Cholera is a life threatening diarrhoeal disease that can rapidly attain acute epidemic and pandemic proportions. The causative organism, *Vibrio cholerae*, is a Gram-negative curved rod with a single polar flagellum and is highly motile (Wachmuth *et al.*, 1994). *V. cholerae* has been classified into various serogroups, based upon the serological cross reactivity of the O-antigens present on their surface (Shimada *et al.*, 1994). The strains belonging to O1 serogroup, which have traditionally been associated with the cholera epidemics have been further classified into two biotypes, Classical and El Tor, based on their phage sensitivity and a few other biological characteristics (Wachmuth *et al.*, 1994). Traditionally, *V. cholerae* non-O1 strains were known to cause only sporadic infections and were not thought to have the potential to cause epidemics (Morris, 1990). However, in 1992 major outbreaks of cholera in India and Bangladesh, were found to be caused by a non-O1 strain of *V. cholerae,* which was subsequently named *V. cholerae* O139 Bengal (Ramamurthy *et al.*, 1993). After about 2 years, these strains were once again displaced by *V. cholerae* O1 El Tor strains, belonging to a different clone from the one, which were in existence before the O139 outbreak (Sharma *et al.*, 1997).

The *V. cholerae* O139 is nearly identical to O1 El Tor excepting that it possesses a capsule, which is distinct from the lipopolysaccharide (LPS) antigen. In one study, evidence was presented to show that O139 strain could have originated from *V. cholerae* O1 El Tor strains (Pajni *et al.*, 1995), through the deletion of about 22Kb of DNA and incorporation of a new piece of DNA within the *rfb* region, which specifies the O-antigen in *V. cholerae* O1 strains (Bik *et al.*, 1995). *V. cholerae* O22 has been shown to be the likely origin of the genes for O139 biosynthesis (Yamasaki *et al.*, 1999 and Dumontier *et al.*, 1998).

To date, about 200 serotypes of *V. cholerae* are known, out of which only O1 and O139 are responsible for the cholera epidemics (Yamai *et al.*, 1997). Strains belonging to serogroups other than O1 and O139, comprise a heterogeneous group which are collectively referred to as the non-O1, non-O139 strains are, found ubiquitously in the aquatic environs are known to be capable of causing sporadic diarrhea (Janda *et al.*, 1988). The clinical association of these organisms with humans, however, is inadequately understood.

There exists in the literature a few reports of major localized outbreaks caused by these strains. An outbreak in Czechoslovakia in 1965 due to *V. cholerae* 05 (Aldova *et al.*, 1968), another one in Sudan in 1968 by O37 serotype (Kamal *et al.*, 1971), an

outbreak in the flight from London to Australia in 1973, (Dakin *et al.,* 1974), one in Lima, Peru in 1994 due to *V. cholerae* O10 and O12 serotypes (Dalsgaard *et al.,* 1995) and so on. In India, an outbreak due to *V. cholerae* O10 was recorded in East Delhi by Rudra *et al.* in 1996. Many of these strains when examined, were found to be resistant to more than one antibiotic.

About 20 years ago the strains of *V. cholerae* were mostly susceptible to the drugs normally used as adjunct therapy in the treatment of cholera (Greenough *et al.,* 1964, Sack *et al.,* 1978 and Islam MR, 1987). However, this situation changed drastically over the years and the reports on the emergence of drug-resistant *V. cholerae* are now appearing with increasing frequency (Mukhopadhyay *et al.,* 1996). Emergence of strains resistant to multiple drugs is a serious clinical problem in the treatment and containment of cholera. Further, the appearance of drug resistant non-O1, non-O139 strains pose a serious problem not only, because the incidences of "cholera" caused by such strains in hospitalized patients are increasing but also because they can act as reservoirs for drug resistant genes.

In 1996, an inexplicable upsurge in the incidence of cholera like illness due to strains belonging to serogroups other than O1 and O139 occurred in Kolkata, India. The diarrhea caused by these strains were clinically indistinguishable from cholera and the nomenclature of, Entero pathogenic *V. cholerae* (EPVC) was proposed to include these serotypes (Sharma *et al.,* 1998). The incidence of EPVC had shown an upward trend 1997 onwards which continued well up to 1998. In the months of July and August in 1998, the EPVCs constituted one third of all *V. cholerae* strains isolated from the hospitalized cases of cholera in Kolkata (Garg *et al.,* 1998).

Even though drug resistant strains of *V. cholerae,* both O1 and non-O1, non-O139 are emerging with increasing frequency there is paucity of information on the underlying basis of drug resistance in *V. cholerae* strains, particularly non-O1, non-O139. Plasmids, which play an important role in the dissemination of drug resistance, have not been found to play major role in *V. cholerae* O1, and is not universally present. In contrast, plasmids large and small, are more ubiquitous in *V.cholerae* non-O1, non-O139 strains. Nonetheless, very little information about these plasmids or the role these plasmids play is available. This thesis incorporates the results of our attempt to rectify this situation by analyzing 93 outbreak strains of *V. cholerae* non-O1, non-O139, isolated in Kolkata, in 1997-98.

In chapter 3, the results of the analysis of 93 non-O1, non-O139 strains to understand the genetic basis of drug resistance, with particular reference to the plasmids carried by them, are presented. It was found that 43 strains carried plasmids out of which 28 strains carried plasmids both large and small, and four had only large plasmids. From the agarose gel profile of these plasmids, it appeared that many of these plasmids had identical molecular size even though they are residents in different hosts. Further, some strains were found to carry more than one large plasmid. Large plasmids from 10 plasmid-bearing strains could be transformed into *E. coli* JM109, transferring resistance to a number of drugs, but not all to the recipient *E.coli* strain. This indicated the presence of other factors responsible for the drug resistance displayed by the parent strains. Among the "vehicles" of transfer of drug resistance, "integrons" are known to occupy an important position. Integrons are "gene-expression" elements that can acquire "open reading frames" usually coding for drug resistance and convert them to functional genes (Hall and Stokes 1993).

When the strains under study were examined for the presence of class 1 integrons, using defined primers, and also by hybridization with appropriately designed probes, class 1 integrons could be detected in 22 of the isolates. Out of these, 14 had both plasmids and integrons. It was found that in 8 strains, integrons were residents on large plasmids, whereas in another 3, class 1 integrons were found to be located both on the plasmid and the chromosome. Six different gene cassettes namely *dfrA1, dfrA5, dfrA15* (for Trimethoprim resistance), *ereA2* (for Erythromycin resistance), the *aac(6')-Ib,* (for Amikacin resistance) and *aadA2* (for aminoglycoside. resistance) were found to be harboured by the integrons. Some of which, namely *dfrA5, dfrA15,* the *aac(6')-Ib* and *ereA2* were detected in *V. cholerae* for the first time.

Besides the drug markers harboured on "integrons" and plasmids (some markers in plasmids were not carried by integrons), the presence of another mobile element, a conjugative transposon called SXT, was discovered in 12 strains. Six out of these 12 strains were also found to harbour large plasmids. Thus in summary, the results described in this chapter showed that plasmids, integrons, SXTs, all play important role in conferring drug resistance to strains of *V. cholerae*; of particular interest was the presence of large plasmids harbouring drug resistance markers, many of which were integron-bourne. In quite a few strains making such strains particularly perilous as far as the spread of drug resistance is concerned.

Plasmid bourne multiple drug resistance in *V. cholerae* O1 El Tor was first reported way back in 1979 in an outbreak in Bangladesh (Glan *et.al.*, 1980). Subsequently over the years, strains of *V. cholerae* belonging to O1, O139 and non-O1, non-O139 serotypes with drug resistant plasmids of various size in this have been reported in the literature. In view of this it was of interest to see if the plasmids, particularly the large ones found in the non-O1, non-O139 strains described in chapter 3, could have been "donated" to them by the O1 strains, which were present prior to the isolation of the non-O1, non-O139 strains. In chapter 4, results of the examination of 78 *V. cholerae* O1 strains, isolated between 1992-1996 in India, for the drug resistant determinants with particular reference to plasmids are presented.

All the strains were found to be multidrug resistant. 23 of the 78 strains carried class 1 integrons and 58 had SXT elements. Quite surprisingly, however, none of the strains were found to carry any plasmid, ruling out this possibility that O1 strain could be the "source" of large plasmids. Further, when the results described in chapter 3 and 4 were compared, it was seen that while *V. cholerae* O1 strain carried only one type of gene cassette in the integrons, non-O1, non-O139 strains carried six different type of gene cassettes providing clear indication that non-O1, non-O139 strains did not "receive" these cassettes from *V. cholerae* O1 strains. Rather these could have come from other pathogenic bacteria present in the environment.

The results presented in chapter 4 considered together with those in chapter 3 also indicated that while in *V. cholerae* non-O1, non-0139 strains the plasmids are stably maintained, it is not so for the O1 strains. In an attempt to obtain a clue as to why it is so and in view of the importance of these plasmids in the spread of drug resistance, it was decided to characterize a large plasmid in detail. Chapter 5 of the thesis describes the experiments carried out to achieve that for the purpose of detailed characterization, one of the two large plasmids present in the clinical non-O1, non-O139 isolate PL107b, was selected and the plasmid designated pKA1. This plasmid lacked class I integron and the host strain harbouring it did not carry the SXT elements. Transformation of this plasmid into *E.coli* JM109 transferred resistance to five antibiotics namely, Ampicillin, Streptomycin, Chloramphenicol, Sulfamethoxazole and Trimethoprim, out of the nine antibiotic resistances, displayed by the mother strain. Through restriction endonucleases digestion and "follow-up" agarose gel electrophoresis, the approximate molecular weight of the plasmid was determined to be between 111-117kb. A library of this plasmid was

generated into pUC19 and the clones were sequenced by primer walking. The sequencing results revealed the presence of all five antibiotic resistant genes in the large plasmid. Beta lactamase gene, *bla* for Ampicillin resistance, *strA* (Amino-glycoside-3-phosphotransferase) and *strB* genes (Streptomycin phosphotransferase) for streptomycin resistance, *floR*, gene a florfenicol exporter for Chloramphenicol resistance, *sulII* (dihydropteroate synthase type II) for Sulfamethoxazole resistance and *dfrA15* (dihydrofolate reductase) for Trimethoprim resistance. Besides this large plasmid carried ParAB system of partitioning which is responsible for correct partitioning of the daughter plasmid among the daughters cells, replication initiation protein, site-specific recombinases transposes and resolvases showing homology with different genes in *E.coli* e.g. flagellar specific ATPase, flagellar biosynthesis genes *fli M, fli K, fli L, nfnB* gene for reduction of mutagenic nitroarenes, *exb* B and *exbD* genes responsible for uptake of enterocholin, *entA* and *entB* for biosynthesis of cofactor carrier enterocholin, *lpx A* and *lpxB* for lipid A biosynthesis, *betT* high affinity choline transport protein, *guaC* GMP reductase, *hofC* putative integral membrane protein involved in biogenesis of fimbriae, protein transport, DNA uptake.

To summarize, this thesis presents the result of an investigation undertaken to analysis the molecular basis of drug resistance in strains of *V. cholerae* non-O1, non-O139 will particular reference to the large plasmids present there in. Even though our study has thrown considerable light on the molecular mechanism underlying the drug resistance in these strains and also on the nature of at least one large plasmid in detail, it could not provide any clue, as we had expected, as to why large plasmids are not usually found in *V. cholerae* O1 strains; Assuming both *V. cholerae* O1 and non-O1, non-O139 strains occupy more or less the same niche, it is inconceivable that large plasmids are not occasionally transmitted to their non-O1, non-O139 cousins.