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Review

Toxigenic *Vibrio cholerae* strains and their associated malaises

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Toxigenic strains of *Vibrio cholerae* belonging to the O1 and O139 serogroups cause cholera, a severe diarrhoeal disease that occurs frequently as epidemics in many developing countries. Although *V. cholerae* is known to be a human pathogen, the bacteria constitute part of the normal aquatic flora in the ecosystem, which includes both epidemic and non epidemic strains that vary in their virulence gene profile. *V. cholerae* O1 and O139 strains are commonly known to carry a set of virulence genes necessary for pathogenesis in human. The major virulence factors of *V. cholerae* include cholera toxin (CT), which is responsible for the profuse watery diarrhoea and a pilus colonization factor known as toxin coregulated pilus (TCP). The presence of virulence-associated genes in the environmental strains provides interesting possibility to understand the pathogenicity of the disease. The emergence of toxigenic *V. cholerae* strains has provided an opportunity to study the coevolution of different serogroups of epidemic *V. cholerae* strains, apparently driven by competition for survival and thereby attaining enhanced fitness. This review attempts to bring together some of the important researches in recent times that have contributed towards understanding the genetic, epidemiology and evolution of toxigenic *V. cholerae*.

Key words: *Vibrio cholerae* O1 and O139, wastewater, virulence-associated factors, environmental strains, mediating gene transfer.

INTRODUCTION

Toxigenic strains of *V. cholerae* belonging to the O1 and O139 serogroups cause cholera, a severe diarrhoea disease that occurs frequently as epidemics and pandemic which is of public health concern in many developing countries. Cholera is thus categorized as one of the "emerging and re-emerging" infections (Satcher, 1995). The disease is an acute dehydrating diarrhoea caused principally by the potent enterotoxin, cholera toxin (CT), produced by these organisms during pathogenesis (Kaper et al., 1995). In southern Asia, parts of Africa and Latin America, cholera is endemic with seasonal epidemics occurring widely and is particularly associated with poverty and poor sanitation.

Cholera is a waterborne disease and the relationship with water ecology is suggested by the close association of *V. cholerae* with water. Although *V. cholerae* is known to be a human pathogen, the bacteria constitute part of the normal aquatic flora in estuarine and brackish waters and are able to persist in the absence of human host (Colwell and Huq, 1994; Colwell and Spira, 1992). This novel feature of the disease raises a question regarding the genetic similarity or diversity of the different toxigenic clones. Strains belonging to other serogroups, collectively referred to as non-O1, non-O139, have also been implicated as etiological agents of moderate to severe human gastroenteritis (Morris and Black, 1985; Janda et al., 1988). Although, the vast majority of the non-O1, non-O139 strains are presumed to be non-pathogenic bacteria constituting part of the normal aquatic flora. *V. cholerae* O1 and O139 strains are commonly known to carry a set of virulence genes necessary for pathoenesis in humans (Kaper et al., 1995; Faruque et al., 1998a, 2004a).

The major virulence factors of *V. cholerae* include cholera toxin (CT), which is responsible for the profuse diarrhoea and a pilus colonization factor known as toxin coregulated pilus (TCP).In addition to CT and TCP, cholera

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pathogenesis is presumed to depend on the synergistic effect of a number of putative accessory virulence-associated factors. These factors include the mannose-sensitive haemagglutinin (MSHA) pilus, the RTX toxin, hemolysins, as well as a few other accessory toxins (Kaper et al., 1995; Faruque et al., 1998a, 2004; Faruque and Mekalanos, 2003a). However, the roles of the accessory virulence factors in cholera pathogenesis are not well established and recent studies are beginning to reveal that at least some of these factors also play a role in environmental fitness of the pathogen (Watnick et al., 1999; Chiavelli et al., 2001). The pathogenic strains of *V. cholerae* have evolved from nonpathogenic environmental strains and horizontal transfer of virulence-related gene clusters play a major role in the process (Faruque et al., 1998b; Faruque and Mekalanos, 2003a). The *ctxAB* genes encoding CT reside in the genome of a lysogenic filamentous phage, CTXΦ (Waldor and Mekalanos, 1996), whereas genes encoding the major colonization factor, TCP, are part of a large cluster of genes also referred to as the TCP pathogenicity island (Kovach et al., 1996; Faruque and Mekalanos, 2003a).

To track the evolutionary events in the origin of pathogenic *V. cholerae* from their non-pathogenic progenitors, it is important to identify intermediate strains that are likely to carry some of the virulence-related genes, but fall short of the complete set of genes required for pathogenesis and epidemic spread. For example, occasionally, environmental non-O1, non-O139 vibrios have been found to carry one or a few of the virulence-associated genes or their homologs (Mukhopadhyay et al., 2001; Faruque et al., 2004a). Understanding the evolution of bacterial pathogens from their non-pathogenic progenytors is challenging. Toxigenic *V. cholerae*, the etiologic agent of cholera, represents a paradigm for this process in that this organism evolved from environmental non-pathogenic *V. cholerae* by acquisition of virulence genes. The aquatic environment of a cholera endemic area harbors strains with various virulence gene profiles and thus constitutes a reservoir of diverse virulence genes (Chakraborty et al., 2000). The ecological settings presumebly favours extensive genetic interactions among *V. cholerae* mediated by phages and mobile genetic elements (Faruque and Mekalanos, 2003a) as well as selection of pathogenic clones leading to clustering of a critical combination of genes required for the emergence of an epidemiologically thriving pathogenic strain. However, the general prevalence of virulence-associated genes among non-O1, non-O139 serogroups of *V. cholerae* and the selection pressures for environmental *V. cholerae* carrying putative virulence genes are not clear. Studies have also identified new putative virulence related gene clusters in *V. cholerae* including genes for a type III secretion system (TTSS) (Dziejman et al., 2005) and the Vibrio seventh pandemic islands (VSP-1 and VSP-2) (Dziejman et al., 2002), the distribution of which in environmental *V. cholerae* strains, is largely unknown. In agreement with these recent findings from genetic analysis of toxigenic *V. cholerae*, molecular epidemiological surveillance of cholera in areas of endemic infection has also revealed temporal changes in the properties of toxigenic *V. cholerae* and a continual emergence of new epidemic clones which often replace existing clones (Faruque et al., 1993, 1994, 1995, 1997a, 1997b; Mitra et al., 1996; Sharma et al., 1997). The probable functions of virulence genes or their homologues in the environment and the ecology of toxigenic *V. cholerae* which support emergence of new epidemic clones and maintains the seasonal pattern of cholera epidemics, have not been adequately explained.

In this paper we attempt to review available information on the possible role on ecology and epidemiology of toxigenic *Vibrio cholerae* as well as the significance of emerging clonal diversity within the serogroup and their virulence genes in the environment.

Classification scheme of toxigenic *Vibrio cholerae*

Vibrio cholerae, a non-invasive gram-negative bacterium (Chatterjee et al., 1998), is classified on the basis of its somatic antigens (O antigens) into serovars or serogroups and there are at least 200 known serogroups (Baumann et al., 1984; Shimada et al., 1993, 1994, 1997). Until 1992, the only serogroup known to cause epidemic cholera was O1. Strains belonging to the O1 serogroup were further classified into two biotypes, namely the classical and El Tor and these can be differentiated by different phenotypic traits and more recently, by precise genetic markers (Kaper et al., 1995). Seven recorded pandemics of cholera have occurred globally and there is firm evidence that at least the 5th and 6th were caused by the classical biotype O1 strains (Blake, 1994). The El Tor biotype is responsible for the ongoing 7th pandemic (Blake, 1994). In 1992, another serogroup, namely O139 started causing outbreaks of cholera in India and Bangladesh (Ramamurthy et al., 1993).

Currently, these 2 serogroups are associated with endemic and epidemic cholera, while the other *V. cholerae* serogroups not associated with epidemics or pandemics are collectively referred to as non-O1, non-O139 *V. cholerae* or also as non-epidemic serogroups (Figure 1). Serogrouping is carried out using specific absorbed antisera or monoclonal antibodies against the 'O' antigen component of the bacterial lipopolysaccharide (Shimada et al., 1994). In addition to this, *V. cholerae* O1 is classified into 3 serotypes, namely Ogawa, Inaba and Hikojima, the last of which is a rare and inadequately described serotype (Shimada et al., 1994). These serotypes are divided into A, B and C antigens. 'A' antigen made of 3-deoxy-L-glycerotetronic acid, B and C antigen has not been characterized yet. The O139 Bengal strain and the O1 serogroup outbreak strains of both the classical and El Tor biotypes show many similarities, but their major differences are significant. The O139 strain is capsulated unlike the O1 strains and has significant dissimilarities in the 'O' antigen component of the bacterial lipopolysaccharide (Johnson et al., 1994).

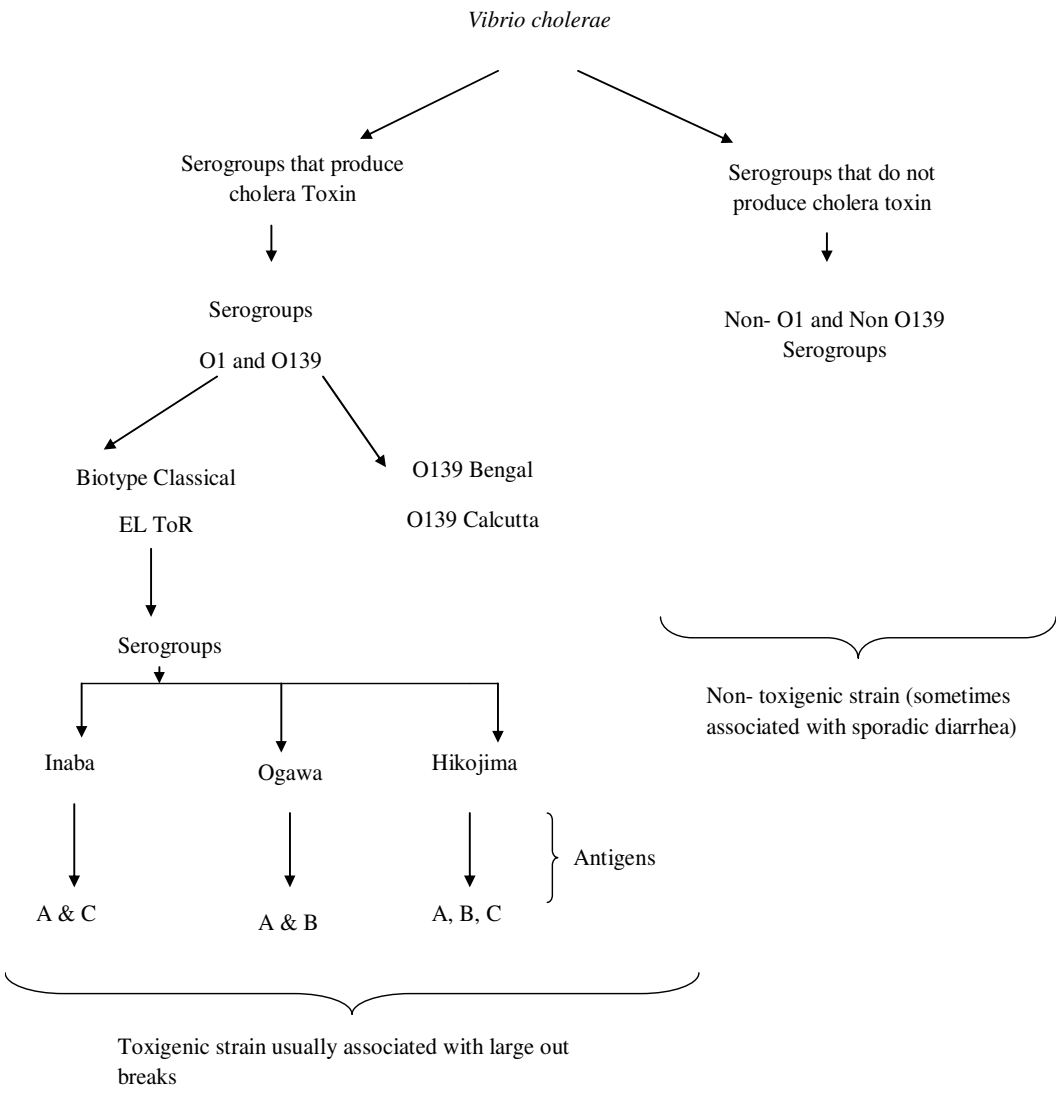

Figure 1. Classification of *Vibrio cholerae* serogroups with toxigenic and non-toxigenic group. (Source: Uma et al. (2003).

Sources of infection

Microbial contamination of water is the largest and most immediate health hazard. Surface water quality is subjected to frequent dramatic changes in microbial quality as a result of the variety of activities on the watershed (Okoh et al., 2005). These changes are caused by discharges of municipal raw waters or treated effluent at a specific point-source into the receiving waters (Okoh et al., 2007). Okoh and co-workers (2007) reported that effluent from treatment plant discharge significant amount of pollution-indictor and pathogenic microorganism, leading to the deterioration in the quality of water.

All infectious agent causing diarrhoea are efficiently spread by the faecal-oral route (Okoh and Osode, 2008). According to epidemiologic investtigation, faecally contaminated water and food are the most common vehicles for cholera infection. As highlighted earlier cholera is a highly epidemic diarrhoeal disease which continues to devastate many developing countries where socio-economic conditions and poor, sanitary systems and public hygiene are rudimentary and safe drinking water is not available (Igbinosa and Okoh, 2008). Okoh et al. (1996) reported the water component along with other wastewaters of the flow stations may be channeled into the saver pits prior to discharge into the environment, usually water bodies such as streams or rivers are potential source of this infection. In rural and sub-urban settings of most developing nations, the use of sewage and wastewater is often the only source of water for irrigation in these area, eating fruit and vegetables that have been irrigated with contaminated water and eaten raw is one likely way that toxigenic *V. cholerae* can be ingested (Okoh et al., 2007; Igbinosa and Okoh, 2008). The practice of direct discharge of effluent into receiving water bodies is of major concern as it could result amongst other things in the

substantial increase in organic load and consequently depletion of the dissolved oxygen content of the receiving water body (Okoh et al., 1996, 2006, 2007; Okoh and Trejo-Hernandez, 2006).

Ecology of toxigenic strains

V. cholerae has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (Hughes et al., 1994). The physicochemical conditions for the survival of toxigenic *V. cholerae* have been studied and the possibility of survival of the organism in an estuarine environment, brackish waters and treated effluents is widely established (Colwell and Spira, 1992; Colwell and Huq, 1994; Okoh et al., 2007, Igbinosa and Okoh, 2009). However, the nature of the survival and persistence of toxigenic *V. cholerae* O1 or O139 in aquatic milieu and the factors involved in the conservation of the CTX element and other pathogenic genes in the aquatic environment are not clear (Colwell and Huq, 1994). The survival may be dependent on several factors occurrence of particular physicochemical conditions, a specific association of the bacteria with aquatic plants or animals, and/or the existence of specific ecological associations involving several components of the aquatic environment. It has been stated that under stress conditions, such as the vibrios are converted to a viable but non-culturable (VBNC) form that cannot be recovered by standard culture techniques and that such VBNC forms are able to cause infection and can revert to the culturable form (Colwell and Huq, 1994).

In areas of endemic infection, cholera epidemics occur in a regular seasonal pattern. It is not clear what determines the seasonal emergence of epidemic *V. cholerae* strains and outbreaks of cholera (Islam et al., 1994). Although, it has been suggested that during interepidemic periods toxigenic *V. cholerae* exists in an unexplained ecological association with aquatic organisms, possibly in the VBNC form, until the next epidemic season, when environmental factors trigger the dormant bacteria to multiply and lead to cholera outbreaks (Islam et al., 1994). However, differences in genetic or phenotypic properties have been often noticed among *V. cholerae* O1 and O139 strains isolated during different epidemics (Mukhopadhay et al., 1998; Siddique et al., 1989; Nakasome et al., 1987). Analysis of restriction fragment length polymorphisms in cholera toxin and rRNA gene restriction patterns of *V. cholerae* strains has also shown clonal diversity among epidemic strains (Faruque et al., 1993, 1994, 1995; Mitra et al., 1996).These events have raised questions about whether seasonal epidemics are caused by periodic appearances of the same strains of *V. cholerae* or are due to a continual emergence of new toxigenic clones from non-toxigenic progenitors. In addition to the O1 and O139 serogroups of *V. cholerae* which normally carry a set of virulence genes, certain environmental isolates belonging to diverse serogroups have also been found to possess virulence genes or their homologues (Chakraborty et al., 2000; Mukhopadhay et al., 2001). This raises question whether the factors which have been described as virulence factors in the context of human infection with *V. cholerae* have other important roles when the bacterium resides in its environmental habitat.

The concept of an aquatic reservoir of *V. cholerae* O1 or O139 implies not only that the vibrios survive, in whatever form, but also that they form an essential component of the ecosystem. Several studies have illustrated the ability of toxigenic *V. cholerae* to associate with a variety of zooplankton, phytoplankton and algae (Islam et al., 1994). The associations prolong survival and presumably the vibrios gain nutrients from the host. However, these studies did not explain whether such association is specific for the epidemic serogroups of toxigenic *V. cholerae* or is a general phenomenon for all other *V. cholerae* serogroups and thus does not seem to be a mechanism for selective enrichment of toxigenic *V. cholerae* (Faruque et al., 1997a, 1997b). Moreover, the benefit imparted to the pathogen by possessing and maintaining the virulence-associated genes with respect to its survival and persistence in the environment is not clear. The role of extracellular enzymes including CT in the environmental micro-ecology of toxigenic *V. cholerae* is uncertain, although it has been suggested that in freshwater systems, local ionic micro-environment can be controlled by toxigenic *V. cholerae* by use of toxin acting on other living cells. However, such hypothesis has yet to be proven with specific experimental data and hence further studies are required to understand the more definitive roles of the virulence-associated factors and environmental selection pressures for toxigenic *V. cholerae* (Chakraborty et al., 2000; Mukhopadhay et al., 2001).

Studies so far suggest that causation of cholera in humans is also linked to a natural process of enrichment of toxigenic *V. cholerae* and partly explains the benefit imparted to the pathogen during the disease in humans (Faruque et al., 1998b, 1998c). However, to understand the general epidemiological behaviour of *V. cholerae*, which includes mechanisms leading to seasonal pattern of epidemics, transient appearance and disappearance of different clones and emergence of new epidemic clones, it is important to study the interactions among the bacteria, genetic elements mediating the transfer of virulence genes, the human host and possible environmental factors (Faruque and Nair, 2002).

Potential for the emergence of new toxigenic strains

The major pathogenic genes in *V. cholerae* are clustered in several regions of the *V. cholerae* chromosome and the structure of these pathogenic gene clusters indicates that they are capable of being propagated horizontally (Harkey et al., 1994; Karaolis et al., 1999; Kovach et al., 1996; Ogierman et al., 1993; Waldor et al., 1996, 1997). This could be attributed to the possibility that environmental strains of *V. cholerae* may have developed the ability to adapt to the intestinal environment through acquisition of

virulence genes (Mukhopadhay et al., 2001). The demonstration of the existence of environmental strains of *V. cholerae* which carry one or more virulence gene or their homologues further supports the possibility of an environmental origin for pathogenic *V. cholerae* (Chakraborty et al., 2000; Mukhopadhay et al., 2001). These environmental strains may constitute reservoirs of virulence genes and participate in gene transfer events leading to emergence of strains carrying a crucial combination of virulence genes. Since acquisition of virulence genes appear to provide increased fitness to the bacteria, the ecosystem for *V. cholerae*, which includes the aquatic milieu as well as the host compartment, should support the origin of pathogenic clones (Faruque and Nair, 2002). Contributing to the apparent dichotomy between toxigenic and non-toxigenic strains is the issue of acquisition of virulence genes in the environment, as most environmental isolates harbor neither *tcp* nor *ctxAB* (Singh et al., 2001). Recombination and acquisition of foreign DNA appear to be common features among vibrios and *V. cholerae* (Heidelberg et al., 2000). Genes for both TCP and CTX can be readily transduced into recipient strains via temperate phages. This is a key issue in the emergence of toxigenic strains, how this occurs in the environment is still under study.

In a recent study by Rahman et al. (2008), 10 environmental strains of *V. cholerae* non-O1, non-O139 that were positive for both TTSS and the TCP island genes and eight of these strains were infected by CTXΦ in the intestine of infant mice were identified. Previous studies showed that the efficiency of CTXΦ infection was considerably higher *in vivo* and this was attributed to more adequate expression of the phage receptor TCP *in vivo* than under laboratory conditions (Waldor and Mekalanos, 1996; Faruque et al., 1998b). Rahman and co-worker (2008) did not detect any Km^R transductants of the environmental strains in the *in vitro* assays and infection with CTXΦ was detectable only in the infant mouse assay. They suggested that infection of these *V. cholerae* strains by CTXΦ was possibly TCP dependent. Hence, these TTSS-positive environmental strains of *V. cholerae* carry TCP island genes and these genes are functional and capable of producing TCP pili; thus, these strains are potentially susceptible to CTXΦ infection under natural conditions, that is, in the human host or other environments where TCP is functionally expressed. The authors propose that these strains are intermediates in the evolution of a group of pathogenic *V. cholerae* strains that carry TTSS in addition to producing TCP and CT. They assume that it further supported by previous study in which clinical TCP^+ and CT^+ strains belonging to O141 serogroup have been found positive for the TTSS (Dziejman et al., 2005).

It has been shown that naturally occurring strains of toxigenic *V. cholerae* O1 and O139 are inducible lysogens of CTXΦ. The phage can be induced *in vitro*, but the induction is not normally associated with cholera pathogenesis in humans (Faruque et al., 1998c). It seems possible that in the natural ecological settings, unidentified environmental factors induce lysogenic CTXΦ in toxigenic *V. cholerae*, resulting in the release of extracellular CTXΦ particles into the aquatic environment. The cell-free phage particles participate in the emergence of novel toxigenic strains of *V. cholerae* through interactions with non-toxigenic strains which exist in the environment and in the human population that consumes the environmental waters. CTXΦ uses TCP as its receptor and hence the phage can infect only *V. cholerae* cells expres-sing TCP. The TCP genes, which are part of a greater genetic element referred to as the TCP pathogenicity island, appear to be the initial genetic factors required for the origination of epidemic strains.

Analysis of the structure of the pathogenicity island suggests that this could be of phage origin, or may be transferred by transducing phages (Chakraborty et al., 2000; Karaolis et al., 1998; Kovach et al., 1996; Faruque et al., 1998c). Since genes responsible for the production of TCP are carried mostly by *V. cholerae* O1 or O139, while other serotypes of *V. cholerae* usually do not carry genes for TCP, it is obvious that the CTX element is also found mostly in the O1 and O139 vibrios, whereas most non-O1 vibrios are usually non-toxigenic. This further supports the assumption that in natural settings, CTXΦ probably plays an important role in the origination of new toxigenic strains of *V. cholerae*. Since *V. cholerae* strains which are TCP positive but CTX negative are not fre-quently isolated during environmental sampling, it is pos-sible that such strains are normally present in very small numbers in the environment, but following conversion by CTXΦ to toxigenicity, the strains are enriched in the gastrointestinal environment and later become detectable as new strains of toxigenic *V. cholerae.* Subsequent increases in the concentration of toxigenic *V. cholerae* in the aquatic environment may lead to epidemic outbreaks of cholera. It has been demonstrated that CTXΦ infects recipient *V. cholerae* strains more efficiently in the intestinal environment, where virulence factors such as TCP are adequately expressed (Faruque et al., 1998b; Waldor and Mekalanos, 1996). While the conversion of non-toxigenic *V. cholerae* is favoured within the gastrointestinal tract of the mammalian host, the natural selection and persistence of the novel toxigenic strains may involve both intestinal and environment factors, the immune status of the host population and antigenic properties of the new pathogenic strain. The induction of CTXΦ lysogens is probably controlled by precise environmental signals such as optimum temperature, sunlight and osmotic conditions and this may account for the observed seasonal outbreaks of cholera in regions of endemic infection (Faruque et al., 1998a).

Molecular epidemiology of toxigenic *V. Cholerae*

Epidemiological surveillance of cholera was limited before the 1970s by the lack of suitable typing systems (Faruque et al., 1998a). Epidemiological information on

the emergence and prevalence of toxigenic *V. cholerae* O139 and its coexistence with the O1 El Tor strains are available primarily from Bangladesh and India through systematic surveillance studies. In the Ganges delta region of India and Bangladesh, epidemics of cholera occur with a regular seasonality, but temporal variation in the prevalence of the 2 epidemic serogroups O1 and O139 have been observed (Faruque et al., 1995, 1997b; Basu et al., 2000). The emergence of *V. cholerae* O139 initially caused a complete displacement of the El Tor biotype strains in these countries. However, *V. cholerae* O139 was again displaced in 1994 by a new genetic variant of the O1 strain and this variant strain dominated until 1996 in India (Faruque et al., 1997a; Basu et al., 2000). In August, 1996, a new variant of the O139 strain emerged and cholera caused by the new O139 genetic variant dominated for a year, until September, 1997 in Calcutta. Similarly in neighboring Bangladesh, during 1994 and till the middle of 1995, in most northern and central areas of the country, the O139 vibrios were replaced by a new clone of *V. cholerae* O1 of the El Tor biotype, whereas in the southern coastal regions, the O139 vibrios continued to exist (Faruque et al., 1997b, 1999; Basu et al., 2000). By late 1995 and through 1996, cases of cholera caused by both *V. cholerae* O1 and O139 were again detected in various regions of Bangladesh. However, since 1996, cholera in Bangladesh was caused mostly by *V. cholerae* O1 of the El Tor biotype, whereas only a few cases were caused by strains of the O139 serogroup.

Recent developments in DNA analysis techniques have introduced several new typing methods and have permitted studies of the epidemiology of toxigenic *V. cholerae* on a larger global perspective (Chen et al., 1991; Cook et al., 1983; Faruque et al., 1993, 1994, 1995, 1992; Wachsmuth et al., 1993, 1994; Waldor and Mekalanos, 1996; Basu et al., 2000). These techniques include the analysis of restriction fragment length polymorphisms (RFLPs) in different genes. The use of gene probes to study RFLPs in the *ctxAB* genes and their flanking DNA sequences, which are part of a larger genetic element (CTX element), indicated that U.S Gulf coast isolates of toxigenic *V. cholerae* are clonal and that they are different from other seventh-pandemic isolates (Kaper et al., 1982). RFLPs in conserved rRNA genes have also been used to differentiate *V. cholerae* strains into different ribotypes. Analysis of isolates from the Latin American epidemic in 1991 showed that they were related to the seventh-pandemic isolates from other parts of the world and that the Latin American cholera epidemic was an extension of the seventh-pandemic (Faruque et al., 1992; Wachsmuth et al., 1993, 1994). Analysis of toxigenic El Tor strains by multi locus enzyme electrophoresis has also been used to group the El Tor strains into major clonal groups. The clones seem to reflect broad geographical and epidemiological associations.

Comparative analysis of the El Tor strains of *V. cholerae* O1 and the epidemic O139 strains suggested that the O139 strains are related to El Tor strains and were derived from them by possible genetic changes in the serotype-specific gene clusters (Faruque et al., 1994; Wachsmuth et al., 1994; Basu et al., 2000). Numerical analysis of ribotype patterns (Faruque et al., 1995) has also revealed that *V. cholerae* strains belonging to the non-O1 and non-O139 serogroups diverge widely from the O1 and O139 *V. cholerae* strains. Molecular analysis of *V. cholerae* strains isolated during the epidemics period of 1961 and 1996 in Bangladesh revealed clonal diversity among strains isolated during different epidemics (Faruque et al., 1993, 1994, 1995, 1997a; Basu et al., 2000). These studies demonstrated the transient appearance and disappearrance of more than 6 ribotypes of classical vibrios, at least 5 ribotypes of El Tor vibrios and 3 different ribotypes of *V. cholerae* O139. Different ribotypes often showed different CTX genotypes resulting from differences in the copy number of the CTX element and variations in the integration site of the CTX element in the chromosome (Faruque et al., 1995, 1997a; Basu et al., 2000). These studies indicated that there had been a continual emergence of new clones of toxigenic *V. cholerae* which replaced existing clones, possibly through natural selection involving unidentified environmental factors and immunity of the host population (Basu et al., 2000; Faruque et al., 2003b).

Virulence associated toxigenic factors in *V. cholerae*

The pathogenesis of cholera is a complex process and involves a number of factors which aid the pathogen to reach and colonize the epithelium of the small intestine and produce the enterotoxin that disrupts ion transport by intestinal epithelial cells. Although production of CT, encoded by the *ctxAB* genes, is directly responsible for the manifestation of diarrhoea, cholera pathogenesis relies on the synergistic action of a number of other genes, including the genes for one or more colonization factors (Kaper et al., 1995). Several bacterial pathogens have acquired clusters of virulence genes that display a typical base composition and these pathogenicity Islands are not present in related non-pathogenic species (Basu et al., 2000).

In *V. cholerae*, the major virulence genes appear to exist in clusters and there are at least 2 regions of the *V. cholerae* chromosome in which genes encoding virulence factors are clustered (Everiss et al., 1994; Harkey et al., 1994; Ogierman et al., 1993; Pearson et al., 1993; Trucksis et al., 1993). These include the CTX element, which has now been shown to be the genome of a filamentous bacteriophage and the TCP-accessory colonization factor (ACF) gene cluster, referred to as the TCP pathogenicity island (Waldor and Mekalanos, 1996; Kovach et al., 1996). The pathogenicity island shares several characteristics with those of other species of pathogenic bacteria. These include the presence of groups of virulence genes, a regulator of virulence genes, a transposable gene, specific attachment sites flanking each end of the island and an integrase with homology to a phage integrase gene

(Karaolis et al., 1998; Kovach et al., 1996). Thus, the TCP pathogenicity island appears to have a phage origin but may now be defective (Kovach et al., 1996).

Since colonization is a requirement to establishing a productive infection by *V. cholerae*, the existence of other possible factors responsible for colonization investigated. This includes the mannose-fructose-resistant cell-associated hemagglutinin (MFRHA) has been implicated as a virulence determinant but its exact role in colonization is not clear (Franzon et al., 1993). The mannose-sensitive hemagglutinin (MSHA), which is expressed mostly by strains of the El Tor biotype, is a flexible pilus composed of subunits (Jonson et al., 1991). Antibodies to some purified outer membrane proteins (OMPs) of *V. cholerae* have been shown to inhibit intestinal colonization in the infant mouse model (Sengupta et al., 1992). The role of other OMPs in virulence has not been established in either animal or human studies (Sengupta et al., 1992). Other factors that have been examined for possible roles in virulence include the core-encoded pilus, which is encoded by the *cep* gene located within the CTX genetic element (Pearson et al., 1993) and several possible adhesions such as the lipopolysaccharide (Chitnis et al., 1982) and a slime agglutinin present on the flagellum (Attridge et al., 1983). Although some of these factors including MFRHA, MSHA and OMPs are suspected to play a role in enhancing adhesion and colonization, possibly in association with other factors, when tested in animal models, their exact role in the virulence of *V. cholerae* in humans is still uncertain.

Studies by Faruque and co-workers (2003b) have shown that the major virulence genes of *V. cholerae* required for pathogenesis in humans and animal models are the genes involved in the production of TCP and CT. The structures of the TCP pathogenicity island and the CTX genetic element are suggestive of horizontal transfer of these gene clusters as a possible mechanism for the origination of new pathogenic clones of *V. cholerae*. It seems possible that the acquisition of the TCP pathogenicity island and the CTX element has allowed specific strains of *V. cholerae* to become adapted to the human intestinal environment (Faruque et al., 1998a).

Vibrio pathogenicity Island (VPI)

An interesting aspect of toxigenic *V. cholerae* is that the receptor for CTXΦ entry into the bacterium is the TCP, which is involved in the colonization of the bacteria on the human gut epithelium as well and the ACF are among the genetic modules that have been acquired by the *V. cholerae* genome from other bacterial donors by horizontal gene transfer (Karaolis et al., 1998). TCP and ACF are borne on the genetic island designated as the Vibrio pathogenicity island (VPI) a characteristic of epidemic and pandemic *V. cholerae* strains.

DiRita et al. (1991) investigated the coregulation of CT and TCP by the ToxR regulatory system, which includes the ToxT protein. The genes encoding ToxT and TCP are located in the same chromosomal region (Brown et al., 1995), together with other ToxR-regulated genes includeing those for ACF (Everiss et al., 1994; Kovach et al., 1994). Molecular study has revealed that although the major subunit of TCP is encoded by the *tcpA* gene, the formation and function of the pilus assembly require the products of a number of other genes located on the chromosome adjacent to the *tcpA* gene and that these constitute the *tcp* gene cluster (Oigerman et al., 1993). At least 15 open reading frames (ORFs) are found in the *tcp* cluster, which is located immediately downstream of the *tagD* gene. The *tcpH* and *tcpI* genes are 2 ToxR-regulated genes that influence TcpA synthesis. Inactivation of *tcpH* results in decreased pilin synthesis, whereas inactivation of *tcpI* leads to increased synthesis of TcpA (Faruque et al., 1998a). Harkey et al. (1994) suggested that regulators such as TcpI, which acts downstream of ToxR and ToxT, may function to fine-tune the expression of the TCP virulent determinant throughout the pathogennic cycle of *V. cholerae*. Hase and Mekalanos (1998) showed that TcpP and TcpH constitute homologues of ToxR and ToxS and cooperate with ToxR and ToxS in the transcriptional activation of the ToxT promoter. Adjacent to and downstream of the *tcp* cluster is located the *acf* gene cluster. The precise nature of the colonization factor is not clear, but *acfD*, 1 of the 4 ORFs (*acfABCD*), encodes a lipoprotein (Parsot and Mekalanos, 1991).

Karaolis et al. (1998) study the relationship between pathogenic and non-pathogenic strains of *V. cholerae* revealed that a pathogenicity island (PAI) is present in the toxigenic strains alone. Karaolis and co-workers (1998) found that ~40 kb VPI has a low GC content of 35%, suggesting acquisition from another source by horizontal gene transfer. Putative integrase and transposase genes and flanking *att* sites were present in VPI and could possibly be of phage origin. Polymerase chain reaction (PCR) and Southern hybridization assays revealed that this gene cluster was absent in non-toxigenic environmental strains of *V. cholerae*, but were invariably present in all epidemic and pandemic strains investigated (Vital Brazil et al., 2002). Boyd et al. (2000) established that the VPI is a necessary element for epidemic and pandemic strains, it was postulated that the evolution of toxigenic strains from non-toxigenic ones must be a multi-step process, the initial step of which would be the acquisition of the VPI. This would lead to the expression of the *tcp*, which would in turn facilitate the acquisition of the CTXΦ, thus providing the genes for cholera toxin. The extent of transfer of these virulence-related elements was demonstrated convincingly when Chakraborty et al. (2000) reported the presence of the toxin co-regulated pilus as well as the cholera toxin genes in environmental isolates that contained neither the O1 nor the O139 antigen of *V. cholerae*. This study proved that virulence genes were not exclusively associated with clinical strains and also set the tone for the hypothesis that environmental strains could act as reservoirs for virulence genes. This track of thought was highlight in a later study by Mukhopadhyay et al. (2001)

where intact or nearly intact VPI islands were found in a set of environmental isolates investigated using PCR assays. The studies also reported the finding of 3 putative unreported *rstR* repressor genes, as well as a new *tcpA* allele. TcpA variants in toxigenic non-O1, non-O139 serogroup isolates have been studied by (Boyd et al., 2002). While TcpA is involved in interaction with the immune system of the host, selection favours diversity at the exposed regions of the protein. This is reflected in the sequence diversity of various *tcpA* variants at the carboxyl region. An assessment of the diverse variants has, however, revealed that they are all capable of functioning as colonization factors (Uma et al., 2003). It appears that the TCP pathogenicity island is the initial genetic element required for the origination of epidemic strains, since CTXΦ uses TCP as its receptor (Waldor and Mekalanos, 1996). Also, the role of TCP as an essential colonization factor inside the host intestine is well recognized (Attridge et al., 1993; Hase and Mekalanos, 1998).

Cholera toxin and CTX phage

Toxigenic *V. cholerae* carries one or more copies of cholera toxin (CT) genes encoded by the genes *ctxA* and *ctxB* (Zhang et al., 1995; Waldor et al., 1996). The A and B subunits of CT are encoded by 2 separate but overlapping open reading frames (ORFs). *V. cholerae* also produces a putative toxin known as zonula occludens toxin (Zot), which increases the permeability of the small intestinal mucosa by affecting the structure of intercellular tight junction, or zonula occludens (Fasano et al., 1991; Baudry et al., 1992). A third toxin that has been described is accessory cholera enterotoxin (Ace) which is capable of inducing fluid accumulation in rabbit ligated ileal loops (Trucksis et al., 1993). In toxigenic *V. cholerae,* CT is encoded by filamentous bacteriophage designated CTXΦ, Which exists as a prophage in the bacterial chromosome. CTXΦ is unusual among filamentous phages because the phage genome encodes the functions necessary for a site specific integration system and thus can integrate into the *V. cholerae* chromosome at a spe-cific attachment site known as *att*RS, forming stable lysogens (Waldor and Mekalanos, 1996; Waldor et al., 1997).

The phage genome consists of 2 regions, RS2 and core. The RS2 represents a site-specific recombination system that allows the lysogenic phage to integrate at specific sites on the host chromosome and the core consists of a retinue of genes, including the *ctxA, ctxB, zot, ace, psh, cep* and *orfU* (Waldor and Makalanos, 1996; Waldor et al., 1997). The RS2 region consists of the *rstR, rstA* and *rstB* genes. The *rstA* gene product is responsible for phage DNA replication, *rstB* is involved in site-specific integration of the phage and *rstR* is a repressor of *rstA* function (Waldor et al., 1997). The *rstR* gene is transcribed in a direction opposite to that of other genes of the phage. The *rstR* gene is flanked by 2 intergenic regions, ig-1 and ig-2. The ig-2 region carries the *rstA* promoter. It has been experimentally inferred that the *rstR* proteins are biotype specific, that is classical *rstA* can be repressed by classical *rstR* but not by El Tor *rstR* (Kimsey and Waldor, 1997). The *rstR* genes exhibit the phenomenon of heteroimmunity, which means that El Tor *V. cholerae* resist superinfection with another El Tor derived CTXΦ, but classical *V. cholerae* will allow the integration of an El Tor-derived CTXΦ (Kimsey and Waldor ,1997). The RS2 module of the phage is preceded immediately by a similar element (Waldor et al., 1997; Campos et al., 1998), the RS1, which has three genes identical in sequence to the corresponding genes in the RS2 module, these are the *rstR*1, *rstA*1, *rstB*1. There is one gene in RS1 that has no counterpart in the RS2 module. This is the *rstC* gene (Waldor et al., 1997; Campos et al., 1998).The RS1 was experimentally proven to be a satellite phage that could exploit the morphogenesis genes of the CTXΦ to exist as a phage itself (Faruque et al., 2002). The function of RstC was elucidated to be that of an antirepressor which could counteract the activity of the *rstR* gene product. RstC was also shown to induce *ctxAB* expression, thus contributing to virulence in *V. cholerae* (Davis et al., 2002).

It has been showed that under suitable conditions toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ particles (Waldor and Makalanos, 1996; Faruque et al., 1998a).The phage can be propagated in recipient *V. cholerae* strains in which the CTXΦ genome either integrates chromosomally at a specific site forming stable lysogens or maintained extrachromosomally as a replicative for of phage DNA (Faruque et al., 1998a). Studies have established that some naturally occurring non-toxigenic strains of *V. cholerae* are infected by CTXΦ and converted to toxigenic strain with epidemic potential (Faruque et al., 1998b). The bacteriophage uses the TCP as a receptor and therefore expression of TCP by the bacterium is a requirement for its susceptibility to the phage. Consequently, a virulence factor of the bacterium in humans also serves as a receptor for CTXΦ, demonstrating a co-evolution of genetic elements mediating the transfer of virulence genes with the pathogenic bacterial species they infect (Faruque et al., 2002).

Expression and regulation of virulence genes in toxigenic *V. cholerae*

The regulation of virulence-associated genes in *V. cholerae* involves multiple systems. Expression of several critical virulence genes in *V. cholerae* is coordinately regulated so that multiple genes respond in a similar fashion to environmental conditions (DiRita et al., 1991; Skorupski and Taylor, 1997). Coordinate expression of virulence genes results from the activity of a cascading system of regulatory factors. The *ctxAB* is a coordinated multi-step process that requires the elaboration of a number of virulence factors (Peterson and Mekalanos, 1988). One such fundamental step is the successful colonization of

the host epithelium by toxigenic bacteria and this is mediated by their filamentous surface structures, the pili or fimbriae. The toxin co-regulated pilus is a well-characterized surface organelle of *V. cholerae*. It is a bundle-forming pilus which is coordinately expressed with CT from which it derives the name toxin coregulated pilus. TCP expression is regulated by ToxR, a transmembrane component that activates CT in response to appropriate environmental signals (Miller and Mekalanos, 1984). TcpA, the protein that constitutes the pilus structure, bears homology to bacterial type-IV pilins involved in colonization (Shaw and Taylor, 1990). The other genes in the cluster are involved in the biogenesis, secretion and export of TcpA (Uma et al., 2003).

Another potential colonization factor of *V. cholerae* designated as *acf* for Accessory Colonization Factor, also regulated by ToxR, is present immediately downstream of the TCP group of genes, this also includes a cluster of genes (Brown and Taylor, 1995).The *ctx* module is synchronized in its function by a regulatory arrangement designated as the cascade system. The regulatory cascade induces the *ctx* module in response to environmental stimuli and thus serves to control virulence in response to environmental conditions like temperature, pH, oxygen concentration, etc. The regulation involves the expression of the toxin genes, the toxin co-regulated pilus and metabolic genes like *aldA* and *tag*. The *tox* genes (*toxT*, *toxS* and *toxR*) are part of the cascade. ToxS is a 'sensory' membranous protein that activates ToxR, also a membrane-spanning protein. ToxT is present in the cytoplasmic matrix and is induced by ToxR; ToxT regulates the expression of the *ctx* and *tcp* genes (Miller and Mekalanos, 1984; Peterson and Mekalanos, 1988; Shaw and Taylor, 1990).

ToxR, a transmembrane protein, is the master regula-tor and is itself regulated by environmental signals. The ToxR protein binds to a tandemly repeated 7-bp DNA sequence found upstream of the *ctxAB* structural gene and increases the transcription of *ctxAB*, resulting in higher levels of CT expression (DiRita et al., 1991; Miller and Mekalanos, 1984; Miller et al., 1987). The activity of ToxR is enhanced by another transmembrane protein, ToxS, which interacts with ToxR. ToxS serves to assemble or stabilize ToxR monomers into the dimeric form (Miller and Mekalanos, 1988). ToxR regulates not only the expression of *ctxAB* but also that of at least 17 distinct genes, which constitute the ToxR regulon. These include the TCP colonization factor (Taylor et al., 1987), the accessory colonization factor (Peterson and Mekalanos, 1988), the OMPs OmpT and OmpU (Miller and Mekalanos, 1988), and 3 other lipoproteins (Parsot et al., 1991). Except for the *ctxAB* genes, other genes in the ToxR regulon are controlled through another regulatory factor called ToxT. ToxR controls the transcription of the *toxT* gene, which encodes a member of the AraC family of bacterial transcription activators (Higgins and DiRita, 1996). The resulting increased expression of the ToxT protein then leads to activation of other genes in the ToxR regulon. Thus, ToxR is at the top of the regulatory cascade that controls the expression of CT and other important virulence factors in *V. cholerae*, while the expression of ToxR itself remains under the control of environmental factors (Skorupski and Taylor, 1997). It has also been recognized that *V. cholerae* has ToxT-dependent and ToxT-independent branches of the ToxR regulon (Champion et al., 1997).

It has also been reported that expression of CT from the RF of CTXΦ is independent of ToxR. This indicates that phage induction may provide another mechanism for the regulation of CT production (Lazar and Waldor, 1998). The coordinate regulation of virulence genes through the ToxR regulon demonstrates that the organism has developed a mechanism of sampling and responding to its environment.*V. cholerae* also possesses a system for expression of genes in response to temperature variation (Parsot and Mekalanos, 1990). Immediately upstream of the toxR gene is the htpG gene, which encodes a heat shock protein (Parsot and Mekalanos, 1990). The toxR and htpG genes, which are transcribed in opposite directions, have their promoters so close that only one RNA polymerase can bind in the intergenic region. This mechanism work in such a way that the normal σ-70 RNA polymerase binds to the toxR promoter and transcribes the toxR gene only at low temperatures. At elevated temperatures, σ-32, the RNA polymerase sigma subunit involved in the transcription of heat shock genes, binds to the htpG promoter, thus repressing the toxR promoter. Expression of certain genes in response to low iron concentration is another distinct regulatory system in *V. cholerae*, that controls additional putative virulence genes including hemolysins and several outer membrane protein (OMP) that are not expressed when cells are grown in iron-rich media (Sigel and Payne, 1982). Sciortino and Finkelstein (1983) have suggested that the intestinal site for growth of *V. cholerae* is a low-iron environment which triggers the expression of these iron-regulated genes *in vivo.* Different regulatory systems in *V. cholerae* apparently allow the bacterium to vary the expression of its genes to optimize survival in different environments, which include the human intestine and the estuarine environment.

Conclusion

In spite of efforts to control cholera, the disease continues to occur as a major public health problem in many developing countries (Igbinosa and Okoh, 2008). Numerous studies over more than a century have made advances in our understanding of the disease and ways of treating patients, but the mechanism of emergence of new epidemic strains and the ecology supporting the regular epidemics, remain mysterious and challenging to researchers in the field. Recent studies of the pathogenicity island, the discovery of bacteriophage encoding CT and

the characteristic system for acquisition of different genes by toxigenic *V. cholerae* have provided an impetus for further study of the organism to understand the molecular basis for the emergence of pathogenesis and natural fact controlling the conservation of particular genetic traits. The emergence of toxigenic *V. cholerae* strains has provided an opportunity to study the coevolution of different serogroups of epidemic *V. cholerae* strains, apparently driven by competition for survival and thereby attaining enhanced fitness. Toxigenic *V. cholerae* provides a natural system to study the coevolution of bacteria and the virulence-associated genetic elements and the mutual benefits imparted to each other in terms of attaining greater evolutionary fitness (Faruque et al., 1998a). Therefore, future challenge for researcher investigating the ecology of toxigenic *V. cholerae* strains should be aimed at explaining more about the emergence of pathogenic strains and factors involved in the natural selection of the species to ensure its continued existence. Also the interaction between regulators and the virulence genes in the pathogenesis of toxigenic *Vibrio* especially in relation to point source pollution such as inadequate treated effluents from wastewater treatment facilities in developing nations should be of interest and these are subjects of intensive investigation in our laboratory.

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REFERENCES

Albert MJ, Siddique AK, Islam MS, Faruque ASG, Ansaruzzaman M, Faruque SM, Sack RB (1993). Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1in Bangladesh. Lancet 341: 704.

Attridge SR, Rowley D (1983). The specificity of *V. cholerae* adherence and significance of the slime agglutinin as a second mediator of *in vitro* attachment. Infect. Dis. 147: 873–881.

Attridge SR, Voss E, Manning PA (1993). The role of toxin co-regulated pili in the pathogenesis of *Vibrio cholerae* O1 El Tor. Microb. Pathog. 15: 421–431.

Baudry B, Fasano A, Ketley J, Kaper JB (1992). Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect. Immun. 60: 428–434.

Baumann P, Furniss AL, Lee JV (1984). Vibrio. In: Kreig NR, Holt JG (Eds.), Bergey's manual of systematic bacteriology. Williams and Wilkins Co., Baltimore MD (1): 518–538.

Basu A, Garg P, Datta S, Chakraborty S, Bhattacharya T, Khan A, Ramamurthy T, Bhattacharya SK, Yamasaki S, Takeda Y, Nair GB (2000). *Vibrio cholerae* O139 in Calcutta, 1992–1998: incidence, antibiograms, and genotypes. Emerg. Infect. Dis. 6: 139-147.

Blake PA (1994). *Vibrio cholerae* and Cholera: In: Wachsmuth IK, Blake PA, Olsvik O (Eds) *Vibrio cholerae* and cholera: Molecular to global perspectives ASM Press, Washington DC pp. 293–295.

Boyd EF, Moyer K, Shi L, Waldor M (2000). Infectious CTXΦ and the Vibrio Pathogenicity Island prophage in *Vibrio mimicus*: evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae*. Infect. Immunol. 68: 1507–1513.

Brown RC, Taylor RK (1995). Organization of *tcp, acf* and *toxT* genes within a ToxT- dependent operon. Mol. Microbiol. 16: 425–439.

Campos J, Fando R, Silva A, Rodriguez BL, Benitez JA (1998). Replication function of the RS1 element associated with *Vibrio cholerae* CTXΦ prophage FEMS Microbiol. Lett. 164: 141–147.

Chakraborty S, Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T, Yamasaki S, Faruque SM, Takeda Y, Colwell RR, Nair GB (2000). Virulence genes in environmental strains of *Vibrio cholerae*. Appl. Environ. Microbiol. 66: 4022–4028.

Champion GA, Neely MN, Brennan MA, DiRita VJ (1997). A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of ToxT mutant strains. Mol. Microbiol. 23: 323-331.

Chatterjee S, Mondal AK, Begum NA, Roychoudhury S, Das J (1998). Ordered cloned DNA map of the genome of *Vibrio cholerae* 569B and localization of genetic markers. J. Bacteriol. 180 (4): 901-908.

Chen F, Evins GM, Cook WL, Almeida R, Bean HN, Wachsmuth IK (1991). Genetic diversity among toxigenic and non-toxigenic *Vibrio cholerae* O1 isolated from the Western Hemisphere. Epidemiol. Infect. 107: 225–233.

Chiavelli DA, Marsh JW, Taylor RK (2001). The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. Appl. Environ. Microbiol. 67: 3220–3225.

Chitnis DS, Sharma KD, Kamat RS (1982). Role of somatic antigen of *Vibrio cholerae* in adhesion to intestinal mucosa. J. Med. Microbiol. 5: 53–61.

Colwell RR, Huq A (1994). Vibrios in the environment: Viable but non-culturable *Vibrio cholerae*. In: Wachsmuth IK, Blake PA, Olsvik O (Eds.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington DC pp. 117–133.

Colwell RR, Spira WM (1992). The ecology of *Vibrio cholerae*. In: Barua D, Greenough III WB (Eds.), Cholera. Plenum Medical Book Co., New York pp. 107–127.

Cook WL, Wachsmuth K, Feeley J, Huq I (1983). The question of classical cholera. Lancet 1: 879–880.

DiRita VJ, Parsot C, Jander G, Mekalanos JJ (1991). Regulatory cascades controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA 88: 5403–5407.

Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ (2002). Comparative genomic anaysis of *Vibrio cholerae:* genes that correlate with cholera endemic and pandemic disease. Proc. Natl. Acad. Sci. USA 99: 1556–1561.

Dziejman M, Serruto D, Tam VC, Sturtevant D, Diraphat P, Faruque SM, Rahman MH, Heidelberg JF, Decker J, Li L, Montgomery K, Grills G, Kucherlapati R, Mekalanos JJ (2005). Genomic characterization of non-O1 non-O139 *Vibrio cholerae* reveals genes for a novel type III secretion system. Proc. Natl. Acad. Sci. USA 102: 3465–3470.

Everiss KD, Hughes KJ, Peterson KM (1994). The accessory colonization factor and toxin-coregulated pilus gene clusters are physically linked on the *Vibrio cholerae* O395 chromosome. DNA Seq. 5: 51–55.

Everiss KD, Hughes KJ, Kovach ME, Peterson KM (1994). The *Vibrio cholerae acfB* colonization determinant encodes an inner membrane protein that is related to a family of signal-transducing proteins. Infect. Immun. 62: 3289–3298.

Faruque SM, Albert MJ (1992). Genetic relation between *Vibrio cholerae* O1 strains in Ecuador and Bangladesh. Lancet 339: 740–741.

Faruque SM, Alim ARMA, Rahman MM, Siddique AK, Sack RB, Albert MJ (1993). Clonal relationships among classical *Vibrio cholerae* O1 strains isolated between 1961 and 1992 in Bangladesh. J. Clin. Microbiol. 31: 2513–2516.

Faruque SM, Alim ARMA, Roy SK, Khan F, Nair GB, Sack RB, Albert MJ (1994). Molecular analysis of rRNA and cholera toxin genes carried by the new epidemic strain of toxigenic *Vibrio cholerae* O139 synonym Bengal. J. Clin. Microbiol. 32: 1050–1053.

Faruque SM, Roy SK, Alim ARMA, Siddique AK, Albert MJ (1995). Molecular epidemiology of toxigenic *V. cholerae* in Bangladesh studied by numerical analysis of rRNA gene restriction patterns. J. Clin. Microbiol. 33:2833–2838.

Faruque SM, Ahmed KM, Siddique AK, Zaman K, Alim ARMA, Albert MJ (1997a). Molecular analysis of toxigenic *Vibrio cholerae* O139 Bengal isolated in Bangladesh between 1993 and 1996: Evidence for the emergence of a new clone of the Bengal vibrios. J. Clin. Microbiol. 35: 2299–2306.

Faruque SM, Ahmed KM, Alim ARMA, Qadri F, Siddique AK, Albert MJ

(1997b). Emergence of a new clone of toxigenic *Vibrio cholerae* O1 biotype El Tor displacing *V. cholerae* O139 Bengal in Bangladesh. J. Clin. Microbiol. 35: 624–630.
Faruque SM, Albert MJ, Mekalanos JJ (1998a). Epidemiology, genetics, and ecoology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. 62: 1301–1314.
Faruque SM, Asadulghani, Alim ARMA, Albert MJ, Islam KMN, Mekalanos JJ (1998b). Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic *V. cholerae* O1 and O139. Infect. Immun. 66: 3752–3757.
Faruque SM, Asadulghani, Saha MN, Alim ARMA, Albert MJ, Islam KMN, Mekalanos JJ (1998c). Analysis of clinical and environmental strains of non-toxigenic *Vibrio cholerae* for susceptibility to CTXΦ; molecular basis for the origination of new strains with epidemic potential. Infect. Immun. 66: 5819–5825.
Faruque SM, Asadulghani, Kamruzzaman M, Nandi RK, Ghosh AN, Nair GB, Mekalanos JJ, Sack DA (2002). RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTXΦ, Infect. Immunol. 70: 163–170.
Faruque SM, Mekalanos JJ (2003a). Pathogenicity islands and phages in *Vibrio cholerae* evolution. Trends Microbiol. 11: 505–510.
Faruque SM, Nair GB (2003b). Molecular ecology of toxigenic *Vibrio cholerae.* Microbiol. Immunol. 46(2): 59-66.
Faruque SM, Nair GB, Mekalanos JJ (2004a). Genetics of stress adaptation and virulence in toxigenic *Vibrio cholerae*. DNA Cell Biol. 11: 723–741.
Fasano A, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM, Kaper JB (1991). *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Natl. Acad. Sci. USA 88: 5242–5246.
Franzon VF, Baker A, Manning PA (1993). Nucleotide sequence and construction of a mutant in the mannose-fucose-resistant hemagglutinin (MFRHA) of *Vibrio cholerae* O1. Infect. Immun. 61: 3032–3037.
Harkey CW, Everiss KD, Peterson KM (1994). The *Vibrio cholerae* toxin-coregulated pilus gene tcpI encodes a homolog of methyl-accepting chemotaxis proteins. Infect. Immun. 62: 2669–2678.
Hase CC, Mekalanos JJ (1998). TcpP is a positive regulator of virulence factor gene expression in Vibrio cholerae. Proc. Natl. Acad. Sci. USA 95: 730-734.
Heidelberg JF, Elsen JA, Nelson WC, Clayton RJ, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson K, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SHO, Colwell RR, Mekalanos JJ, Venter JC, Fraser C (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406: 477–483.
Higgins DE, DiRita VJ (1996). Genetic analysis of the interaction between *Vibrio cholerae* transcription activator ToxR and ToxT promoter DNA. J. Bacteriol.178: 1080–1087.
Hughes KJ, Everiss KD, Harkey CW, Peterson KM (1994). Identification of a *Vibrio cholerae* ToxR-activated gene (*tagD*) that is physically linked to the toxin coregulated pilus (*tcp*) gene cluster. Gene 148: 97–100.
Igbinosa EO, Okoh AI (2008). Emerging *Vibrio* species: An unending threat to public health in developing countries Res. Microbiol. 159: 495-506.
Igbinosa EO, Okoh AI (2009) Impact of charge wastewater effluents on the physico-chemical qualities of a receiving watershed in atypical rural community. Int. J. Environ. Sci. Tech. 6(2): 175-182.
Islam MS, Drasar BS, Sack RB (1994). The aquatic environment as reservoir of *Vibrio cholerae*: a review. J. Diarrhoeal Dis. Res. 11: 197–206.
Janda JM, Powers C, Bryant RG, Abbott SL (1988). Current perspective on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. Clin. Microbiol. Rev. 1: 245– 267.
Johnson JA, Salles CA, Panigrahi P, Albert MJ, Wright AC, Johnson RJ, Morris Jr JG (1994). *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* El Tor but has important differrences. Infect. Immunol. 62: 2108–2110.
Jonson G, Holmgren V, Svennerholm AM (1991). Identification of a mannose-binding pilus on *V. cholerae* El Tor. Microb. Pathog. 11: 433–441.
Kaper JB, Bradford HB, Roberts NC, Falkow S (1982). Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. J. Clin. Microbiol. 16:129–134.
Kaper JB, Morris JG, Levine MM (1995). Cholera. Clin. Microbiol. Rev. 8: 48–86.
Kaper JB, Moseley SL, Falkow S (1981). Molecular characterization of environmental and non-toxigenic strains of *V. cholerae*. Infect. Immun. 32: 661–667.
Karaolis DKR, Johnson JA, Bailey CC, Boedeker EC, Kaper JB, Reeves PR (1998). A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. Proc. Natl. Acad. Sci. USA 95: 3134–3139.
Kimsey HH, Waldor MK (1998). CTXΦ immunity: application in the development of cholera vaccines. Proc. Natl. Acad. Sci. USA 95: 7035–7039.
Kovach ME, Hughes KJ, Everiss KD, Peterson KM (1994). Identification of a ToxR- activated gene, tagE, that lies within the accessory colonization factor gene cluster of *Vibrio cholerae* O395. Gene 148: 91–95.
Kovach ME, Shaffer MD, Peterson KM (1996). A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. Microbiol. 142: 2165–2174.
Lazar S, Waldor MK (1998). ToxR-independent expression of cholera toxin from the replicative form of CTXΦ. Infect. Immun. 66: 394–397.
Mekalanos JJ (1983). Duplication and amplification of toxin genes in *Vibrio cholerae* Cell 35: 253–263.
Miller VL, Mekalanos JJ (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. Proc. Natl. Acad. Sci. USA 81: 3471–3475.
Miller VL, Mekalanos JJ (1988). A novel suicide vector and is use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. 170: 2575–2583.
Mitra R, Basu A, Dutta D, Nair GB, Takeda V (1996). Resurgence of *Vibrio cholerae* O139 Bengal with altered antibiogram in Calcutta, India. Lancet 348: 1181.
Morris JG, Black RE (1985). Cholera and other vibrios in the United States. N. Eng. J. Med. 312: 343–350.
Mukhopadhyay AK, Basu A, Garg P, Bag PK, Ghosh A, Bhattacharya SK, Takeda Y, Nair GB (1998). Molecular epidemiology of reemergent *Vibrio cholerae* O139 Bengal in India. J. Clin. Microbiol. 36: 2149–2152.
Mukhopadhyay AK, Chakraborty S, Takeda Y, Nair GB, Berg DE (2001). Characterization of VPI pathogenicity island and CTXΦ prophage in environmental strains of *Vibrio cholerae.* J. Bacteriol. 183: 4737–4746.
Nakasone N, Iwanaga M, Eeckels R (1987). Characterization of *Vibrio cholerae* O1 recently isolated in Bangladesh. Trans. R. Soc. Trop. Med. Hyg. 81: 876–878.
Ogierman MA, Zabihi S, Mourtzios L, Manning PA (1993). Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. Gene 126: 51–60.
Okoh AI, Babalola GO, Bakare MK (1996). Microbial densities and physicochemical qualities of some crude oil flow stations saver pit effluents in the Niger Delta Area of Nigeria. Sci. Tot. Environ. 187: 73-78.
Okoh AI, Barkare MK, Okoh OO, Odjadjare E (2005). The cultural microbial and chemical qualities of some waters used for drinking and domestic purpose in a typical rural setting of Southern Nigeria. J. Appl. Sci. 5(6): 1041-1048.
Okoh AI (2006). Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants. Biotech. Mol. Bio. Rev. 1(2): 38-50.
Okoh AI, Trejo-Hernandez MR (2006). Remediation of petroleum hydrocarbon polluted systems: Exploiting the bioremediation strategies. Afr. J. Biotech. Vol. 5(25): 2520-2525.
Okoh AI, Odjadjare EE, Igbinosa EO, Osode AN (2007). Wastewater treatment plants as a source of microbial pathogens in the receiving watershed. Afr. J. Biotech. 6(25): 2932-2944.
Okoh AI, Osode AN (2008). Enterotoxigenic Escherichia coli (ETEC): A recurring decimal in infants' and travelers' diarrhea. Rev. Environ. Health. 23(2): 135-148.

Parsot C, Mekalanos JJ (1990). Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modified by the heat shock response. Proc. Natl. Acad. Sci. USA 87: 9898–9902.

Parsot C, Taxman E, Mekalanos JJ (1991). ToxR regulates the production of lipoproteins and the expression of serum resistance in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA 88: 1641–1645.

Pearson GDN, Woods A, Chiang SL, Mekalanos JJ (1993). CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. USA 90: 3750–3754.

Peterson KM, Mekalanos JJ (1988). Characterization of the *Vibrio cholerae* ToxR regulon: Identification of novel genes involved in intestinal colonization. Infect. Immunol. 56: 2822–2829.

Rahman MH, Biswas K, Hossain MA, Sack RB, John J, Mekalanos JJ, Faruque SM (2008). Distribution of genes for virulence and ecological fitness among diverse *Vibrio cholerae* population in a cholera endemic area: tracking the evolution of pathogenic strains DNA and Cell Biol. 27(7): 347–355.

Ramamurthy T, Garg S, Sharma R, Bhattacharya SK, Nair GB, Shimada T, Takeda T, Karasawa T, Kurazano H, Pal A, Takeda Y (1993). Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India; Lancet 341: 703–704.

Satcher D (1995). Emerging infections: getting ahead of the cure; Emerg. Infect. Dis. 1: 1–6.

Sciortino CV, Finkelstein V (1983). *Vibrio cholerae* expresses iron-regulated outer membrane proteins *in vivo*. Infect. Immun. 42: 990–996.

Sengupta DK, Sengupta TK, Ghose AC (1992). Major outer membrane proteins of *Vibrio cholerae* and their role in induction of protective immunity through inhibition of intestinal colonization. Infect. Immun. 60: 4848–4855.

Sharma C, Nair GB, Mukhopadhyay AK, Bhattacharya SK, Ghosh RK, Ghosh A (1997). Molecular characterization of *Vibrio cholerae* O1 biotype El Tor strains isolated between 1992 and 1995 in Calcutta, India: evidence for the emergence of a new clone of the El Tor biotype. J. Infect. Dis. 175: 1134–1141.

Shaw CE, Taylor RK (1990). *Vibrio cholerae* O395 tcpA pilin gene sequence and comparison of predicted protein structural features to those of type 4 pilins. Infect. Immunol. 58: 3042–3049.

Shimada T, Arakawa E, Itoh K, Okitsu T, Matsushima A, Asai Y, Yamai S, Nakajato T, Nair GB, Albert MJ, Takeda Y (1994). Extended serotyping scheme for *Vibrio cholerae*. Curr. Microbiol. 28: 175–178.

Shimada T, Nair GB, Deb BC, Albert MJ, Sack RB, Takeda Y (1993). Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet 341: 1347.

Siddique AK, Zaman K, Mazumder Y (1989). Simultaneous outbreaks of contrasting drug resistant classic and El Tor *Vibrio cholerae* O1 in Bangladesh. Lancet 1: 396.

Sigel SP, Payne SM (1982). Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio cholerae*. J Bacteriol 150:148–155.

Singh DV, Matte MH, Matte GR, Jiang S, Sabeena F, Shukla BN, Sanyal SC, Huq A, Colwell RR (2001). Molecular analysis of *Vibrio cholerae* O1, O139, and non-O1 and non-O139 strains: Clonal relationships between clinical and environmental isolates. Appl. Environ. Microbiol. 67: 910–921.

Skorupski K, Taylor RK (1997). Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol. Microbiol. 25: 1003–1009.

Taylor RK, Miller VL, Furlong DB, Mekalanos JJ (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA 84: 2833–2837.

Trucksis M, Galen JE, Michalski J, Fasano A, Kaper JB (1993). Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. Proc. Natl. Acad. Sci. USA 90:5267–5271.

Uma G, Chandrasekaran M, Takeda Y, Nair GB (2003). Recent advances in cholera genetics Curr. Sci. 85(11): 1538-1545.

Vital Brazil JM, Alves RM, Rivera IN, Rodrigues DP, Karaolis DK, Campos LC (2002). Prevalence of virulence associated genes in clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 1999. FEMS Microbiol. Lett. 215: 15–21.

Wachsmuth IK, Bopp CA, Fields PI, Carrilo C (1991). Difference between toxigenic *Vibrio cholerae* O1 from South America and US Gulf Coast. Lancet 337: 1097–1098.

Wachsmuth IK, Evins GM, Fields PI, Olsvic O, Popovic T, Bopp CA, Wells JG, CarrilloC, Blake PA (1993). The molecular epidemiology of cholera in Latin America. J. Infect. Dis. 167: 621–626.

Wachsmuth IK, Olsvik O, Evins GM, Popovic T (1994). Molecular epidemiology of cholera. In Wachsmuth K, Blake PA, Olsvik O (Eds.), *Vibrio cholerae* and cholera: molecular to global perspectives. American Society for Microbiology, Washington, D.C pp. 357–370.

Waldor MK, Mekalanos JJ (1996). Lysogenic conversion by a filamentous bacteriophage encoding cholera toxin. Sci. 272: 1910–1914.

Waldor MK, Rubin EJ, Pearson GDN, Kimsey H, Mekalanos JJ (1997). Regulation, replication and integration functions of the *Vibrio cholerae* CTXΦ are encoded by region RS2. Mol. Microbiol. 24: 1910–1914.

Watnick PI, Fullner KJ, Kolter R (1999). A role for the mannose sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J. Bacteriol. 181: 3606–3609.

Zhang RG, Scott DL, Westbrook MI, Nance S, Spangler BD, Shipley GG, Westbrook EM (1995). The three-dimensional crystal structure of cholera toxin. J. Mol. Biol. 251: 563–573.