2. Review of literature
2. REVIEW OF LITERATURE

2.1. Vibrio cholerae and cholera

*Vibrio cholerae* is a comma shaped, non-spore forming, facultative anaerobic, gram negative, motile organism with single polar flagellum (Barua, 1992). The infection in human results from ingestion of contaminated water or food and the incubation period to cause diarrhea ranges from 12 h to 72 h depending upon inoculation size (Levine *et al*., 1981). The dreaded diarrheal disease ‘cholera’ is characterized the passages of rice watery stool leading to severe loss of electrolytes, dehydration, and voluminous shock. Death can happen from cholera if the patient is left untreated for long time (Kaper *et al*., 1995). The term Cholera has been derived from the Greek words “Chole” (bile) and “Rein” (to flow). In India, the sanskrit word “Visuchica” from ‘Sushruta Samhita’ is thought to denote cholera (Barua, 1992). Cholera in human is caused by *V. cholerae* strain belonging to serogroup O1 and O139 (Morris, 1995). These strains secrete cholera toxin (CT) as major toxin to cause cholera (Kaper *et al*., 1995).

Outbreaks of cholera can be traced back in time to early recorded descriptions of enteric infections. However, it was not until 1854, when the classical epidemiological study of John Snow who mapped transmission of cholera through water in London (Parkes, 2013). The organism *V. cholerae* was first identified in 1854 in Italy by Pacini, who found a large number of curved bacteria in the intestinal contents of cholera victims which he termed *Vibrio*
cholera (Pollitzer, 1959). This pioneering work was overshadowed by the work of Robert Koch, who studied the stool sample of an Egyptian patient in 1883 and designated the causative agent as *Kommabazillen* (Pollitzer, 1959). To acknowledge the pioneering work of Pacini, the organism was renamed as *Vibrio cholerae* (Kaper et al., 1995).

The small intestine is the primary site of infection where quick massive loss of fluid and salts leads to tachycardia, hypotension and vascular collapse (Morris and Black, 1985). The treatment of cholera involved the rapid intravenous or oral replacement of fluid and electrolytes (Carpenter, 1992) which is being continued with certain modifications (Bhattacharya, 2003). To follow the replacement, administration of isotonic maintenance solution (here glucose) is generally continued until the diarrhoea ceases. The formulation was based on the observation that during cholera diarrhoea functionality of glucose mediated co-transport of sodium and water across the mucosal surface remained unchanged because action of cholera toxin (CT) poisoned the other channels important for sodium and water absorption in normal condition (Philips, 1964; Rohde and Cash, 1973). Use of chemotherapeutic agents like tetracycline and erythromycin had been shown to be effective in reducing the duration of symptoms (Bhattacharya, 2003).

### 2.2. The cholera pandemics

Cholera, a curse for centuries, is thought to have originated before the time of Hippocrates and Buddha (Barua, 1992). Africa and South Asia is
A highly endemic region for cholera. Every year, 1.4 to 4.3 million people affected by cholera which includes death of ~28,000-1,42,000 people (WHO, 2016). In 2014, many cases were reported from different parts of the world which included 55% of cases from Africa, 30% from Asia and 15% from the Americas. Recently, more than 10,700 cholera cases and 170 deaths have been reported in five countries in the WHO Eastern Mediterranean and African regions (WHO, 2016). During the first six cholera pandemics (1817 and 1923), *V. cholerae* classical biotype was thought to be the causative agent (Blake, 1994; Dziejman *et al.*, 2002). A major epidemic outbreak was recorded in the Indonesia in 1935 which was caused by El Tor biotype (*V. cholerae* MAK757). Subsequently, in 1937, another El Tor biotype was also isolated in Makassar, Indonesia (*V. cholerae* M66-2) during outbreak of cholera. Both of the outbreaks were not pandemics but the MAK757 and M66-2 isolates were subsequently labelled as pre-seventh pandemic El Tor (Barua, 1992; Banerjee *et al.*, 2014). The seventh cholera pandemic was caused by the El Tor biotype which replaced the classical strains. This was the only pandemic that had originated outside of India namely in Sulawesi, Indonesia, in 1961. After a silence, El Tor again caused a massive outbreak in 1991 in Peru, the first cholera epidemic in Latin America since 1895 (Seas *et al.*, 2000). In 1992, a new variant of the seventh pandemic strain appeared in Madras that spread rapidly in Asia which raised questions about the beginning of an eighth pandemic of cholera (Siddique *et al.*, 1996). This new pathogenic serotype was referred to as *V. cholerae* O139 ‘Bengal’, the O1 antigen of the prototypic
seventh pandemic strain having been replaced by the O139 antigen (Albert, 1994). The ongoing seventh pandemic is the longest of the cholera pandemics. It is still believed that El Tor and its derivatives are better adapted as compared to their classical predecessors (Faruque et al., 1998; Safa et al., 2010).

2.3. Large cholera outbreaks in 21st century

Cholera is burning problem in the developing countries. In Zimbabwe, one of the most severe cholera outbreaks occurred in 2008-2009 causing 98,592 reported cases and 4,288 reported deaths (Sardar et al., 2013). During 2009–2011, almost 220 people died of the 25,431 reported cases of cholera in Mozambique (Gujral et al., 2013). This cholera cases was due to emergence of *V. cholerae* O1 El Tor variant strains (Langa et al., 2015). The world witnessed a large cholera outbreak in Haiti started in 2010 in which 8,534 deaths and 6,97,256 cholera cases have been reported by the Haitian Ministry of Public Health and Population (WHO, 2016). A close relationship between the Haitian isolates and variant *V. cholerae* El Tor O1 strains isolated in Bangladesh in 2002 and 2008 was found on the basis of single-nucleotide variations and the presence and structure of hyper variable chromosomal elements (Chin et al., 2011). Recently, Haitian variant of *V. cholerae* O1 Ogawa causing outbreaks and sporadic cases of cholera in South India has been reported (Bhattacharya et al., 2016). Analysis of genomic variation of the Haitian isolates indicates a more distant relationship with circulating South American isolates (Chin et al., 2011).
2.4. Serological classification of *V. cholerae*

Serological assay has been used to diagnose the nature strains of *V. cholerae* on the basis of somatic antigenic variability. Two major antigens present on the surface of *V. cholerae* are somatic ‘O’ and flagellar ‘H’ antigens (Gardner and Venkatraman, 1935). The major surface antigen which determine the serogroup/serotype specificity of the organism is the ‘O’ antigen which is dependent on the cell surface lipopolysaccharide (LPS) structures (Jann *et al*., 1975). *V. cholerae* was classified into more than 206 serogroups based on its somatic antigenic specificity (Yamai *et al*., 1997) in which O1 and O139 have been associated with epidemic disease (Kaper *et al*., 1995). It has been reported that O antigen also serves as receptor for VP4 phage for El Tor type strain (Xu *et al*., 2013). In contrast to this, the importance of the ‘H’ antigen for species identification is limited as presence of common H-epitopes among all *Vibrio* species (Shinoda *et al*., 1976).

In many cases, *V. cholerae* O1 may undergo a smooth to rough strain transformation which was characterized by the removal of O-antigen from core lipid A (Rochetta *et al*., 1999). Loss of serological specificity occurred due to this the smooth rough transformation.

2.4.1. *V. cholerae* O1

*V. cholerae* O1 serogroup has been associated with epidemic and pandemic cholera (Kaper *et al*., 1995). The O1 serogroup has been further subdivided into three serotypes Ogawa, Inaba and Hikojima in which
Hikojima is rare. All three serotypes carry a common antigenic determinant referred as the ‘A’ antigen. Additionally, existence of two other antigens, ‘B’ and ‘C’ were reported to be present among strains with different serotypes (Sakazaki and Tamura, 1971). The O1 antigen biosynthesis gene cluster was originally named as rfb (Manning et al., 1994) which has been changed to wbe (Reeves et al., 1996; Yamasaki et al., 1999).

*V. cholerae* O1 strains have been divided into two biotypes *i.e.* classical and El Tor (Kaper et al., 1995). The basis of differentiation of biotypes is based on certain biochemical and phenotypic traits of this organism. These include hemolysis of sheep erythrocytes, chicken erythrocytes agglutination, Voges-Proskauer reaction and sensitivity to polymyxin B (50-U disk) and lysis by biotype specific *Vibrio* phages. El Tor strains were known to cause i) lysis of sheep erythrocyte, ii) agglutinate chicken erythrocyte, iii) positive reaction to Voges-Proskauer (VP) test (Kaper et al., 1995) iv) polymyxin B resistant (50-U disk) and v) Resistant to lysis mediated by classical Gr. IV vibriophage. Classical strains can be identified by its sensitive phenotypes towards i) polymyxin B (50-U disk) ii) classical Gr. IV vibriophage and iii) negativity to VP test.

Besides these two canonical biotypes of *V. cholerae* O1, strains of O1 serogroup was identified that shared phenotypes and genotypes of classical and El Tor strains (Nair et al., 2002). Strains with El Tor biotype traits carried classical allele of *ctxB* gene and these strains were later considered as El Tor variant strain (Nair et al., 2006). El Tor variant strains were also isolated from
several areas in Asia and Africa. In Kolkata, El Tor variant strains appeared in 1990. Complete replacement of prototype El Tor strains by the El Tor variant strains occurred since 1995 (Ghosh-Banerjee et al., 2010).

Amount of cholera toxin (CT) produced both in vitro and in vivo by *V. cholerae* O1 El Tor variant strains revealed that El Tor variant strains produced a much larger amount of CT than prototype El Tor strains. The amount of CT produced by El Tor variant strains was more or less similar to that produced by classical strains (Ghosh-Banerjee et al., 2010). Emergence and spread of tetracycline resistant *V cholerae* O1 El Tor variant strains in the tribal areas of Odisha has been reported recently (Kar et al., 2015).

### 2.4.2. *V. cholerae* O139

Sudden outbreak of cholera-like diarrhoea was reported in Madras at the end of year 1992 (Ramamurthy et al., 1993). The causative agent was *V. cholerae* non-O1. The organism was characterized later and type as O139 and named as ‘Bengal’ to link its origin for coastal area of Bay of Bengal organism (Shimada et al., 1993; Bhattacharya et al., 1993). Studies indicated that the O139 strains emerged from El Tor strains upon due to the exchange of genetic materials (Karaolis et al., 1994; Bik et al., 1995). Epidemiological analysis like zymovar analysis, ribotyping, and pulsed-field gel electrophoresis showed that *V. cholerae* O139 ‘Bengal’ strains were closely related to O1 El Tor strains (Popovic et al., 1995; Berche et al., 1994; Albert, 1994).
2.4.3. *V. cholerae* non-O1, non-O139

The “non-O1, non-O139” strains of *V. cholerae* were identified on the basis of biochemical tests. These strains do not agglutinate either with O1 or with the O139 specific antisera. They are also known as ‘non-cholera vibrios’ (NCVs) or non-agglutinable vibrios (NAGs) (Kaper *et al*., 1995) which are found in estuarine environment. Majorities of non-O1, non-O139 serogroups had never been involved in cholera. These strains were reported to be responsible for sporadic outbreaks of diarrheal cases (Cheasty *et al*., 1999; Morris and Black, 1985). The symptoms varying from mild to severe cholera-like diarrhoea, sometimes with vomiting, fever and bloody stools (Blake *et al*., 1980). However, large outbreaks of cholera like diarrhea was reported earlier caused by *V. cholerae* O5 and O37 serogroups in Czechoslovakia and Sudan, respectively (Aldová *et al*., 1968; Kamal, 1971; Yamamoto *et al*., 1995).

2.5. Virulence factors of *V. cholerae*

Three different pathogenicity islands were associated with virulence of *V. cholerae*. These pathogenicity islands were shown to be mobile integrative genetic elements (MIGEs) transferred horizontally from other bacteria (Carpenter *et al*., 2015). The pathogenicity islands are i) Vibrio Pathogenicity Island-I included TCP pathogenicity island, accessory colonisation factor (ACF), and virulence gene regulator ToxT. Genes were located in a 40 kb region, designated as Vibrio Pathogenicity Island (VPI-I) (Karaolis *et al*., 1998). *V. cholerae* strains of epidemic lineages possessed VPI whereas most of the
non-O1, non-O139 strains were shown to be devoid of VPI-I (O’shea et al., 2004), ii) Vibrio Pathogenicity Island-II, a novel 57.3 kb, consisting of 52 open reading frames, was discovered in V. cholerae O1 (Jermyn et al., 2002). It included the presence of a bacteriophage-like integrase (int), and the direct repeats sequences iii) Vibrio Seventh Pandemic Island-I (VSP-I) and II (VSP-II) have been shown to be present in El Tor and O139. V. cholerae VSP-I encodes several genes of unknown function and XerCD-like integrase. The VSP-II encodes a ribonuclease H1 homologues protein, type IV pilus, DNA repair protein, 2 transcriptional regulators, 2 methyl accepting chemotaxis proteins and a P4-like integrase (O’Shea et al., 2004). A novel variant of VSP-I was found to be present only in non-O1, non-O139 strains of V. cholerae and Vibrio mimicus (Grim et al., 2010).

2.5.1. Toxigenic factors

2.5.1.1. Cholera Toxin

Cholera toxins (CT) encoded by the ctxAB loci were located on a ~7 kb DNA fragment (Mekalanos, 1983). The genes, ctxAB, were acquired by V. cholerae the lysogenic conversion of a filamentous prophage, named as CTX (CTXΦ) (Waldor and Mekalanos, 1996). The CTXΦ prophage was shown to be composed of two regions designated as ‘Core’ and RS2. In the 4.5-kb “Core” region, genes encoding the toxins (ace, zot and ctxAB), a core encoded pilus (cep) and an ORF of unknown function (orfU) were found to be located. A ~2.4 kb region namely RS2 element was located into the close vicinity to the ‘Core’ region (Waldor and Mekalanos, 1996). The RS2 element was composed of
ORFs namely \( \text{rstR} \), \( \text{rstA} \) and \( \text{rstB} \). Recently, another element namely, RS1 satellite phage was reported to promote diversity of \( \text{V. cholerae} \) El Tor strains by aiding CTX prophage loss (Kamruzzaman \textit{et al.}, 2014). Classical strains lack RS-1 element (Davis \textit{et al.}, 2000) in their genome. It was shown that integration of CTX prophage to \( \text{V. cholerae} \) genome depended on Xer recombination system (Das, 2014). Though, CTX prophage was shown to be primarily associated with \( \text{V. cholerae} \) strains of O1 and O139 serogroups. Very limiting number of \( \text{V. cholerae} \) non-O1, non-O139 were identified to carry \text{ctx} genes and these strains were identified from shrimp aquaculture as well as diarrheal cases (Madhusudana and Surendran, 2013).

Cholera toxin (CT) is the prime virulence factor of \( \text{V. cholerae} \) and encoded by the \text{ctxAB}. Extensive research was carried out on CT, describing its function and role in cholera (Spangler, 1992; Finkelstein, 1992, Kaper \textit{et al.}, 1995). Presence of a toxigenic factor in cell free culture filtrate of \( \text{V. cholerae} \) was reported by De (1959) who proved its capacity to show diarrheagenic potential in animal models. The enterotoxin, CT is typical of the A-B_5 type ADP-ribosylation subunit group of toxins. It is consisted of one enzymatic subunit (subunit A) and five identical binding subunits (subunit B) (Gill, 1976; Gill \textit{et al.}, 1981; Lai \textit{et al.}, 1976; Ohmoto \textit{et al.}, 1976; Sixma \textit{et al.}, 1991). Functional analysis revealed that ‘A’ subunit (molecular wt. 27 kDa) possessed a specific enzymatic functions and ‘B’ subunit (molecular wt. 11.6 kDa) aided to bind the holotoxin to the eukaryotic cell receptor monosialosylganglioside (GM_1) (Gill and King, 1975). The mechanism of
action of CT has been extensively worked out. Following cellular entry, ‘A’ subunit gets proteolytically cleaved to produce ‘A1’ (molecular wt. 21.8 kDa) and ‘A2’ (molecular wt. 5.4 kDa) fragments by reduction of the disulfide bond (Tomasi and Montecucco, 1981). The ‘A1’ fragment could bind with NAD$^+$ (Cassel and Pfeuffer, 1978; Galloway and Heyningen, 1987) and catalyze the ADP-ribosylation of G$\text{sa}$. The modified G$\text{sa}$ was found to be associated with GTP to form an active tertiary complex with adenylate cyclase which catalyses the production of cAMP from ATP. The ‘A1’ subunit of cholera toxin has an unfolded but not disordered structure at physiological temperature which is important for the non-productive reassembly of CT holotoxin in the endoplasmic reticulum (Taylor et al., 2015). For the modification of its G$\text{sa}$ by ‘A1’ subunit, a folded conformation is thus necessary which regained by ADP-ribosylation factor-6 (ARF6) and lipid raft carrying GM$_1$ of the membrane of host cell (Ray et al., 2012; Banerjee et al., 2014). The increased cAMP concentrations also increased Cl$^-$ secretion by intestinal crypt cells and decreased NaCl absorption by villus cells (Field, 1981). The net movement of electrolytes from inside the cells into the lumen resulted in heavy water flow into lumen. The large volume of water overrides the absorptive capacity of the intestine, resulting into diarrhea.

2.5.1.2. The Haemolysin/ Cytolysin

The hemolysin was purified by Honda and Finkelstein, 1979. Initially, haemolysin/cytolysin is made as an 82 kDa inactive protein which is further processed to a 65 kDa active cytolyisin (Yamamoto et al., 1990) capable to lyse
sheep erythrocytes. Hemolysis of sheep erythrocytes was traditionally used to distinguish between the El Tor and classical biotypes of *V. cholerae* although more recent El Tor isolates showed poorly haemolytic activities on sheep erythrocytes (Barrett and Blake, 1981; Gallut, 1974). Genes encoding this haemolysin (*hlyA*) are present in classical, El Tor and non-O1, non-O139 strains of *V. cholerae* (Brown and Manning, 1985). Classical strains has an 11-bp deletion in the *hlyA* locus leading to the synthesis of a truncated product of 27 kDa protein (Alm et al., 1988; Alm and Manning, 1990). The purified haemolysin induced fluid accumulation in ligated rabbit ileal loops (Ichinose et al., 1987). Cytolysin was associated with some proteins as it can induce cytotoxic effect on Y-1 adrenal and CHO cells (McCardell et al., 1985, and Spira et al., 1986). *V. cholerae* non-O1, non-O139 strains carrying El Tor variant haemolysin genes *hlyEAT* has been reported from the Chesapeake Bay, Maryland, USA (Ceccarelli et al., 2015).

### 2.5.1.3. The repeat in toxin (*rtx*) gene cluster

RTX toxin gene cluster was identified in *V. cholerae* El Tor strains that showed cytotoxic activity in Hep-2 cells *in vitro* (Lin et al., 1999). This gene cluster contains four genes namely *rtxA*, *rtxC*, *rtxB* and *rtxD*. The cluster is physically linked to the cholera toxin (CTX) element in the *V. cholerae* genome (Lin et al., 1999). In case of classical strain, there was a deletion on *rtxC* resulting into elimination of cytotoxic activity (Lin et al., 1999).
2.5.1.4. The toxin linked cryptic plasmid (pTLC element)

The toxin linked cryptic plasmid (pTLC) was identified as 4.7 kb cryptic plasmid present in classical strains of *V. cholerae* and tightly linked with CTX genetic element. It has been considered that the pTLC helped in the formation of complete attRS site in the genome of *V. cholerae* to aid CTX prophage lysogenization (O’Shea *et al.*, 2004; Faruque *et al.*, 2007).

2.5.2. Colonization factors

2.5.2.1. Toxin co regulated pilus (TCP)

Toxin co-regulated pilus (TCP) was discovered by Taylor *et al.*, in 1987 as a prime colonization factor of *V. cholerae* O1 and expression of the TCP was found to be coregulated with the expression of CT. Later, role of expression of the TCP was found to in O139 (Waldor and Mekalanos, 1994a) and some clinical non-O1, non-O139 strains (Nandi *et al.*, 2000). A new tcpA allele (*tcpA-env*) in the environmental non-O1, non-O139 strains (Mukhopadhyay *et al.*, 2001) has also been reported. The TCP shares 82.83% homology between El Tor and classical strains (Iredell and Manning, 1994; Rhine and Taylor, 1994). The TCP was shown to be composed of repeating subunits of the 20.5 kDa TcpA pilin protein which was the major subunit encoded by *tcpA*. The TCP fibres was then formed by lateral association of individual pilus to develop a bundle like structure (Taylor *et al.*, 1987). The *tcpA* was located on the larger chromosome with other genes required for biosynthesis of TCP. The locus was named as TCP Pathogenicity Island (Karaolis *et al.*, 1998). The TCP
belongs to the type-4 family of pili which is involved in colonizing process of diverse bacterial species (Strom and Lory, 1993). Till date cognate receptor for binding of TCP to host cells are not known. It has been considered that the TCP binds directly to the intestinal epithelia rather mediating the bacterial interaction via direct pilus-pilus contact, and this allows micro-colony formation (i.e. bacterial-bacterial interaction) on the epithelial cell surface in intestine. This is one of the critical steps in intestinal colonization (Kirn et al., 2000; Jude and Taylor, 2011). All genes responsible for the assembly components of the TCP were shown to be located on a large (~40 kb) pathogenicity island i.e. V. cholerae pathogenicity island (VPI) (Karaolis et al., 1998) where as large cluster of tcp genes are organized into an operon (tcp ABQCRDSTEF) (Brown and Taylor, 1995). The synthesis and regulation of expression of TCP have worked out in details which revealed a complex regulation of expression is under the control of ToxR/S and ToxT systems (Ogierman et al., 1993; Kaufman et al., 1993). Recently, VexH, a pump of resistance-nodulation-division (RND) efflux systems has been also reported to induce TCP expression (Taylor et al., 2012).

2.5.2.2. Flagella

V. cholerae remains motile by a single polar, sheathed flagellum which has been reported to be considered as an important virulence phenotype (Follet and Gordon, 1963). A membrane sheath covers the flagellar core and is contiguous with the outer membrane of the organism. The bacterial flagellum consists of three distinct parts: a hollow filament with a length of 15–20 μm
composed of flagellin; the hook, which connects the filament to the motor complex; and the membrane embedded motor complex consisting of a rotor and a stator (Morimoto and Minamino, 2014). Serine 26 residue of PomB, a subunit of stator, is important for hypermotility of *V. cholerae* (Halang *et al*., 2015). Beside its role in motility, it have also been suggested that flagellum serves as adhesin (Attridge and Rowley, 1983; Eubanks *et al*., 1977; Hranitzky *et al*., 1980). The non-motile mutants have reduced virulence in rabbit model of cholera (Richardson, 1991). However, they do not show reduced colonization in infant mouse model (Klose and Mekalanos, 1998).

### 2.5.2.3. Other colonization factors and toxins

The mannose-sensitive hemagglutinin (MSHA) is a flexible pilus which has a subunit molecular mass of 17 kDa (Jonson *et al*., 1991). It is generally expressed on the cell surface of *V. cholerae* O1 El Tor, O139 and certain non-O1, non-O139 strains. The MSHA are shown to contribute to the ability of *V. cholerae* O1 El Tor and O139 strains to adhere to crustaceans, zooplanktons and to form biofilms for their survival in the environment (Moorthy and Watnik, 2004).

Both the classical and El Tor biotypes of *V. cholerae* produce a cell-associated hemagglutinin (HA), which is not inhibited by mannose, fucose or other sugars. The gene encoding this hemagglutinin was cloned by Franzon and Manning in 1986, and named as MFRHA. The nucleotide sequence of 693 bp ORF predicted a functional 26.9 kDa protein (Franzon *et al*., 1993). The
MFRHA has been reported as a cationic outer membrane protein (OMP) held by charge interactions with the LPS (Manning et al., 1991).

Certain *V. cholerae* strains belonging to either classical or El Tor biotypes were known to produce a soluble HA protease. It was characterized as a zinc metalloenzyme with potential to cause to nick cholera enterotoxin and cleaved fibronectin, mucin and lactoferrin (Finkelstein et al., 1983). This proteinaseous factor has not been shown to be associated with colonization potential of the strains. In fact, covalent modification of other toxins, the degradation of the protective mucus barrier and disruption of intestinal tight junctions were the functions associated with the protein (Benitez and Silva, 2016).

*V. cholerae* has been shown to encode another colonization factor by a set of genes called accessory colonization factor (*acf* genes) (Peterson and Mekalanos, 1988). Role of Acf was shown to be important for the colonization as mutation in *acf* exhibited reduced colonization potential (Taylor et al., 1987).

In 1991, Fasano et al., reported for the first time about zonula occludens toxin (Zot) with potential to alter the permeability of the intracellular tight junction (zonula occludens).

Another potential enterotoxin of *V. cholerae* known as accessory cholera enterotoxin (*ace*), encoded by *ace* gene, a 96-residue peptide with a predicted molecular masses $M_r$ of 11.3 kDa (Trucksis et al., 1993). The Ace has a striking
similarity to eukaryotic ion transporting ATPases. Ace forms an ion channel by aggregating and inserting into eukaryotic membrane (Trucksis et al., 1993).

Certain non-O1, non-O139 *V. cholerae* strains produce 17 amino acid heat-stable enterotoxin (NAG-ST). It shares 50% protein sequence homology to the ST (Stable toxin) of enterotoxigenic *E. coli* (Takeda et al., 1991). It was shown that non-O1, non-O139 strains of *V. cholerae* producing the toxin caused diarrhoea in volunteers (Morris, 1990).

A novel non-membrane damaging cytotoxin (NMDCY) with molecular mass of 35 kDa in size was identified in *V. cholerae* O1 (Saha et al., 1996). Culture filtrate of *V. cholerae* strains producing NMDCY induced rapid rounding of cultured HeLa, CHO and Vero cells. The toxin also showed enterotoxin activity in the rabbit ileal loop assay (Basu et al., 1999).

### 2.5.3. Regulation of virulence factors and the regulatory molecules

Much research has been focused on elucidating the regulatory pathways that controls CT and TCP expression in *V. cholerae*. The regulation of the *ctx* and the *tcpA* was shown to involve very complex mechanisms and that were linked to functional attributes of multiple transcriptional activators and environmental signals. The direct transcriptional activator of the *ctx* and *tcpA* is the AraC-like protein ToxT (DiRita et al., 1991). The *toxT* has been shown to encode in VPI of *V. cholerae*. The laboratory conditions that promoted expression of regulate CT and TCP *in vitro* were reported as well as
their transcriptional regulator ToxT (DiRita et al., 1996). Induction of the toxT transcription was thought to initiate virulence factor expression within the intestine. Bi-carbonate from bile was found to activate toxT dimerization (Thomson and Withey, 2014), a required condition for function of the ToxT. It was reported that an unstructured region in the ToxT N-terminal domain (region between 100th to 110th residues) was involved in multiple aspects of virulence gene regulation (Thomson et al., 2015). The toxT promoter has been shown to be activated by two transmembrane proteins, namely the ToxR and the TcpP. Both the ToxR and the TcpP contain amino-terminal cytoplasmic domains which is homologous to the OmpR family of transcriptional activators transmembrane segments, and carboxy terminal periplasmic domains (Hase and Mekalanos, 1998). The ToxR and the TcpP are found to be associated with ToxS and TcpH, respectively. All the genes were reported to be co-transcribed and localized to the membrane for enhancing their activity (DiRita and Mekalanos, 1991). The TcpH and the Tsp serve to protect full-length TcpP from spurious proteolysis by the YaeL (Teoh et al., 2015). The ToxS stabilizes the ToxR by protecting the virulence regulator from premature proteolysis (Almagro-Moreno et al., 2016). Both the ToxR and the TcpP have been shown to be required for toxT transcription in V. cholerae, although overexpression of the TcpPH allows for toxT transcription in a toxR null strain (Hase and Mekalanos, 1998). The ToxR binds to a region between -100 to -69, while TcpP binds to a region between -51 to -32 with respect to the start site of toxT transcription (Krukonis et al., 2000). ToxR has no potential to activate
toxT directly. The ToxR binding to the toxT promoter allows recruitment of TcpP which subsequently activates toxT transcription (Murley et al., 1999). Two transcriptional activators, the AphA and the AphB, were identified that activated the promoter of tcpPH synergistically (Skorupski and Taylor, 1999). The AphB functions synergistically with the AphA to activate the expression of tcpPH in response to environmental stimuli and it also appeared to contribute to the differences in virulence gene expression between the two biotypes, (classical and El Tor) of V. cholerae (Kovacikova and Skorupski, 2002). Researchers showed that oxygen-limiting conditions enhanced the dimerization and activity of the AphB, which lead to the induction of TcpP-ToxR interaction (Fan et al., 2014). Biotype-specific regulation of the tcpPH transcription can be correlated with biotype-specific virulence factor expression (Murley et al., 1999). A single base pair difference (A to G substitution at position -65) between the classical and El Tor tcpP promoters was identified. Exchange of this base pair resulted into conversion of a classical strain to express virulence factors like an El Tor, and vice versa (Kovacikova and Skorupski, 2000). The toxT gene is located downstream of the tcpA gene cluster, with which toxT is cotranscribed. So, with the regulation of the tcpA and the toxT, once synthesized, can autoregulate its own synthesis (Brown and Taylor, 1995). The ToxR regulon was shown to control at least 17 distinct genes in addition to ctxAB. These include the TCP colonization factor, the accessory colonization factor, the OmpT and the OmpU, and three other lipoproteins (Faruque et al., 1998). The ToxR is also known to activate leuO
expression that contributes bile resistance phenotype in *V. cholerae* (Ante *et al.*, 2015). Expressions of the *toxR* is also positively regulated by a global regulator, CsrA (Mey *et al.*, 2015).

The expression of the CT and the TCP in *V. cholerae* is complicated as additional factors influencing the ToxR/TcpP/ToxT cascade. In *V. cholerae* the crp or the cya mutant strains, the *ctx* and the *tcp* genes were shown to be transcribed even under non-virulence inducing conditions which suggested that CRP negatively regulated the *ctx* and the *tcp* transcription (Skorupski and Taylor, 1997). It was established that the cAMP-CRP complex negatively regulated the *tcpP* and the *tcpH*, by binding to the AphA/AphB binding site on the *tcpPH* promoter region (Kovacikova and Skorupski, 2002).

A *V. cholerae hns* mutant strain exhibited high-level constitutive expression of the CT and the TCP suggesting that the H-NS also negatively regulated virulence factor expression (Nye *et al.*, 2000). High level the *toxT*, the *tcpA*, and the *ctx* transcription occurred in the *hns* mutants even in the absence of sufficient amount of the ToxR, the TcpP, or the ToxT, suggesting that H-NS acted as a “silencer” that allowed these virulence regulators to induce virulence gene transcription only under appropriate environmental signals (Klose, 2001). The ToxR also antagonizes *hns* transcriptional regulation which has impact in colonization and biofilm formation (Kazi *et al.*, 2016).
2.6. Quorum sensing and biofilm formation

Like social organisms, bacterial cells interact with one another to carry out group behaviors. To accomplish these collective tasks, an individual bacterium in the group needs to communicate and coordinate with its neighbors or else its own effort would be obsolete. One of the most characterized microbial intercellular signaling processes is called quorum sensing (QS), which includes the production, secretion, and detection of cell density-dependent chemical signals, known as autoinducers (AIs) (Ng and Bassler, 2009; Rutherford and Bassler, 2012; Schuster et al., 2013; Williams et al., 2007). Quorum sensing in V. cholerae has been shown to be highly important for maintaining infectious cycles in humans, controlled expression of phenotypic factors critical to pathogenesis, such as production of virulence factors, biofilm formations, and protease secretion (Reidl et al., 2002, Kierek et al., 2003; Zhu and Mekalanos, 2003). Previously, two QS pathways were established in V. cholerae (Miller et al. 2002). The first system was composed of the autoinducer signal CAI-1, made through expression of the CqsA, and the CqsS, a transmembrane histidine kinase (Higgins et al., 2007; Miller et al., 2002; Ng, et al., 2011; Wei et al., 2011, 2012). The second system was composed of the signal AI-2, which was synthesized by the LuxS, and another histidine kinase receptor the LuxPQ (Chen et al., 2002; Miller et al., 2002; Schauder et al., 2001). Recent study proposed abilities of V. cholerae to sense at least four different signals including the CAI-1 and the AI-2 using four parallel receptors the CqsA/S, the LuxS/PQ, the VpsS and the CqsR to regulate a common
response regulator, LuxO, and to modulate its QS response (Jung et al., 2015; Jung et al., 2016).

At low cell density, in the absence of the AIs and the CqsS, the LuxQ transferred a phosphate group to the response regulator LuxO via the phosphotransfer protein LuxU (Lenz et al., 2004). Phosphorylated LuxO induced the transcription of the genes for four small regulatory RNAs (sRNAs) called the qrr1-4 (quorum regulatory RNAs). The qrr sRNAs, along with the Hfq, a sRNA chaperone, destabilized the mRNA encoding the hapR (Lenz et al., 2004). Expression of the qrr levels are required to direct maintainence of the expression of the quorum-sensing regulated target genes (Svenningsen et al., 2009). In the absence of the HapR, the ctx and the tcp as well as polysaccharide genes (vps), required for biofilm formation, were expressed (Zhu et al., 2002; Miller et al., 2002; Vance et al., 2003; Zhu and Mekalanos, 2003; Hammer and Bassler, 2003).

At high cell densities, inactivation of the LuxO caused de-repression of the hapR (Miller et al., 2002; Lenz et al., 2004). Expression of the HapR suppressed the aphA and the aphB transcriptions, activators of the tcpPH operon via an additional regulatory loop using the toxR promoter (Kovacikova et al., 2002; Xu et al., 2010). The HapR also repressed the vps (Vibrio polysaccharide synthesis) operon resulting into less biofilm formation (Zhu et al., 2002; Hammer and Bassler, 2003, Yildiz et al., 2004). In addition to this, the HapR up-regulated the expression of the hapA, encoding secreted hemagglutinin (HA)/protease. The HA protease was earlier reported to be
responsible for the detachment of the organisms from the intestinal epithelium (Finkelstein et al., 1992, Jobling and Holmes, 1997). Loss or down-regulation of the HapR was demonstrated to be helpful for survival and colonization of *V. cholerae* as such deactivation caused increase of CT and TCP expression. Loss of the HapR resulted into low HA protease production leading to lower extent of detachment of bacteria from the intestinal epithelium. A natural frame-shift mutation in the *hapR* was reported in several toxigenic *V. cholerae* strains including reference El Tor N16961 and classical O395 strains leading to alteration of quorum sensing pathways (Zhu et al., 2002). Other than leading to HA protease, the cytotoxin protein that caused disease symptoms was reported to be hemolysin encoded from *hlyA* locus. Recent report has suggested that the *hlyA* was repressed by the quorum sensing regulated transcription factor *hapR* (Tsou and Zhu, 2010) at the transcriptional as well as at the post translational levels. Interestingly, absence of any of the four pathways have only a minor, if any, effect on virulence of this pathogen. In contrast, mutants lacking all four receptors have been reported to display avirulent phenotype (Jung et al., 2015).

2.7. Ecology

The genus *Vibrio* contains more than 50 species that are found free-living in aquatic habitats such as marine coastal waters, estuaries, sediments, and aquaculture settings as well as in association with marine organisms such as mollusc, coral, fish, zooplankton, and shrimp (Thompson et al., 2004). However, the nature of the survival and the persistence of toxigenic *V.*
*V. cholerae* in an aquatic ecosystem and the factors involved in the conservation of the pathogenic genes in the aquatic environment are yet to be clearly elucidated. Under stressed conditions like nutrient limited situation *V. cholerae* strains were shown to undergo to a dormant state referred to as a viable but non-culturable (VBNC) form (Chaiyanan *et al*., 2001). Recent report showed that *V. cholerae* cells carrying mutations in the genes required for quorum sensing and biofilm formation displayed altered VBNC formation (Kamruzzaman *et al*., 2010). The VBNC bacteria were again transformed into a cultivable state once the environment becomes favorable (Colwell *et al*., 1985). The importance of the VBNC bacteria, their morphology, metabolic and genetic properties, pathogenicity, resuscitation and identification have been widely reviewed (Ozcakir, 2007).

In an aquatic environment, *V. cholerae* attaches itself on the chitineous surface and has the ability to utilize chitin, composed of β1,4-linked N-acetyl glucosamine (GlcNAc) residues, by secreting chitinase, to convert chitin into GlcNAc (Meibom *et al*., 2004). The organism is well adapted to reside in human intestine, a widely different for nutrient source as compared to environmental conditions. These factors are known to have immense effect on the survival of any microorganism. Carbon, nutrient source and oxygen influence bacterial growth (Braun and Linda, 2005). In the intestine, a variety of amino sugars, neutral sugars and sugar acids are available which include glucose, galactose, ribose, fucose, mannose, N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine, glucosamine, galactosamine, gluconate (Gnt),
galactonate glucuronate, galacturonate, sialic acid etc. (Peekhaus and Conway, 1998a). Thus utilization of certain sugars may be considered to have enough influence on *V. cholerae* survival and pathogenesis *in vivo* (Nesper et al., 2002). It was reported earlier that inability to utilize Gnt or GlcNAc caused severe virulence attenuation in *V. cholerae* when tested in appropriate animal models (Patra et al., 2012; Ghosh et al., 2011).

2.8. **Utilization of carbon sources by different metabolic pathway by bacteria**

Bacterial cells utilize carbohydrates as the source of carbon and produce energy through different metabolic pathways. In bacteria, the following pathways are primarily known for metabolism of carbohydrate *i.e.* the Glycolysis or the Embden-Meyerhof-Parnas (EMP) pathway, the Pentose Phosphate (PP) Pathway, the Entner-Doudoroff (ED) pathway and the Ashwell pathway.

2.8.1. **Glycolysis or Embden-Meyerhof-Parnas (EMP) pathway**

Glycolysis is the sequence of reactions that metabolizes one molecule of glucose to two molecules of pyruvate with the concomitant net production of two molecules of ATP. A key discovery was made by Hans Buchner and Eduard Buchner in 1897, accidentally when they were manufacturing cell-free extracts of yeast for therapeutic use. The complete glycolytic pathway was elucidated by 1940, largely through the pioneering contributions of Gustav Embden, Otto Meyerhof, Carl Neuberg, Jacob Parnas, Otto Warburg, Gerty
Cori, and Carl Cori. Glycolysis is also known as the Emden-Meyerhof pathway (Semenza and Turner, 1992).

The six-carbon glucose is metabolized into two molecules of the three-carbon pyruvate in ten steps. In the preparatory phase of glycolysis, ATP is utilized to convert glucose molecule into fructose 1,6-bisphosphate which is further converted to two glyceraldehyde 3-phosphate molecules. In the payoff phase, each of the two molecules of glyceraldehyde 3-phosphate is converted to two molecules of pyruvate. In the glycolysis stage, two molecules of ATP per molecule of glucose are generated. Hexokinase, PFK-1, pyruvate kinase serve to control the flow of carbon through the pathway (Lehninger, 2005).

### 2.8.2. Pentose phosphate (PP) pathway

Next to glycolysis, the pentose phosphate pathway (PP) was discovered. Work on the PP was carried out by the famous scientist named Otto Warburg in Berlin-Dahlem. The pentose phosphate (PP) pathway, also called hexose monophosphate (HMP) shunt, produces NADPH that carries reducing energy; and generate pentoses like ribose that is necessary for the biosynthesis nucleic acids (Semenza and Turner, 1992).

The first reaction of PP pathway is initiated by the dehydrogenation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase to form 6-phosphoglucono-δ-lactone which is converted into sefructose 6-phosphate and glyceraldehydes 3-phosphate in many steps to be further catabolized through the EMP pathway (Stincone et al, 2015).
2.8.3. Entner-Doudoroff (ED) pathway

The ED pathway was discovered in 1952 in *Pseudomonas saccharophila* (Entner and Doudoroff, 1952), later in *E. coli*. The ED pathway is thought to predate the EMP pathway the evolutionary timescale (Romano and Conway, 1996). Previously ED pathway was considered to be present in gram negative bacteria. Now it is known to be present in diverse groups of organism ranging from archea to eubacteria (Peekhaus and Conway, 1998a). Recently, presence of ED pathway was evident in eukaryotes like cyanobacteria and plants (Chen et al., 2016). The ED pathway can be considered as an alternative to EMP pathway for the utilization of six carbon sugar. The overall scheme of ED pathway are quite similar to EMP pathway in which 6 carbon sugars are phosphorylated and subsequently undergone to aldol cleavage to make 3 carbon intermediates. The differences between these two pathways lie in cleaving the nature of substrate. In the EMP pathway fructose 1,6 bisphosphate is cleaved by fructose bisphosphate aldolase to yield one molecule of glyceraldehyde-3-phosphate and one molecule of dihydroxy acetone phosphate whereas in the ED pathway 2-keto-3-deoxy-6 phosphogluconate (KDPG) serves as a substrate for aldol cleavage by KDPG aldolase (*eda*) leading to yield of one molecule of glyceraldehydes-3-phosphate and one molecule of pyruvate which are further metabolized through common pathway that is shared by the EMP and the ED pathway leading to yield of energy by substrate level phosphorylation which is essential for bacteria for their growth and maintaining other physiological
activities (Conway, 1992). In addition to providing a carbon source for biosynthesis, the classical EMP and the ED pathways fulfill the energetic demand by generating ATP during glucose metabolism (Stettner and Segre, 2013). The EMP pathway yields two ATP per glucose whereas the ED pathway produces only one molecule of ATP. On the basis of simple stoichiometry of reactants and products, the EMP pathway yields twice as much ATP than the ED pathway. However, to generate one molecule of ATP, the ED pathway requires less enzymatic protein to achieve the same glucose to ATP conversion rate as compared to the EMP pathway (Flamholz et al. 2013). The ED pathway was also reported to be thermodynamically less constrained as compared to the EMP pathway (Flamholz et al. 2013; Stettner and Segre, 2013).

In few organisms the ED pathway is employed in a catabolic direction which may be either constitutive (Zymomonas mobilis) or inducible by particular sugar (Pseudomonads), and forms the central metabolic pathway (Conway, 1992). In numerous bacteria including enterics, the ED pathway operates as a linear parallel pathway which is induced by particular sugar, and the EMP pathway forms the core of central carbohydrate metabolism for these bacteria (Conway, 1992). In few methylootrophs, the ED pathway plays an anabolic role (Conway, 1992). The ED pathway has been reported to be the main route to utilize glucose in Xanthomonas campestris (Schatschneider et al., 2014) and Pseudomonas putida (Chavarria et al., 2013).
The ED pathway have been shown to play significant role in pathogenesis of many bacteria. In case of *Pasteurella pestis*, addition of gluconate in the culture medium increases production of virulence factors (Mortlock *et al.*, 1962). An apparent induction of three folds of the genes of the ED pathway in *Neisseria gonorrhoeae* have been observed in presence of 10% serum (Britigan *et al.*, 1985). In *Yersinia enterocolitica*, Gnt supported growth and the synthesis of heat stable enterotoxin (YST) (Amirmozafari *et al.*, 1993). Utilization of Gnt through the ED pathway is an important for *E. coli* to colonize in the mammalian intestine (Sweeney *et al.*, 1996a). Gnt metabolism via ED pathway appeared to be an important source for intracellular growth of *Salmonella typhimurium* within macrophages (Eriksson *et al.*, 2003). The ED pathway has an effect on the colonization potential of *H. pylori* in mice (Wanken *et al.*, 2003). Gnt assimilation through the ED pathway plays important role during growth of the organism inside alveolar macrophage (Bruggemann *et al.*, 2006).

2.9. Gluconate (Gnt) utilization system

Catabolism of Gnt via the ED pathway was established elaborately in *E. coli*. The enzymes namely the Edd (6-phosphogluconate dehydratase) and the Eda (KDPG aldolase) play key role in Gnt utilization. Studies carried out with *E. coli* MG1655 showed that Gnt was the major carbon source for large intestinal survival. Gnt utilization was important for both initiation and maintenance stages of colonization in the mouse model (Chang *et al.*, 2004). Gnt uptake and initial catabolism of Gnt to Gnt 6-phosphate (Gnt6P) in *E. coli*
through GntI and GntII systems have been well documented (Tsunedomi et al., 2003). During initial stage, Gnt gets converted to Gnt6P which further metabolizes through the ED or PP pathways to pyruvate. The GntI system of *E. coli* has been shown to be composed of gntT, gntU, gntK and gntR encoding high and low affinity permeases, thermo-resistant Gnt kinase and regulatory molecule, respectively. The GntI system was thought to be the main system for Gnt utilization in *E. coli*. The genes gntW and gntV were later identified to encode another high affinity permease and thermo-sensitive Gnt kinase, respectively and formed part of the GntII system in *E. coli* (Tsunedomi et al., 2003). It was reported that the GntI system genes of *E. coli* were positively regulated by cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex and negatively regulated by GntR (Peekhaus and Conway, 1998b). In a recent study, dual role of the gntH to activate genes of the GntII system with concurrent repression of the GntI system have been reported (Tsunedomi et al., 2003). Presence of a third Gnt transporter gene, gntP, was also reported in *E. coli*, and the gene was under catabolite repression but not induced by the presence of Gnt (Peekhaus and Conway, 1998a).

Several alternate mechanisms for uptake and initial catabolism of Gnt by *E. coli* have been shown to exist. Although, physiological basis of the presence of multiple systems in *E. coli* is largely unknown, deactivation of the gntP resulted into altered specificity towards intestinal niche to colonize in animal model was documented (Sweeney et al., 1996b). Mutation in the *eda* showed severe decrease in colonization ability of *E. coli* in mouse model.
In intestinal mucosa, primary source for Gnt has been suggested to be Gnt6P derived from sloughed dead epithelial cell contents (Sweeney et al., 1996b). Presumptive Gnt6P specific phosphatases present in the mucus layer subsequently converts Gnt6P to gluconate. In V. cholerae, the Gnt utilization system has not been well worked out. Previously, Patra et al., 2012 showed that Gnt was utilized through ED pathway in V. cholerae which induced expression of virulence index genes in vitro. Inactivation of edd of V. cholerae lead to significant reduction of CT production, attenuation of colonization potential and lowering of fluid accumulation in rabbit ileal loop assay (Patra et al., 2012).

2.10. Prevention and Immunity to cholera

Even, today globally, 1 billion people still do not have access to safe water and remain at risk of cholera (Harris et al., 2012). Levine and his group contributed significantly in the development of cholera vaccine (Levine, 1980; Levine et al., 1981; Levine et al., 1979). A group of scientist from Sweden developed an oral combination vaccine consisting heat or formalin killed V. cholerae cells with 1 mg of purified cholera toxin B subunit (Svennerholm and Holmgren, 1986). The vaccine was used in field trial in Bangladesh and was found to be safe and protective (Clemens et al., 1986). V. cholerae O1 El Tor Inaba JBK70 and the classical Ogawa CVD101 were the first recombinant attenuated strains which were used as vaccines. The strain JBK70 did not carry both the A and B subunits of CT while CVD101 lack of A subunit of CT (Levine et al., 1988). In the trial, volunteers immunized with a single oral dose
of JBK70 showed significant protection against cholera. *V. cholerae* classical Inaba 569B was attenuated to develop CVD103-HgR vaccine (Ketley *et al*., 1993; Levine *et al*., 1988). The vaccine strain *V. cholerae* CVD103-HgR was tested for safety and immunogenicity by different trials conducted in different countries worldwide (Cryz *et al*., 1990; Ketley *et al*., 1993; Levine and Kaper, 1993; Levine *et al*., 1988; Su-Arehawaratana, 1992; Suharyono *et al*., 1992; Jackson and Chen, 2015).

Initially, all the commercially available vaccines were used to protect cholera infection caused by *V. cholerae* O1. These vaccines did not confer protection against infection caused by *V. cholerae* O139 strains (Alberts *et al*., 1994). However, many studies were carried out to develop vaccine against O139 infection (Ledon *et al*., 2003; Tacket *et al*., 1995; Coster *et al*., 1995). Among the live cholera vaccines, CVD112 and Bengal 15 were the most prosperous to offer protection against *V. cholerae* O139 (Tacket *et al*., 1998; Waldor and Mekalanos, 1994b; Waldor and Mekalanos, 1996; Coster *et al*., 1995). Vaccine CVD112 was derived from *V. cholerae* O139 that contained deletions in *ctxA*, *zot*, *ace* and *cep* (Tacket *et al*., 1998; Waldor and Mekalanos, 1996). Currently, 2 oral killed vaccines, prequalified for use by WHO, are licensed and commercially available. These two prequalified vaccines are Dukoral and Shanchol. Dukoral (WC-rBS, Crucell, Sweden) contains several biotypes and serotypes of *V. cholerae* O1 supplemented with 1 mg per dose of recombinant cholera toxin B subunit (Harris *et al*., 2012). Shanchol (Shantha Biotechnics-Sanofi Pasteur, India) contains several biotypes and serotypes of
V. cholerae O1 and V. cholerae O139 without supplemental cholera toxin B subunit (Harris et al., 2012). Shanchol is the bivalent vaccine that is internationally available; mORCVAX (VaBiotech, Vietnam) is the locally produced Vietnamese version of this vaccine (Harris et al., 2012).

2.11. Antibiotics and antimicrobial agents

Antibiotics/ antimicrobial agents can be administered ideally within 4 h of initiation of cholera diarrhea. Currently practised antibiotics include tetracycline, doxycyclins, norfloxacin, ciprofloxacin, azithromycin, erythromycin etc. Antibiotic therapy should be based on prevailing local resistance patterns (Harris et al., 2012). Oral rehydration therapy (ORT), vaccines and antibiotics are available for cholera treatment, but a growing concern is the emergence of multidrug resistant V. cholerae strains belonging to O1 and O139 serogroups (Kitaoka et al., 2011, Pang et al., 2016; Folster et al., 2014; Jain et al., 2016). Due to emergence of MDR phenotype novel therapeutic approaches is urgently needed (Ghosh and Ramamurthy, 2011).

Extensive research is going on the development or identification of new antimicrobial compounds or small molecules that target component(s) of regulatory cascades (Clatworthy et al., 2007, Raju et al., 2012, Anthouard and DiRita, 2013, Zahid et al., 2015, Maxson and Mitchell, 2016). Occurrence of multidrug resistance is common among different human pathogens therefore discovery of virulence specific and organism specific (growth inhibitory) safe drugs will be more advantageous. These pathogen specific drugs may have the potential to diminish infection where vaccinations are not available or
would not be able to build up a timely protection. Anti-virulence drugs are designed to neutralize a pathogen by eliminating its virulence potential, provides several advantages (Clatworthy, 2007; Maeda et al., 2012). On the other hand, growth suppressive compounds specific to the particular pathogen are also being used. Therefore, targeting virulence as well as growth specific drugs have been reported to have minimal effects on the endogenous microbiota as extensive uses of antibiotics lead to dysbiosis resulting into to acute and chronic intestinal problems (McFarland, 2008; Sekirov et al., 2010). Recent time, discovery of potential small molecule targeting virulence regulatory cascade growth inhibitory properties are in limelight as small-molecule inhibitors can pass the cell membrane easily and modulate gene regulation (Anthouard and DiRita, 2013). To identify such virulence specific active small molecules, the major impediment is non-availability of HTS assays appropriately designed with proper target gene(s)/ phenotypes. A promising compound ‘virstatin’ was identified having the potential to attenuate CT expression by V. cholerae and that without altering growth of the target organism (Hung et al., 2005). Virstatin was identified by a screening assay (screening of 50,000 compounds) targeting reduction of cholera toxin (CT) expression as CT is one of the major virulence factors of V. cholerae (Hung et al., 2005). Virstatin was found to reduce expression of toxin co-regulated pilus (TCP), the other most important virulence factor of V. cholerae. Virstatin acts by inhibiting dimerization of the transcriptional activator ToxT (Shakhnovich et al., 2007a). New studies are emerging to predict potent
inhibitors on the basis of *in silico* analysis on high resolution X-ray cryptographic data on target regulatory protein like ToxT of *V. cholerae* (Mondal *et al.*, 2014). For the treatment of cholera, toxtazin A and toxtazin B and B', representing two novel classes of *toxT* transcription inhibitors have been identified a HTS assay by a screening of almost 63,000 compounds (Anthouard and DiRita, 2013). In another report, cyclo (Phe-Pro) was identified which inhibited the production of the virulence factors cholera toxin (CT) and toxin-coregulated pilus (TCP) in O1 El Tor *V. cholerae* N16961 during growth under virulence gene-inducing conditions (Bina and Bina, 2010). *V. cholerae* has been shown to produce a cyclic dipeptide, cyclo (phenylalanine–proline)(cFP), that functions to repress virulence factor production. The results revealed that cyclo (valine-valine) (cVV) inhibited virulence factor production by a ToxR-dependent process that resulted in the repression of the virulence regulator (Vikram *et al.*, 2014).

The use of natural compounds as inhibitory agents for virulence factor production is a new approach to overcome increased antimicrobial resistance in pathogenic bacteria. Sub-bactericidal concentration of Anethole, a component of sweet fennel seed, could suppress virulence potential of toxigenic O1 El Tor strains of toxigenic *V. cholerae* (Zahid *et al.*, 2015). Another, plant extract compound named 6-Gingerol from Ginger plant was extracted and found to inhibit binding of CT with GM₁ receptor present on the intestinal epithelial cells (Saha *et al.*, 2013). Capsaicin, a compound extracted from red chili, reduced the expression of major virulence-related genes such
as the ctxA, the tcpA and the toxT (Chatterjee et al., 2010) but enhanced the expression of hns gene. Therefore, repression of CT production by capsaicin or red chili might be due to the repression of virulence genes transcription by H-NS (Yamasaki et al., 2011). Further studies also showed that the presence of toxT alleles in non-O1, non-O139 V. cholerae resulted in virstatin resistance (Shakhnovich et al., 2007b). Therefore to enrich our arsenal for sudden attack by MDR strains of V. cholerae, a proper and novel HTS assay is thus needed for identification of small molecules which could be used either therapeutic or prophylactic or both purposes.