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

The most notable event in the epidemiology of cholera occurred during late 1992, when a new serogroup of epidemic *V. cholerae*, nominated O139, emerged in the coastal areas of India and Bangladesh and spread to neighboring countries, causing the beginning of a possible eighth pandemic of cholera (4, 5). Strains that have epidemic and pandemic potential, as mentioned above, belong to the serogroups O1 and O139 (2, 6).

Before 1961 most epidemics had been caused by the classical biotype. However, with the passage of time the classical biotype went missing from the scenario and the El Tor emerged as the major biotype that caused cholera in humans (7). It was concluded that transition from sixth to seventh cholera pandemic resulted in a change from *V. cholerae* O1 classical to O1 El Tor biotype (8). Several evidences suggest that O139 is closely related to and is deriveed from the El Tor biotype of *V. cholerae* O1 by the replacement of genes encoding the O139 antigen and acquisition of the ability to produce a capsule (9-13).

During this time, the El Tor biotype was responsible for most of the outbreaks; however, the classical biotype still was responsible for the isolated cases until 1992. These cases included a large outbreak in West Pakistan in 1968 and the appearance of the classical biotype in Bangladesh in 1979, with a continuous presence until the end of 1992 (14). However, since 2001, series of reports have been published revealing clinical isolates, from as early as 1990s, that are of El Tor biotype background but they possess some classical biotype traits (10, 15-20).

As a result, 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 (8).

Classical and El Tor are differentiated primarily based on a number of phenotypic properties such as susceptibility to polymyxin B, chicken cell (erythrocytes) 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 assumed to have existed long before their human host. The pathogenic clones therefore, have evolved from the aquatic environments and attained the ability to colonize the human intestine by the acquisition of genetic determinants, then a few strains showed pathogenic characters (22). Two principle properties of *V. cholerae* are taken into account in assessing the public health significance. These properties consist of possession of the O1 or O139 antigen, that acts as a marker of epidemic potential and production of Cholera Toxin (CT) which is responsible for the

severe diarrhea (2). However molecular analysis have revealed that in addition to CT gene, all the toxigenic *V. cholerae* strains carry the gene encoding a factor known as toxin-coregulated pilus (TCP) and *tox*R 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 elements (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.

The major virulence factors, cholera enterotoxin (CT) and the colonization factor toxin-coregulated pilus (TCP), are required for the infection by both biotypes (26, 27).

2.2. CTX φ in V. cholerae biotypes

The CTX genetic element is related to *ctx*AB operon that encodes the A and B subunits of CT. The studies have revealed that the CTX genetic determinant corresponds to the genome of a lysogenic filamentous bacteriophage called CTXΦ. The dissemination of this bacteriophage may be associated with the derivation of toxigenic *V. cholerae* strains from nontoxigenic progenitors (28). Multiple copies of CTX prophage are arranged randomly in El Tor strains of *V. cholerae*, however, the number and arrangement of the CTX elements and their associated repetitive sequences can vary (7, 29). CTXΦ-DNA is generally found integrated at either one on chromosome I (El Tor biotype) or two (classical biotype) loci on both chromosomes within the *V. cholerae* genome (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). This core region is flanked by one or more copies of a repetitive sequence termed as RS1 (32). Divergence between repetitive sequences has been proven by different analysis and revealed that two nearly identical sequences are present designated as RS1 (2.7 kb) and RS2 (2.4 kb), that are generically referred to as the RS sequence (32).

The RS sequences contain three nearly identical open reading frames (ORFs) that in RS2 were designated as rstR, rstA and rstB. RS1 contains an additional ORF designated rstC (33). The rstR and flanking sequences from El Tor strains ($rstR^{ET}$) and the corresponding regions of the classical prophage ($rstR^{class}$) are biotype specific (34).

It was determined that only in toxigenic *V. cholerae* O1 El Tor and O139 strains, cholera toxin prophage region (CTXΦ) is often flanked by RS1 element containing *rst*C gene (figure 1). The RS1 sequence which is closely related to CTXΦ and is often interspersed with CTX prophages in El Tor strains were not detected in classical *V. cholerae*. The CTX prophage arrangements in classical strains will not yield extra chromosomal CTX DNA and thus will not yield virions (35).

At the upstream of the CTX genetic element is a toxin-linked cryptic (TLC) element, and on the other side of CTX is a region encoding an RTX toxin (*rtx*A), together with its activator (*rtx*C) and transporter (*rtx*BD) genes (36). RTX gene cluster in El Tor *V. cholerae* encodes a cytotoxic activity for HEp-2 cells in vitro. The toxin, RtxA, resembles members of the RTX toxin family as it contains a GD-rich repeated motif. Like other RTX toxins, its activity depends on an activator, RtxC, and an associated ABC transporter system, RtxB and RtxD. In *V. cholerae* strains of the classical biotype, a deletion within the gene cluster removes rtxC and eliminates cytotoxic activity. Other strains, including those of the current cholera pandemic, contain a functional gene cluster and display cytotoxic activity (36). Cholera toxin, the principal virulence factor of *V. cholerae*, is composed of two functional units, an enzymatic A subunit of 27 kDa and an intestinal receptor-binding B subunit consisting of five identical 11.6 kDa peptides (37). Although the sequences of the *ctx*A gene encoding cholera toxin A subunit from classical and El Tor strains are identical, the sequence of *ctxB*, the gene encoding the B subunit of the El Tor biotype varies from that of the classical biotype by two nucleotides at positions 115 and 203, which results in differences in two amino acids (cytosine in the classical and thymine in El Tor biotype) (17). Recently, a new variant called 'El Tor variant' has emerged, where *V. cholerae* O1 shows the typical El Tor biotype however, it produces cholera toxin of the classical type (6, 9, 17, 38). The prototype seventh pandemic strains of the El Tor biotype with *ctx*B sequence of El Tor strains have been completely replaced by El Tor variant in Bangladesh and has spread in other countries in Asia and Africa (38-41). Subsequent to the isolation of the El Tor variant in Bangladesh which is reported by Nair and colleagues (38), El Tor variant strains have been isolated from several countries and areas in Asia and Africa (9, 39, 42, 43). Recently published results indicate that some of the clinically isolated El Tor variants produce different levels of cholera toxin that produce higher levels than classical biotype strains (44).

A retrospective analysis of *V. cholerae* O1 strains over a period of more than a decade established that the hybrid CTX prophage with El Tor *rst*R and classical *ctx*B replaced El Tor type completely since 1995 in Kolkata, India and other areas (41).

2.3. Vibrio Pathogenicity Island in V. cholerae biotypes

The crucial component of the infection strategy is the colonization of the brush borders in the small intestine that is assumed to be mediated by a rigid pilus colonization factor, TCP, since it is under the same genetic control as CT (27).The Vibrio pathogenicity island (VPI) is one of the initial genetic factors required for the emergence of epidemic *V. cholerae*. It contains several gene clusters, including the *tcp* gene cluster that encodes the type IV pilus structure known as TCP that is an essential colonization factor (23, 27) and acts as the CTXΦ receptor (31). The VPI appears to be phage-encoded and can also form a plasmid replicative form (45, 46). The VPI also contains genes (*tcp*P, *tcp*H) that encode proteins that regulate virulence, (Figure 2) (47-51). It was indicated that VPI has the same specific chromosomal insertion site in 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).

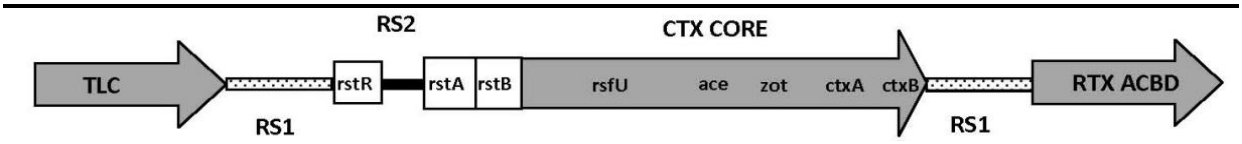

Figure 1. Schematic arrangement of the structure of the CTX genetic element and the flanking regions of strain N16961(19, 33).

In the central segment of VPI, the *tcp*I-*tcp*P and *tcp*H-*tcp*A intragenic spaces have particularly high levels of variation, however all the intergenic regions in this segment have higher levels than do intergenic regions in the left and right segments in classical and El tor biotypes (26).

The *tcp*A sequence from El Tor strain N16961 is identical to that of O139 strain MO3 (53) but shows significant deflection from the classical biotype gene, especially in the portion encoding the C-terminal region of the pilin, where epitopes are recognized by the protective monoclonal antib-odies map (54-56). Although 75% similarity at the nucleotide level have been observed in the major pilin protein TcpA known to be different significantly between the El Tor and classical biotypes (53, 57). The variation in TcpA especially at its C terminus enables the observed biotype spec-ific differrences in the antigenic epitopes and in protection (56). This specificity resides around the disulfide loop between the amino acid 120 and 186 where the majority of changes affecting the distribution of charged amino acid are localized (Figure 3).

It is reported that the *tcp* regions of classical and El Tor are highly conserved (98% identity). Significant variation have been observed only within the *tcp*I-*tcp*P (89% identity) and *tcp*H-*tcp*A (87% identity) intergenic regions and in the C-terminus coding domain of *tcp*A (77% identity)(58).

The VPI-2 with size of 57±3kb, exhibits all the characteristics of a pathogenicity island and is present in pathogenic *V. cholerae* while non-pathogenic isolates do not harbor this region. The VPI-2 encodes several gene clusters such as a restriction modification system like *hsd*R and *hsd*M and genes required for the utilization of amino sugars such as nan-nag region.

It is determined that toxigenic *V. cholerae* O1 serogroup El Tor or Classical biotypes contained VPI-2, whereas non-toxi-genic 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 in haemolysin encoding *hly*A gene from classical and El Tor strains revealed the deletion of11 bp sequence in classical strains that results a truncated protein of 27kDa without haemolytic functionality in classical strains, 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 region were unique to seventh pandemic El Tor isolates (62). The VSP-I and VSP-II showed several characteristics of pathogenicity islands. The VSP-I spans 16 kb region covering 11 ORFs, with a GC content of 40% 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 transcriptional regulator and a ribonuclease H1 (62).

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

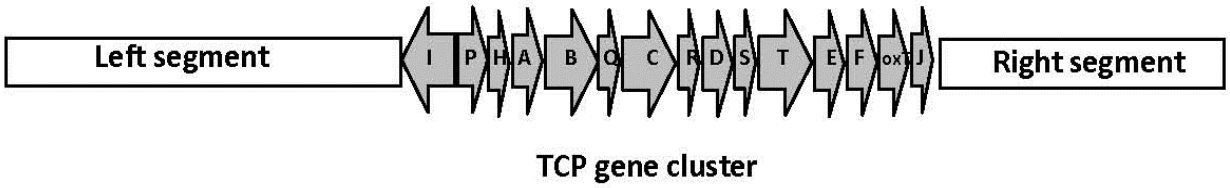

Figure 2. Schematic presentation of VPI (39.5kb) in *V. cholerae* El Tor strain N16961. The ORFs and gene clusters are shown (47).

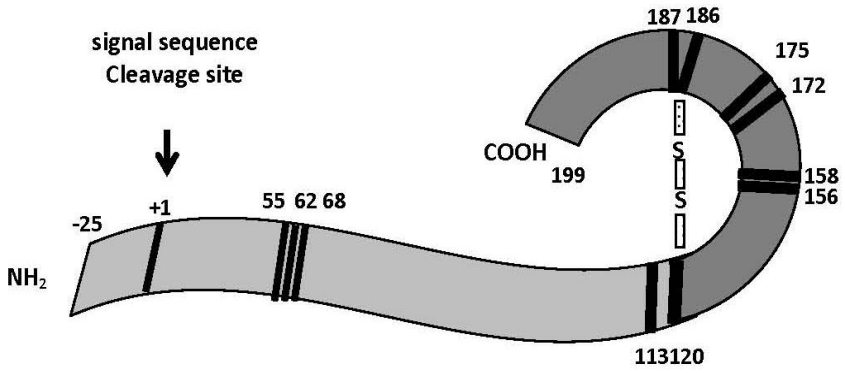

Figure 3. Differences in charged aa within TcpA from El Tor and classical strains. The variant aa are shown (shaded boxes) and correspond to:Asp^{62}→Asn; Lys^{68}→Gln; Asp^{113}→Gly; Ala^{138}→Glu; Ala^{156}→Asp; Glu^{158}→Ala; Lys^{172}→Ala; Asp^{175}→Asn; Lys^{187}→Thr, for classical and El Torstrains, respectively. The disulfide bridge is formed between the Cys residues (black bars) at aa 120 and 186 of mature TcpA(18).

2.7. Expression of virulence genes in biotypes

The production of the virulence factors CT and TCP is controlled by a complex transcriptional regulatory cascade (63), which 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 under the control of two other regulatory proteins, AphA and AphB (67). Expression conditions of *ctx*, *tcp* and *tox*T genes in El Tor biotype contain complex growth medium, the incubation of cultures at 37°C under motionless conditions for 4 h, followed by overnight incubation with shaking at 37°C. In contrast, the classical biotype is regulated by the environmental signals, including pH, temperature, osmolarity, and amino acids (68). It is shown that the variation in TcpP and TcpH production is due to the differences in DNA sequence between the classical and El Tor TcpP, H promoters and the resulting interaction of AphB with the *tcp*P, H promoter (69). It has also been shown that the timing of the transcription of *tcp*P, H is different between the classical and El Tor biotypes (70). It is also determined that a total of 524 genes (13.5% of the genome) were found to be differentially expressed in the two biotypes (63). The expression of genes encoding proteins required for biofilm formation, chemotaxis, and transport of amino acids, peptides, and iron is higher in the El Tor biotype. Differences in the expression of these genes may contribute to the enhanced survival capacity of the El Tor biotype in environmental reservoirs. In contrast, the expression of genes encoding virulence factors was greater in the classical than El Tor biotype. In addition, the *vie*SAB genes, that were originally identified as regulators of *ctx*A transcription, were expressed at a five fold higher level in the classical biotype (63). A large fraction (20.8%) of the genes that are differentially expressed in the classical versus the El Tor biotype are controlled by *Vie*A, that were originally identified as the regulators of *ctx*A 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 additional genotypic and phenotypic tests should be performed to characterize the variants. Raychoudhuri and colleagues proposed a modification of the existing biotyping scheme with several molecular marker genes by, A 2008 (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 scheme 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 ctxB	classical	El Tor	classical	El Tor / classical
rtxC	-	+	+	+/-
tlc	+	+	+	+/-
Allele of tcpA	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

All authors contribute in writing different parts of this manuscript.

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