally, there is evidence that not all of the gene cassettes but only a few associated with CIs are significantly expressed.

1.5.1.3. Gene cassettes in the integron

As described in the earlier section, more than 130 gene cassettes in MIs are known for antibiotic resistance genes [Cambray et al., 2010]. For example, resistance to chloramphenicol is due to acetylation of the antibiotic (*catB* gene) and for the aminoglycosides, due to modification of antibiotic by acetylation (*aacA* and *aacC* genes) and adenylation (*aadA* and *aadB* genes). The β-lactamases encode three distinct families; class A (*blaP* genes), class B metallo β-lactamase (bla_{IMP} genes) and class D (*oxa* genes) which inactivate the β-lactam drugs. The *dfrA* and *dfrB* genes code for dihydrofolate reductase conferring trimethoprim resistance. Apart from antibiotic resistance genes, many unknown ORFs have also been reported and these ORFs are assigned letters in the order of their identification like *orfA*, *orfC* and *orfD* [Recchia and Hall, 1995].

In contrast to MIs, CIs contain highly diverse cassettes, mostly of unknown functions. In 2007, 1677 cassettes were identified by the analysis of Vibrionales genome [Boucher et al., 2007]. Among these, 75% of the cassette pool corresponded to accessory genes of unknown functions. Remaining 25% of the cassettes contained genes like phage-related proteins, toxin-antitoxin (TA) systems, resistance gene cassettes, DNA modification systems, virulence and experimentally confirmed restriction modification systems, lipases, dNTP pyrophosphohydrolases, polysaccharide biosynthesis and sulphate–binding proteins [Robinson et al., 2005; Nield et al., 2004; Smith and Siebeling, 2003]. Many *dfr* cassettes in different environmental isolates of *V. splendidus* have been found while *catB9* (encoding chloramphenicol resistance), *carb7*, *carb9* (encoding carbenicillin resistance) and *qnr* (encoding resistance to quinolones) cassettes have been identified in *V. cholerae* CI [Le Roux et al., 2009; Fonseca et al., 2008; Petroni et al., 2004; Melano et al., 2002; Rowe-Magnus et al., 2002]. Altogether, CIs are involved not only in the acquisition of antibiotic resistance genes but also widely in the adaptation of bacteria in different environments [Baharoglu et al., 2013; Cambray et al., 2010].

1.5.1.4. Toxin- Antitoxin (TA) genes

The gene content of SI can be ~3% of the bacterial genome content [Chen et al., 2003; Heidelberg et al., 2000]. The cassette array of SI implies either existence of selective pressure to maintain the gene cassettes or mechanisms that promote their persistence in the absence of selection [Cambray et al., 2010]. These addiction modules are selfish genetic elements. Two classes of addiction modules are known toxin/antitoxin (TA) and restriction methylation systems (RMS) [Van Melderen et al., 2009; Kobayashi, 2001; Gerdes et al., 1997]. TA loci are commonly found on plasmids or within prophages where they have been found to enhance plasmid and phage maintenance by preventing the multiplication of plasmid-free or phage-free progeny in bacterial populations. TA system consists of two ORFs and is organized as an operon (Figure 1.5). One ORF codes for stable toxin while the other codes for unstable antitoxin. Disruption in the expression of TA system leads to the accumulation of free toxin due to loss of neutralizing activity of antitoxin thereby inhibits the multiplication of bacterial growth (devoid of plasmids/ prophages). The

TA systems have been proposed to stabilize chromosomal regions by preventing the accidental deletions when located in unstable segments like MGEs. There are reports of SIs containing the TA cassettes. Thirteen TA cassettes have been reported in the *V. choerae* N16961 SI [Pandey and Gerdes, 2005; Row-Magnus et al., 2003; Heidelberg et al., 2000]. Functional analysis of TA cassettes in SI was also carried out in other groups of *Vibrios* like *V. vulnificus, V. metschnikovii* and *V. fischeri* [Szekeres et al., 2007; Christensen-Dalsgaard and Gerdes, 2006; Row-Magnus et al., 2003]. Similarly, another addiction module RMS consists of long half-life restriction enzymes and comparatively short half-life modification methylases which maintain plasmid stability by post-segregational killing [Kobayashi, 2001]. A bacterium acquiring RMS begins to depend on this system for its survival, as the methylase protects the host genome along with RMS cassettes by methylating the specific sequences that could be recognized and cleaved by a restriction enzyme. Loss or disruption of expression of RMS systems results in cell death through the cleavage of the chromosome by the restriction enzymes. Thus even in the absence of antibiotic exposure, the resistance-conferring gene cassettes linked with this RMS system will be maintained [Kobayashi, 2001]. *Xanthomonads* and *Pseudomonads* SIs contained RMS systems which may stabilize the cassette array in the absence of selection [Row-Magnus et al., 2003; Vaisvila et al., 2001].

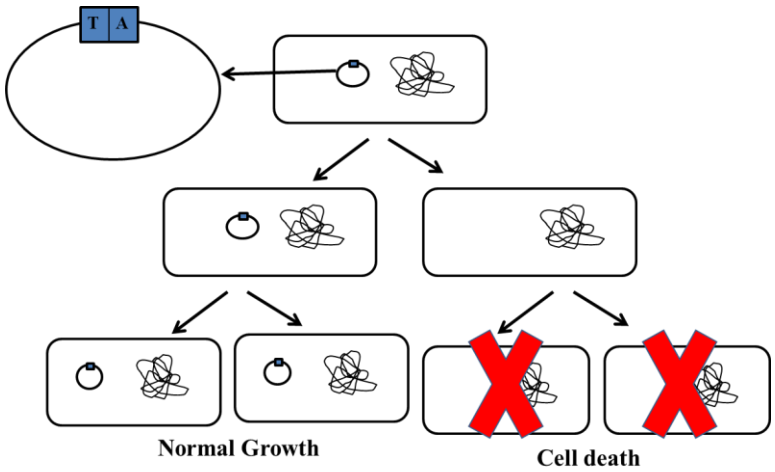

Figure 1.5. Toxin-antitoxin system T: toxin; A: antitoxin. It consists of stable toxin and unstable antitoxin. Disruption in the expression of TA system leads to the accumulation of free toxin due to the loss of neutralizing activity of antitoxin thereby leading to cell death.

1.5.2. Integrative and Conjugative Elements

Integrative and Conjugative Element (ICE), is a conjugative, self-transmissible integrating element that shows similarity to conjugative transposons. ICEs are a group of MGEs that contain programs to determine their excision and integration and play important role in genome flexibility of numerous Gram-positive and Gram-negative bacteria [Ceccarelli et al., 2011]. These elements can integrate into the bacterial genomes, replicate as part of host chromosome, get excised and then disseminate to new host genomes by conjugation (Figure 1.6). Thus these elements have combined features of other MGEs such as 1) phages/transposons which integrate into and excise from the host chromosome but do not have the ability to get transferred, 2) conjugative plasmids which have the ability to transfer from one bacterium to another bacterium by conjugation. Unlike plasmids, ICE elements are not capable of autonomous replication, so they integrate into host chromosome for their replication and get transferred intracellularly or intercellularly [Burrus and Waldor, 2003]. ICEs help bacteria to adapt to new ecological conditions, to colonize in new niches, to survive in stress conditions like antibiotic exposure and play a very important role in reshaping bacterial genomes [Burrus and Waldor, 2004]. It is an important vehicle for spreading of antibiotic resistance in bacteria like *V. cholerae*, *Providencia alcalifaciens, P. rettgeri, Haemophilus influenza, Enterococcus faecalis, Pseudomonas spp.* and *Bacteroides spp*. [Woznaik and Waldor, 2010; Beaber et al., 2002; Waldor et al., 1996]. This MGE harbours a diverse array of genes such as antibiotic resistance genes, genes for complex degradation pathways for toxic compounds and genes for tolerance to heavy metals. Usually, ICEs have modular structures with organized clustered genes [Burrus and Waldor, 2003]. All ICEs contain three distinct components that facilitate their integration, excision, conjugation and its regulation. Those ICE elements which belong to the same ICE family usually share the same integration site and augment intra-species variability. There are various hot regions in the bacterial genome that provide a dramatic illustration of how ICEs generate intra-species diversity at a particular locus and inter-genus locus variability [Burrus and Waldor, 2003]. The recombinase and insertion sequences facilitate ICE evolution by acquisition and dissemination of genomic islands due to the effect of variable conditions [Schubert et al., 2004]. ICEs have been

difficult to identify experimentally as they are associated with the bacterial chromosome. However, *in silico* analysis of various bacterial genomes revealed that ICEs are found in many bacterial species. The SXT/R391 ICE family one of the largest family as it has more than 100 elements, was identified experimentally or bioinformatically [Bi et al., 2012]. Some other previously identified families are Tn916, Tn4371, CTnDot/ERL and ICE6013. One of the well-studied examples of ICE family is SXT element described in the next section.

1.5.2.1. SXT element

SXT element was first identified in *V. cholerae* O139 strain MO10 isolated from an epidemic in Madras, India in 1992 [Waldor et al., 1996]. As this element was identified in *V. cholerae* MO10, the name was given SXT^{MO10} [Hochhut et al., 2001a; Waldor et al., 1996]. It was found to encode resistance property for chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim [Waldor et al., 1996]. SXT element was also reported from other *Vibrios* i.e. *V. cholerae* O1, *V. cholerae* non-O1/non-O139 [Kutar et al., 2013; Mohapatra et al., 2008; Burrus et al., 2006; Hochhut et al., 2001b]. There are many known SXT elements which bear different antibiotic resistance genes i.e. SXT^{MO10}, SXT^{ET}, SXT^{HN1}, SXT^{KN14}, SXT^{MCV09} and SXT^{LAOS} (http://db-mml.situ.edu.cn/ICEberg).

1.5.2.1a. Structure of SXT elements

The SXT elements show modular organization and harbor three modules responsible for their maintenance (integration and excision component), dissemination (conjugation component) and regulation [Burrus and Waldor, 2004]. This conserved backbone can acquire other genes through insertion sequences, transposons and recombinases.

The integration of SXT element in the bacterial chromosome is site-specific (Figure 1.6a). Chromosomal integration and excision occurs at 5'end of *prfC* region (encoding peptide chain releasing factor 3), through non-replicative circular intermediate [Hochhut and Waldor, 1999]. The *prfC* region, the major integration module of *V. cholerae* and other γ-protobacteria, is also responsible for the integration of closely related ICEs including R391, R997 and pMERPH. In the absence of *prfC*

region, SXT can preferentially integrate into others alternative sites, such as the 5' end of *pntB* which encode a pyrimidine nucleotide transhydrogenase [Burrus and Waldor, 2003; Pembroke et al., 2002; Hochhut et al., 2001b]. The SXT-encoded recombinase Int is mainly responsible for integration and excision from the chromosome (Figure 1.6b). The *setC* and *setD*, two loci of SXT element, are required for *int* expression by regulating host promoter [Burrus and Waldor, 2003; Beaber et al., 2002]. A repressor molecule SetR from *V. cholerae* SXT, controls the dissemination of SXT DNA. MosT and MosA toxin-antitoxin proteins promote the maintenance of SXT element [Van Melderen et al., 2009; Magnuson, 2007; Hayes, 2003].

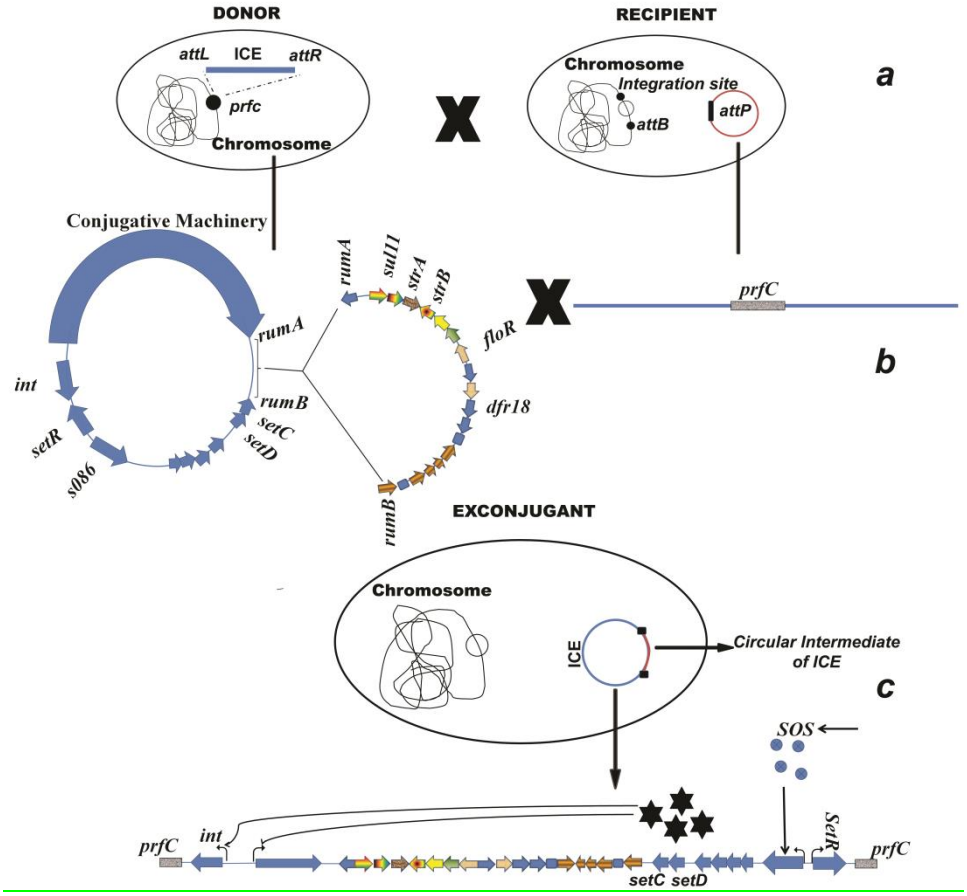

Figure 1.6. Horizontal gene transfer in a bacterial population containing SXT element: a. Donor bacterium transferring chromosomal ICE SXT element to recipient bacterium by conjugation. b. Circular intermediate of SXT element contains conjugative machinery, UV repair DNA polymerase genes (*rumA*, *rumB*), regulatory genes (*int*, *setC*, *setD*, *setR*). Between *rumA* and *rumB*, antibiotic resistance genes *sulII*, *strA*, *strB*, *dfr18* are located. This circular intermediate gets integrated at *prfC* (encoding peptide chain releasing factor 3) region of chromosomal DNA in bacterium; c. Effect of SOS response on SXT regulation and expression: SOS response in bacterial cell mediates autoproteolysis of setR due to activation of recombinase A. This, in turn, activates *setC* and *setD* leading to increased

expression of integrase gene *int* (responsible for integration and excision). [Source: Bhardwaj et al., 2014]

For the dissemination of ICE, they encode proteins that promote conjugation between the donor and the recipient bacteria. Conjugative transfer is initiated at the 299 bp OriT locus that is nicked by a putative relaxase called TraI to produce single-stranded DNA. The conjugative transfer of SXT element is dependent on SXT relaxase TraI that binds covalently to well conserve SXT *oriT* [Ceccareli et al., 2008].

1.5.2.1b. SXT/R391family

Till date, a total of 28 ICE families have been defined based firstly on integrase similarity and secondarily on core structure synteny [Bi et al., 2012]. SXT/R391 family of ICEs is the biggest and most widespread of these 28 families with >100 elements being identified from this group to date. SXT/R391 ICEs are major contributors for the dissemination of drug resistance in *V. cholerae* [Wozniak et al., 2009; Hochhut et al., 2001b]. These ICEs are also present in non-*Vibrio* pathogens [Badhai et al., 2013; Harada et al., 2010; Osorio et al., 2008]. The ICE element, R391, encoding resistance to mercury and kanamycin from a clinical isolate of *Providencia rettgeri* and the SXT element had similar integration mechanisms into the 5' end of the *prfC* gene [Coetzee et al., 1972; Hochhut et al., 2001b]. Nucleotide sequence analysis of 99.5 kb SXT and 89 kb R391 revealed a close similarity between these elements which shared about 65 kb of DNA. This conserved sequences included the machinery for mobility of these elements including the genes for conjugative transfer, integration, excision and regulation of these events [Beaber et al., 2002]. These ICEs have five hotspots for DNA insertion and four minimal gene set modules: *int-xis* (integration and excision module); *mob* (DNA mobilization and processing module); *mfp* (mating pair formation module); *reg* (regulation module) which are required for the integrity of these elements [Wozniak and Waldor, 2010].

1.5.3. Plasmids

Antimicrobial resistance in bacteria has been largely attributed to gene exchange due to horizontal transfer of plasmid-located resistance genes. Naturally occurring plasmids range in sizes from few kilobases to several hundred kilobases and in copy

number from one to several hundred per cell [Novick, 1987]. Copy number is controlled by a plasmid-coded system that determines the rate of initiation of replication. Naturally occurring plasmids are transferred and maintained in the progeny cells through the partitioning mechanisms. Plasmids have been shown to harbor the resistance to the major classes of antimicrobials, including β-lactams, aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, macrolides and quinolones [Carattoli, 2009]. Plasmids acquire mobile genetic elements (integron, insertion sequences and transposons) that mobilize the antimicrobial resistance genes, toxin genes, degrading enzymes and transfer genes. Plasmids promote the horizontal transfer of resistance determinants among bacteria of different species, genera and kingdoms, depending on their narrow or broad host range, conjugative properties and efficiency of conjugation [Thomas and Nielsen, 2005].

1.5.3.1. Features and characteristics of resistance plasmids

Plasmids conferring multidrug resistance are generally larger in size, self-conjugative and encode mechanisms for controlled copy number and rate of replication [Nordstrom, 2006]. The minimum portion of a plasmid that replicates with a characteristic copy number of the parent plasmid is called basic replicon [Kollek et al., 1978]. Replicon contains an origin of replication (*Ori*) and specific replication initiator protein (Rep) that binds the *Ori* and their regulating factors [Couturier et al., 1988; Kollek et al., 1978]. Two plasmids with the identical replicons cannot be propagated by replication in same bacteria. This phenomenon is known as plasmid incompatibility. Promiscuous broad host range plasmids mostly carried portioning systems or restriction methylation systems that ensure stable inheritance during the cell division.

1.6. Mobile genetic elements imparting drug resistance in *Vibrio* and *Shigella*

As mentioned in the earlier section, drug resistance is prevalent in *Vibrio* spp. as well as in *Shigella* spp. Various mobile genetic elements play an important role in acquisition and dissemination of drug resistance. The spread of drug-resistant genes in these organisms is facilitated by HGT through the ICEs, plasmids and integrons.

1.6.1. Mobile genetic elements in *Vibrio* spp.

The SXT element harbored various genes responsible for resistance to sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin [Waldor et al., 1996]. *V. cholerae* O1, O139, non-O1 and non-O139, *V. fluvialis* and other *Vibrio* species were reported to carry the SXT elements or different siblings of SXT elements [Kutar et al., 2013; Mohapatra et al., 2008; Ahmed et al., 2005; Waldor et al., 1996]. The acquisition of the SXT family of antibiotic resistance elements has shaped the pandemic spread of *V. cholerae*. This study has also shown that this family was first acquired at least ten years before its discovery [Mutreja et al., 2011].

Apart from the SXT element, *Vibrios* also harbor integrons. In *Vibrios,* class 1 integron was well reported [Kitoka et al., 2011; Adabi et al., 2009; Ceccarelli et al., 2006; Srinivasan et al., 2006]. Class 2 integron was also reported in *Vibrio* spp. [Sá et al., 2010; Opintan et al., 2008; Ahmed et al., 2006a]. Both classes carry multiple gene cassettes encoding resistance genes, for example, *dfr* (trimethoprim resistance). Super-integron was also first reported in *Vibrio cholerae* N16961 that harbored various gene cassettes including the antibiotic resistance genes [Mazel et al., 1998].

Plasmids are also a key element for the horizontal gene transfer of drug resistance genes in *Vibrios*. The quinolone resistance proteins (qnrs) residing on the plasmids are known as plasmid-mediated quinolone resistance (PMQR) factors. The PMQR determinants were frequently reported from *Vibrio* species. 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 elements and plasmids [Fonseca and Vincente, 2013]. The *aac(6') Ib-cr* gene was reported from *V. fluvialis* [Chowdhury et al., 2011] and *V. parahaemolyticus* [Aedo et al., 2014]. Apart from PMQR, there are plasmids which harbor the resistance genes for tetracycline, ampicillin, kanamycin, streptomycin, gentamicin and trimethoprim [Glass et al., 1983]. A conjugative plasmid (p3iANG) in *V. cholerae* O1 isolate carried a set of three class 1 integrons harboring *dfrA15, blaP1* and *qacH-aadA8* cassettes which code resistance phenotype for trimethoprim, beta-lactam, quaternary ammonium compounds and aminoglycoside. This plasmid also harbored chloramphenicol (*cat1*), sulphonamide (*sul2*) and tetracycline (*tetG*) resistance genes in between the spacer region of two integrons [Ceccareli et al., 2006].

1.6.2. Mobile genetic elements in *Shigella* spp.

Resistance to some of the antibiotics in *Shigella* has been attributed to class 1 and class 2 integrons. Class 1 integrons harbored the *dfrA* (trimethoprim resistance), *aadA* (aminoglycoside resistance), *catB* and *cmlA* (chloramphenicol resistance) and bla_{OXA} (beta lactam resistance) gene cassettes [Xu et al., 2016b; Ahmed and Shimamoto, 2015; Eftekhari et al., 2013; Dubois et al., 2007]. An atypical class 1 integron harbored the bla_{OXA30} and *aadA1* genes that conferred resistance to ampicillin and streptomycin respectively [Pan et al., 2006]. This atypical class 1 integron was first found on Pathogenicity Island carrying *Shigella* Resistance Locus (SRL), on the chromosome of *S. flexneri* 2a strain YSH6000 and carried various genes such as *aadA1-*bla_{oxa}*-cat-tetA-tetC*, that were the resistance determinants for ampicillin, streptomycin, chloramphenicol and tetracycline [Pan et al., 2006; Turner et al., 2003; Luck et al., 2001].

Class 2 integron typically harbours *dfrA1, sat1* (streptothricin resistance) and *aadA1* gene cassettes [Zhu et al., 2011a; Gassama-Sow et al., 2010; Gu et al., 2008; Dubois et al., 2007]. Class 2 integrases are non-functional and the majority of the cassette arrays on class 2 integrons are usually constant. There are also some reports which showed the unusual class 2 integron that harbored the *dfrA1* or *dfrA1-dfrA12* or *dfrA1-sat1-aadA1-orfX* gene cassettes [Gassama-Sow et al., 2006; Oh et al., 2003].

It has also been demonstrated that all the recent emergence of *S. sonnei* infections are due to a small number of clones that dispersed globally from Europe within the last 500 years [Holt et al., 2012]. Four distinct lineages of *S. sonnei* were identified. Lineage III is globally most prevalent and becoming dominant in Asia, Africa and South America. *S. sonnei* belonging to lineage III carried a distinct class 2 integron which conferred resistance to trimethoprim, streptothricin and streptomycin. Many of these lineages also harbored a genetic locus on a small plasmid that conferred resistance to tetracycline and sulphonamides. Similar studies were also carried out in *S. flexneri*, but no correlation was found in the lineages and intercontinental spread of antibiotic resistance elements/loci [Connor et al., 2015]. The systematic review of mobile genetic element-mediated drug resistance reported from *Vibrio* and *Shigella* species in the past ten years is listed in Table 1.2.

Table 1.2. Prevalence of mobile genetic elements in *Vibrio* spp. and *Shigella* spp.

Sr. No.	strains	Year/ sample/place	Presence of Mobile genetic elements	Drug resistance gene cassettes	Remarks	Reference
1	*Vibrio parahaemolyticus* V36	2010, Shrimp sample from Hong Kong	Plasmid harbouring complex class 1 integron	IS*CR1-bla*$_{PER-1}$*-gst-abct-qacEΔ1-sul1*	-	Li et al., 2015a
2	*Vibrio cholerae* O1 El Tor IDH02365	2009, clinical sample, Kolkata, India	Plasmid, SXT	Not identified	Plasmid carried resistance genes for ampicillin, kanamycin, neomycin	Rajpara et al., 2015
3	*Vibrio cholerae* O139	1993 to 2009, clinical and environmental samples, china	Class1 integron, SXT	*dfrA12-orfF- aadA2, aar3-dfrA27-aadA16, arr2-aadA3C, aadA2*	-	Yu et al., 2012
4	*Vibrio cholerae* L34, VC97,VC98	1991 and 1979, Brazilian Amazon region and Ghana, Africa	Class 2 integron	*sat2-aadA1-ybeA*	-	Da Fonseca et al., 2011
5	*Vibrio cholerae* non-O1/non-O139	Clinical sample, China	Class 1 integron	*arr3-dfrA27-aadA16*	-	Sun et al., 2010
6	*Vibrio cholerae*	2004 to 2006, clinical sample, Iran	Class 1 integron, SXT element	*aadA2*	-	Adabi et al., 2009
7	*Vibrio cholerae* O1 El Tor	2007, clinical sample, Eastern India	Class 1 integron, SXT element	-	-	Jain et al., 2008
8	*Vibrio cholerae O1*	2006, Clinical sample, Accra, Ghana	Class 1 integron, Class 2 integron, SXT element	*dfrA1-sat-aadA1*	-	Opintan et al., 2008
9	*Vibrio cholerae O1* VC627	1998, clinical sample, Amazon region, Brazil	Class 1 integron	*aadA2-qnrVC2*	-	Fonseca et al., 2008
10	*Vibrio cholerae* non-O1/non-O139	1979 to 1988, clinical and environmental sample, Varanasi, India	Class 1 integron, SXT element	*aadA1, aadA2, aadA5, dfrA15, dfrA18, sulII and strAB*	-	Mohapatra et al., 2008
11	*Vibrio parahaemolyticus* V4	2013, China	Plasmid	*traB-traV-traA*-IS*Ecp1-bla*$_{CMY-2}$*-blc-sugE-encR-orf1-orf2-orf3-orf4-dsbC-traC*	-	Li et al., 2015b
12	*Vibrio cholerae* V122	2012, Hong Kong	Plasmid	*qnrVC7*	-	Po et al., 2015

13	*Vibrio cholerae O139* ICDC 1447	2005, clinical sample, China	Plasmid	*catII, tetR, tetD,mel, mph2, aadA, qacEΔ1, sulI, floP* and bla_{TEM}	-	Wang et al., 2015
14	*Vibrio cholerae* 2012EL-2176	2012,clinical sample, Haiti	Plasmid	*aac(3)-IIa*, bla_{CMY-2}, $bla_{CTX-M-2}$, bla_{TEM-1}, *dfrA15*, *mphA*, *sul1*, *tetA, floR*, *strAB*, and *sul2*	-	Folster et al., 2014
15	*Vibrio parahaemolyticus*	Shrimp sample, Hong Kong	Plasmid, Class 1 integron	*qnrVC6,* bla_{PER1} *and aacA3-catB2-dfrA1- aadA1*	-	Liu et al., 2013a
16	*Vibrio cholerae*	2005-2007, clinical sample, Wardha, India	SXT element	-	Resistance to Sulphamethoxazole, streptomycin, trimethoprim, chloramphenicol	Pande et al., 2012
17	*Vibrio fluvialis*	2009, West Bengal, India	Plasmid, Class 1 integron	*qnrA1, arr3,* bla_{SHV}, bla_{TEM}, $bla_{CTX-M-3}$, bla_{OXA1}, bla_{OXA7}, *aadA1, strA, aadB, aac(6_)-Ib-cr*	-	Chowdhury et al., 2011
18	*Vibrio fluvialis*	1998 to 2002, clinical sample, India	Class 1 integron, plasmid, and SXT element	*dfrA15, dfrA1, aadA7,aac(3')Ib* and *orfC*	-	Srinivassan et al., 2006
19	*Vibrio cholerae* O1 El Tor	1998 to1999, Clinical sample, Eastern Africa	SXT related ICE and plasmid	*floR, sulII, strA* and *dfrA1*	-	Pugliese et al., 2009
20	*Vibrio cholerae* O139	2004, Clinical sample, China	Conjugative plasmid	-	Plasmid carried resistance genes for ampicillin, streptomycin, gentamicin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole	Pan et al., 2008
21	*Vibrio splendidus*	Environmental sample, US and France	Plasmid	*qnrS*	-	Cattoir et al., 2007
22	*Vibrio cholerae* O1 El Tor	2009,clinical sample, Kolkata, India	SXT element, Plasmid	-	carried resistance for chloramphenicol, trimethoprim, sulfamethoxazole , streptomycin and cotrimoxazole	Kutar et al., 2013

23	*Vibrio cholerae* O1	2005 to 2007, clinical sample, Iran	SXT element	*dfrA, floR, strB* and *sulII*	-	Rahmani et al.,2012
24	*Vibrio cholerae O1*	1992 to 2007, Clinical sample, India	SXT element, Class 1 integron	*aadA, strA*	-	Goel and Jiang, 2010
25	*Vibrio cholerae O1*	2002 to 2008, Clinical sample, Dhaka	SXT element	*dfr6 and qnrVC3*	-	Kim et al., 2010
26	*Shigella flexneri*	2001 to 2011, Jiangsu Province, China	class 1, class 2, and the atypical class 1 integrons	*dfr*A5, *aad*A1, *dfr*A1- *aad*A1, *dfr*A17-*aad*A5, and *aac*A4-*cml*A1 , bla_{OXA30}-*aad*A1 on class 1 integron *dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Xu et al.,2016b
27	*Shigella flexneri*	2009 to 2012, clinical sample, Jinan, China	class 1, class 2, and the atypical class 1 integrons	dfrV, *dfr*A17-*aad*A5, bla_{OXA30}-*aad*A1 on class 1 integron *dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Ma et al., 2015
28	*Shigella flexneri*, *S. boydii* and *S. sonnei*	2011to 2013, clinical sample, Gabon , Africa	Atypical class 1 integron and class 2 integron	bla_{OXA30}-*aad*A1	-	Schaumburg et al., 2015
29	*Shigella flexneri*, *S. dysenteriae* and *S. sonnei*	Food sample, Egypt	Class 1 integron, class 2 integron and plasmid	*qnr*B, *qnr*S, *aac*(6')-*Ib-cr,* *dfr*A1-*sat*1-*aad*A1 and *dfr*A1-*sat*1 on class 2 integron *aad*B-*cat*B3, *dfr*A12-orf-*aad*A2 and *est*X-*aad*A1 on class 1 integron	-	Ahmed and Shimamoto, 2015
30	*Shigella flexneri* 1b	2005 to 2013, clinical sample, China	Atypical class 1 integron and class 2 integron	bla_{OXA30}-*aad*A1 on class 1 integron *dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Cui et al., 2015
31	*Shigella spp.*	2007 to 2010, clinical sample, Kolkata, India	Atypical class 1 integron, class 2 integron and plasmid	*dfr*A1-*sat*1-*aad*A1 and *dfr*A1-*sat*1 on class 2 integron bla_{OXA30}-*aad*A1 on class 1 integron *qnr*S, *aac*(6')-*Ib-cr*	-	Ghosh et al., 2014
32	*Shigella flexneri*	1981 to 1983 and 2009 to 2010, clinical sample, China	Atypical class 1 integron and class 2 integron	*dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Wang et al., 2014
33	*Shigella sonnei*	2011 to 2013, clinical sample, Bhutan	Class 2 integron	*dfrA1, sat1* and *aadA1*	-	Ruekit et al.,2014

34	*Shigella flexneri*, *S. boydii* and *S. sonnei*	2005 to 2009, clinical sample, China	class 1, class 2, and the atypical class 1 integrons	*dfr*A17-*aad*A5, *arr3*-*aac*A4, *dfr*A12-orfF-*aad*A2 on class 1 integron bla_{OXA1}-*aad*A1 on atypical class 1 integron *dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Yang et al., 2014
35	*Shigella flexneri* and *S. sonnei*	1998 to 2008, clinical sample, Hungary	Class 1 integron and class 2 integron	*aad*A1 or *dfr*A1-*aad*A1 on class 1 integron *dfr*A1-*sat*1-*aad*A1 or *dfr*A1-*sat*1 on class 2 integron	-	Nogrady et al., 2013
36	*Shigella sonnei*	2001 to 2011, Clinical sample, Bangladesh	Class 1 integron and class 2 integron	*dfr*A5 on class 1 integron *dfr*AI-*sat*1-*aad*A1-orfX or *dfr*A1-*sat*2 on class 2 integron	-	Ud-Din et al., 2013
37	*Shigella* spp.	2009 to 2010, Clinical sample, Iran	Class 1 integron and class 2 integron	*dfr*A7, *dfr*A17, *aad*A5 and *cat*B3 on class 1 integron *dfr*AI-*sat*1-*aad*A1 or *dfr*A1-*sat*2 on class 2 integron	-	Eftekhari et al., 2013
38	*Shigella flexneri* and *S. sonnei*	Clinical sample, China	Class 1 integron and class 2 integron	*dfr*A17-*aad*A5, *dfr*A12-orfF-*aad*A2 of class 1 integron and *dfr*A1-*sat*1-*aad*A1 of class 2 integron	-	Zhu et al., 2011b
39	*Shigella flexneri*	2005 to 2006, Clinical sample, China	Atypical class 1 integron, class 1 integron and class 2 integron	*dfr*A12-orfF-*aad*A2 or *dfr*A17-*aad*A5 on class 1 integron bla_{OXA30}-*aad*A1 on Atypical class 1 integron and *dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Zhu et al., 2011a
40	*Shigella sonnei*	1982-1987, 1993, 1995, 1997 and 1998 , Clinical sample, Taiwan,ROC	Class 1 integron , class 2 integron, and plasmid	estX-psp-*aad*A2- *cml*A1-*aad*A1-qacH, *dfr*17-*aad*A5, *aad*A1 and *dfr*12-orfF-*aad*A2 on class 1 integron *dfr*A1-*sat*1-*aad*A1 on class 2 integron bla_{TEM}, *cat*1, *tetB,tetA* and *sul*2 genes on plasmid	-	Chang et al., 2011
41	*Shigella sonnei*	1999 to 2008, Seoul	Class 1 integron and class 2 integron	*dfr*A12-*aad*A2 on class 1 integron *dfr*A1-*sat*1-*aad*A1 or *dfr*A1-*sat*1 on class 2 integron	-	Jin et al., 2010
42	*Shigella spp.*	1990 to 2002, clinical sample, France	Atypical class 1 integron and class 2 integron	bla_{OXA30}-*aad*A1 on class 1 integron *dfr*A1-*sat*1-*aad*A1 on class 2 integron	-	Dubois et al., 2007

43	*Shigella spp.*	2000 to 2004, clinical sample, Japan	Class 1 integron and class 2 integron	estX-*aad*A1 on class 1 integron *dfr*A1-*sat*1-*aad*A1 or *dfr*A1-*sat*1 on class 2 integron	-	Ahmed et al., 2006b
44	*Shigella flexneri*	1998, clinical sample, China	plasmid	$bla_{CTX\text{-}M\text{-}55}$	-	Wang et al., 2013
45	*Shigella flexneri*	China	Plasmid	*aac-6'-Ib-cr* and *qnrS*	-	Pu et al., 2016
46	*Shigella spp.*	1998 to 2013, clinical sample, China	Plasmid	*aac-6'-Ib-cr* and *qnrS*	-	Pu et al., 2015
47	*Shigella flexneri*	2006 to 2011, Clinical sample, Bangladesh	Plasmid	*sul2*	-	Iqbal et al., 2014
48	*Shigella spp.*	2001 to 2008, clinical sample, China	Plasmid	*qepA* and *aac(6)-Ib-cr*	-	Yang et al., 2013a
49	*Shigella sonnei*	Clinical sample	Plasmid	$bla_{CTX\text{-}M\text{-}55}$	-	Lee et al., 2013
50	*Shigella spp.*	2006 to 2009, clinical sample, US	Plasmid	*qnrS1, qnrB19, qnrB6* and *aac*(6')-*Ib-cr*	-	Folster et al., 2011
51	*Shigella sonnei*	2005 to 2006, clinical sample, US	Plasmid	*mphA*	-	Howei et al., 2010
52	*Shigella flexneri 3a*	Clinical sample, India	Plasmid	-	Plasmid mediated resistance for streptomycin and sulfamethoxazole	Barman et al., 2010
53	*Shigella sonnei*	Clinical sample, Vietnam	Plasmid	*bla*CTX-M-24	-	Nguyen et al., 2010
54	*Shigella spp.*	2001 to 2004, Clinical isolates, India	Plasmid	aac(6)-Ib-cr	-	Pazhani et al., 2008
55	*Shigella flexneri*	2006, Clinical isolates, Argentina	Plasmid	CMY-2	-	Rapoport et al., 2008
56	*Shigella sonnei*	2004 to 2005, clinical isolates, Lebanon	Plasmid and Class 2 integron	*bla*-CTX-M-15, *bla*-TEM-1 *dfr*A1, *sat*1 and *aad*A1 on class 2 integron	-	Matar et al., 2007
57	*Shigella spp*	2001 to 2002, clinical sample, Bangladesh	Plasmid	-	Carried ESBL	Rahman et al., 2007
58	*Shigella dysenteriae*	1999 to 2004, clinical sample, Bangladesh	Plasmid	-	Carried ampicillin and co-trimoxazole	Talukder et al., 2006

1.7. Rationale of the study

Diarrhoeal infections caused by *Shigella* and *Vibrio spp* are a major health threat in developing countries like India. In addition, there is a problem of antibiotic resistance and multidrug resistance that pose serious clinical problems like excessive morbidity and mortality. Therefore, to control the spread of the disease, continuous surveillance is important to monitor drug resistance patterns in a given geographical region/population. It is also of great consequence to understand the basic mechanisms that lead to MDR phenotypes. Understanding of drug resistance mechanisms could provide new treatment options to disarm potential pathogens and management of these diseases. For example, development of efflux pump inhibitors to use as adjuvants in antibiotic therapy or use of β- lactamase inhibitors like clavulanic acid along with β-lactam antibiotics.

The major genetic factors/mechanisms for MDR have long been recognized, including integrons, transposons, multidrug efflux, hyper-mutability, plasmids and promiscuous drug resistance. Within many individual isolates, the complexity of resistance is increasing, with multiple determinants being gained, amplified and lost. Therefore, understanding the role of the mobile genetic elements could provide possible reasons for dissemination and dispersal of antibiotic resistance in any population of pathogenic bacteria causing diarrhoeal outbreaks at diverse geographical location.

1.8. Objectives of the study

The objective of this work was to unravel and understand the role of mobile genetic elements in the acquisition and dissemination of drug resistance genes in the context of molecular epidemiology in *Vibrio* spp. and *Shigella* spp.

- Study of clonal relationships of the acquired and confirmed bacterial isolates (*Vibrio* spp. and *Shigella* spp.)
- Determination of antibiotic susceptibility profiles of these bacterial isolates with appropriate antibiotics.
- Screening of these isolates for the presence of mobile genetic elements [plasmids, integrons and conjugative transposons (SXT elements)] and determination of their transferable resistance traits.

- Detection of inherent/chromosomal borne drug resistance mechanisms like efflux pump and mutation in topoisomerases that could be working in synergy with these mobile genetic elements.
- Sequencing and detailed analysis of genes isolated from integrons and plasmids by tools like BLAST/Alignments/ORF finder and GenBank submissions of these sequences.