

Genetic Determinants Differences between *Vibrio cholerae* Biotypes

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Vibrio cholerae O1 are classified into two biotypes, classical and El Tor based on susceptibility to bacteriophages and some biochemical properties, each encoding a biotype-specific genetic determinants. Before 1961, most epidemics had been caused by the classical biotype. However, with the passage of time, the classical biotype missed from the scenario and the El Tor emerged as the major biotype causing the cholera in humans. The present cholera global pandemic is attributed to a change among seventh pandemic strains and emergence of *V. cholerae* O139, *V. cholerae* O1 El Tor hybrid, and *V. cholerae* O1 El Tor with altered cholera toxin subunit B. The *V. cholerae* biotypes are not only different in phenotype but also human infections caused by them are different clinically. Infection with classical *V. cholerae* O1 more frequently produces severe infection than does El Tor, suggesting that the genetic and phenotypic differences between the two biotypes may also be reflected in their pathogenic potential. Considering the recent emergence of "hybrid biotype" and "El Tor variant" in different areas and in our country, we reviewed differences in genetic structure of *V. cholerae* biotypes.

Keywords: Genetic determinants, El Tor variant, hybrid biotype

1. Background

Cholera is described by a severe watery diarrhea caused by toxigenic *Vibrio cholerae* (1). As yet seven distinct pandemics of cholera have recorded since the diagnosis of the first one in 1817 (2). *V. cholerae* is classified into more than 200 serogroups, on the basis of somatic antigen called as the lipopolysaccharide O antigen. At the first and until in recent times, serogroup O1 was supposed to responsible serogroup for all cholera epidemics and endemics, have occurred. Serogroup O1 has two main serotypes Ogawa and Inaba. The Hujikoma serotype has been rarely reported. These serotypes have been further characterized into two well established biotypes called El Tor and Classical based on susceptibility to bacteriophages and some biochemical properties (3).

The most significant event in the epidemiology of cholera occurred throughout late 1992, when a new epidemic serogroup of *V. cholerae*, nominated O139, appeared in the coastal regions of India and Bangladesh and spread to neighboring countries, probably, lead to initiating of a eighth pandemic of cholera (4, 5). The serogroups O1 and O139, as mentioned above, contained strains that possess epidemic and pandemic potential (2, 6).

Until 1961 most epidemics had been caused by the Classical biotype. However, with the passing of time the Classical biotype went missing and the El Tor appeared as the major biotype that caused cholera in humans (7). It was concluded that passing from sixth to seventh cholera pandemic resulted in changing from *V. cholerae* O1 Classical to O1 El Tor biotype (8). Several evidences suggest that O139 is closely related to and is derived from the El Tor biotype of *V. cholerae* O1 by the replacement of genes encoding the O139 antigen and acquisition of the capability to produce a capsule (9-13).

Within this time, the El Tor biotype was the agent of most outbreaks; however, the Classical biotype as yet was

responsible for the isolated cases until 1992. These cases involved a wide outbreaks in West Pakistan in 1968 and the emergence of the Classical biotype in Bangladesh in 1979, with a constant presence until 1993 (14). However, since 2001, some clinical isolates emerged that possessed El Tor biotype background but revealed some Classical biotype characters (10, 15-20).

As a result, the current global pandemic of cholera is attributed to a change between seventh pandemic strains and emergence of *V. cholerae* O139, *V. cholerae* O1 El Tor hybrid, and *V. cholerae* O1 El Tor with different cholera toxin subunit B (8).

Classical and El Tor are distinguished primarily based on several phenotypic properties such as susceptibility to polymyxin B, chicken cell agglutination (CCA), haemolysis of sheep erythrocytes, Voges-Proskauer (VP) test and phage susceptibilities (2, 9).

The *V. cholerae* biotypes are not only different in phenotype but also human infections caused by them are different clinically. Infection with classical *V. cholerae* O1 is more frequently severe than El Tor, suggesting that the genetic and phenotypic differences between the two biotypes may also be reflected in their pathogenic potential (21).

2. Context

2.1. Genetic determinants in pathogenic *V. cholerae*

V. cholerae, similar to other bacteria is supposed to have been alive extended before their human host. The pathogenic clones therefore, have evolved from the aquatic environments and obtained the potency to colonize the human intestine by the acquisition of genetic determinants, then a few strains showed pathogenic properties (22). Two principle properties of *V. cholerae* that resulted in assessing as the public health significance consist of the acquisition of O1 or O139 antigens, that acts as an epidemic potential indicator and Cholera Toxin (CT) production which is responsible for the severe diarrhea (2). However genetic analysis have shown that in addition to CT gene, all the toxigenic

V. cholerae strains carry the gene encoding toxin-coregulated pilus (TCP) and *toxR* gene which regulates the expression of CT and TCP proteins (23). All the virulence genes in *V. cholerae* do not act individually but they are part of larger genetic structures (24).

Genetic determinants in pathogenic *V. cholerae* consist of CTX prophage (cholera enterotoxin), TCP island or Vibrio Pathogenicity Island (VPI-1, 2), Vibrio Seventh Pandemic Island (VSP-1 and VSP-2), Integrin Island and RTX (repeats in toxin) toxin gene cluster (25). These determinants vary among different *V. cholerae* serogroups and biotypes.

In the both biotypes, cholera enterotoxin (CT) and the toxin-coregulated pilus (TCP) as colonization factor are the significant virulence factors that are necessary for the infection (26, 27).

2.2. CTX ϕ in *V. cholerae* biotypes

The CTX genetic element is linked to *ctxAB* operon that encodes the A and B subunits of CT. The studies have revealed that the CTX genetic determinant relates to the genome of a lysogenic filamentous bacteriophage called CTX ϕ . The dissemination of this bacteriophage may be associated with the derivation of toxigenic *V. cholerae* strains from nontoxigenic progenitors (28). In El Tor strains of *V. cholerae*, numerous copies of CTX prophage are arranged randomly but the number and arrangement of the CTX elements and their associated repetitive sequences can be different (7, 29). The DNA of CTX ϕ is usually integrated at either one locus on chromosome I or two loci on both chromosomes within the *V. cholera* genome of El Tor and Classical biotypes, respectively (8, 30, 31).

The CTX element is composed of two main regions termed Core and RS sequence. The core is the principle part that encodes different virulence factors such as CT, zonula occludens toxin (Zot), accessory cholera enterotoxin (Ace), core encoded pilin (Cep) and an open reading frame of unknown function (OrfU). The core region is flanked by one or further copies of a repetitive sequence termed as RS1 (32). Divergence between repetitive sequences has been proven by different analysis and revealed that two almost identical sequences are present determined as RS1 (2.7 kb) and RS2 (2.4 kb), that are generically referred to as the RS sequence (32).

Three approximately identical open reading frames (ORFs) located on RS sequences that in RS2 were defined as *rstR*, *rstA* and *rstB*. An additional ORF existed in RS1 and designated *rstC* (33). The *rstR* and flanking sequences are biotype specific in El Tor (*rstR*^{ET}) and Classical (*rstR*^{class}) strains (34).

It was determined that only in toxigenic *V. cholerae* O1 El Tor and O139 strains, cholera toxin prophage region (CTX ϕ) is often flanked by RS1 element containing *rstC* gene (figure 1). The RS1 sequence which is closely linked to CTX ϕ were not detected in Classical *V. cholera* and often dispersed with CTX prophages in El Tor strains, then the CTX prophage arrangements in Classical strains will not produce extra chromosomal CTX DNA element and virions (35).

A toxin-linked cryptic (TLC) element and RTX toxin (*rtxA*) with its activator (*rtxC*) and transporter (*rtxBD*) genes, are located at the upstream and downstream of the CTX genetic element,

respectively (36). The product of RTX gene cluster in El Tor *V. cholerae* have a cytotoxic activity against HEp-2 cells in vitro. RtxA toxin resembles other RTX toxin family and contains a GD-rich repeated motif in its structure. RtxC, an activator, and RtxB -RtxD, ABC transporter system, are necessary for RtxA activity. In *V. cholerae* strains of the Classical biotype, as a result of a deletion in gene cluster, eliminates *rtxC* and cytotoxic activity. Other strains, that the responsible of the current cholera pandemic, possess a functional gene cluster and demonstrate cytotoxic activity (36). Cholera toxin, the major virulence factor of *V. cholerae*, is consisted of two functional units, an enzymatic subunit A, (27 kDa) and receptor-binding subunit B composed of five identical 11.6 kDa peptides (37). Although the sequences of the *ctxA* gene encoding cholera toxin A subunit is identical between Classical and El Tor strains, however, the sequence of *ctxB*, the gene encoding the B subunit of CT is different in two nucleotides at positions 115 and 203, among the El Tor and Classical biotypes that result in differences in two amino acids (cytosine in the Classical and thymine in El Tor biotype) (17). The El Tor variant that has emerged recently, is a *V. cholerae* O1 that shows the typical El Tor biotype properties but, produces cholera toxin of the Classical biotype (6, 9, 17, 38). In Bangladesh, The seventh pandemic prototype with *ctxB* sequence of El Tor strains have been completely replaced by El Tor variant and has disseminated in other countries in Asia and Africa (38-41). Nair and et al., 2006 reported the isolation of the El Tor variant in Bangladesh (38), subsequently, this variant strains have been isolated from several countries and regions in Asia and Africa (9, 39, 42, 43). Recently published reports represent that some of the clinically isolated El Tor variants produce higher levels of cholera toxin than classical biotype strains (44).

A retrospective study of *V. cholerae* O1 strains over a period of more than a decade established that the hybrid CTX prophage with El Tor *rstR* and Classical *ctxB* completely replaced El Tor type since 1995 in Kolkata, India and other areas (41).

2.3. Vibrio Pathogenicity Island in *V. cholerae* biotypes

TCP, a rigid pilus colonization factor, is a critical component of the infection strategy and colonization of *V. cholerae* in the brush borders of the small intestine and is under the same genetic control as CT (27). The Vibrio pathogenicity island (VPI) is one of the primary genetic elements which is necessary for the emergence of epidemic *V. cholerae*. It includes several gene clusters, involving the *tcp* gene cluster that produces the type IV pilus known as TCP that is a major colonization factor (23, 27) and functions as the CTX ϕ receptor (31). The VPI seems to be encoded by filamentous phage and can also form a replicative plasmid (45, 46). The VPI also contains *tcpP*, *tcpH* genes which encode proteins that regulate virulence, (Figure 2) (47-51). It was indicated that VPI has the similar specific insertion site in chromosome of both Classical and El Tor strains (26). The VPI of El Tor biotype is 41,272 bp and encodes 29 predicted proteins, whereas in the Classic biotype it is 41,290 bp (26). The TCP is a polymer of repeating subunits of the major pilin protein TcpA that is found within the Vibrio pathogenicity island (52).

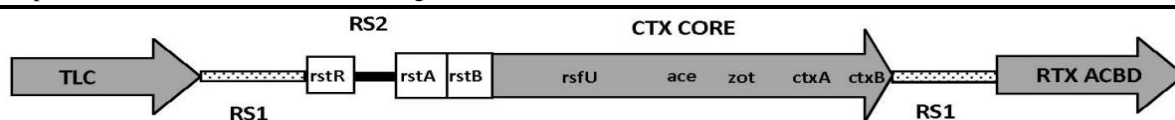


Figure 1. Schematic representation of the CTX genetic element and the flanking regions in strain N16961(19, 33).

In the central segment of VPI, intragenic spaces of the *tcpI-tcpP* and *tcpH-tcpA* have high levels of variation, however all the intergenic regions in this part have higher levels than the left and right segments in Classical and El Tor biotypes (26).

The sequence of *tcpA* from El Tor strain N16961 is same to that of O139 strain MO3 (53) but shows significant deflection from the Classical biotype gene, particularly in the segment encoding the C-terminal region of the pilin, where contained epitopes that recognized by the protective monoclonal antibodies (54-56). Although 75% nucleotide similarity have been observed in the major pilin protein TcpA known to be different significantly among the El Tor and Classical biotypes (53, 57). The variation in TcpA mainly at its C terminus provides the observation of biotype specific differences in the antigenic epitopes and antibody protection (56). This specificity locates around the disulfide loop between the amino acid 120 and 186 where the majority of changes influencing the distribution of charged amino acid are localized (Figure 3).

It is reported that the *tcp* cluster of Classical and El Tor are highly similar (98% identity). Considerable variation have been detected only within the *tcpI-tcpP* (89% identity) and *tcpH-tcpA* (87% identity) intragenic regions and in the C-terminus coding domain of *tcpA* (77% identity) (58).

The VPI-2 with size of 57 ± 3 kb, displays all the features of a pathogenicity island and is present in pathogenic *V. cholerae* while non-pathogenic isolates do not harbor this region. The VPI-2 contains several gene clusters such as a restriction/modification system like *hsdR* and *hsdM* and genes are necessary for the usage of amino sugars such as nan-nag region.

It is determined that toxigenic *V. cholerae* O1 serogroup El Tor or Classical biotypes carried VPI-2, whereas non-toxicogenic isolates lacked this island (59).

2.4. MSHA in *V. cholerae* biotypes

One of the main features that distinguishes El Tor biotypes from the Classical is the expression of a cell-associated mannose-sensitive Hemagglutinin (MSHA) (60). This hemagglutinin has been associated with the expression of a pilus and is proposed to be a colonization factor for El Tor strains (60).

2.5. HlyA in *V. cholerae* biotypes

Comparison of nucleotide sequences of *hlyA* gene, that encodes haemolysin, between Classical and El Tor strains revealed the deletion of 11 bp sequence in Classical strains results in producing a truncated protein (27kDa) without haemolytic functionality, while in El Tor strains the HlyA is intact 82kDa with biological activity (61).

2.6. VSP in *V. cholerae* biotypes

Two genomic regions were assigned to the *V. cholerae* isolates related to seventh pandemic including island-I (VSP-I) and VSP-II. These regions were special to seventh pandemic El Tor isolates (62). The VSP-I and VSP-II showed several properties of pathogenicity islands. The VSP-I covers 16 kb region containing 11 ORFs, with a 40% GC content in contrast to 47% for the entire genome (62). The VSP-II region with the size of 7.5kb encompasses eight ORFs, that encode a regulator of transcription and a ribonuclease H1 (62).

These structures encode genes with hypothetical functions that are supposed to be required for evolutionary fitness and epidemic spread of the seventh pandemic clone were found particularly among El Tor biotype isolates not in the Classical (25, 62).

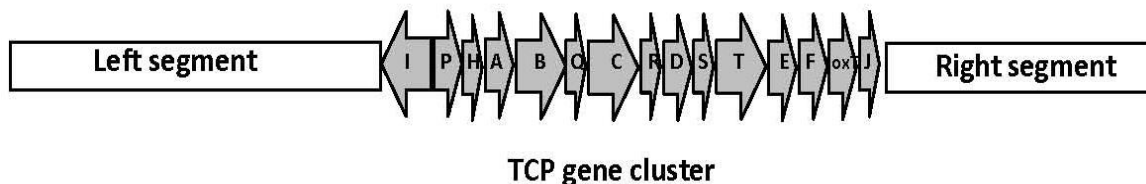


Figure 2. Schematic structure of VPI (39.5kb) in *V. cholerae* El Tor strain N16961. (47).

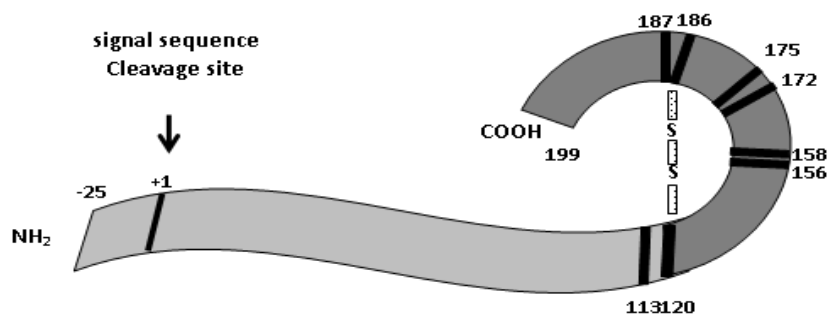


Figure 3. Differences in charged aa among El Tor and Classical TcpA. The different aa are shown (black boxes) and contained : Asp¹¹³→Gly; Ala¹⁵⁶→Asp; Glu¹⁵⁸→Ala; Lys¹⁷²→Ala; Asp¹⁷⁵→Asn; Lys¹⁸⁷→Thr, for Classical and El Tor strains, respectively. The disulfide bond is formed between the Cys residues at aa 120 and 186 of TcpA(18).

2.7. Expression of virulence genes in biotypes

The production of major virulence factors is controlled by a complex cascade of transcriptional regulators (63). This cascade is positively controlled by the regulatory proteins ToxR, ToxS and TcpP, TcpH, that in turn control the expression of regulator ToxT (51, 64-66). The expression of TcpP and TcpH is also regulated by two other regulatory proteins, AphA and AphB (67). The conditions for expression of *ctx*, *tcp* and *toxT* genes in El Tor biotype contain: complex growth medium, the incubation of cultures at 37°C without motion for 4 h, followed by overnight incubation at 37°C with shaking. In contrast, environmental signals, including pH, temperature, osmolarity, and amino acids regulate the gene expression in Classical biotype (68). It is shown that the sequence differences in promoters of TcpP, H between the Classical and El Tor biotypes affect the interaction of AphB with them and result in variation of TcpP and TcpH production. (69). The timing of the transcription of *tcpP*, H is also different between the Classical and El Tor biotypes (70). It has also been determined that a total of 524 genes (13.5% of the genome) expressed differentially between two biotypes (63). In the El Tor biotype, the expression of proteins which required for biofilm formation, chemotaxis, and transport of amino acids, peptides and iron is higher. Differences in the expression of these genes may cause to the increased survival ability of the El Tor biotype in environmental reservoirs. In contrast, the expression of virulence factors was greater in the Classical than El Tor biotype. In addition, the expression of *vieSAB* genes, as regulators of *ctxA* transcription, are at a five fold higher level in the Classical biotype (63). A large portion (20.8%) of the genes that are differentially expressed in the Classical against the El Tor biotype are regulated by *VieA*, that were originally identified as the regulators of *ctxA* transcription in the Classical biotype(63).

2.8. Biotyping of *V. cholerae* O1

As mentioned above, current tests for distinguishing biotype are not sufficient to complete the identification and supplementary genotypic and phenotypic tests should be performed to characterize the variants. Raychoudhuri and colleagues; 2008 proposed a modification of the existing biotyping scheme with several molecular marker genes (Table 1) (9). We suggest that biotyping will play an important role in understanding the epidemiology and infection severity of the emerging strains of *V. cholerae* O1 in future.

Table 1. New procedure for biotyping of *V. cholerae* O1(9)

Feature	Biotype			
	Classical	El Tor	El Tor variant	Hybrid
Voges-proskauer test	-	+	+	+/-
Susceptibility to polymyxin B (50U)	+	-	-	+/-
Agglutination of Chicken cell	-	+	+	+/-
Lysis by classical IV phage	+	-	-	+/-
Lysis by El Tor phage V	-	+	+	+/-
Epitype of CT	CT1	CT2	CT1	CT1/CT2
Genotype of <i>ctxB</i>	classical	El Tor	classical	El Tor / classical
<i>rtxC</i>	-	+	+	+/-
<i>tlc</i>	+	+	+	+/-
Allele of <i>tcpA</i>	classical	El Tor	El Tor	Variable
RS element	RS2	RS1, RS2	RS1, RS2/RS2	RS1, RS2/RS2

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors Contribution

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