Borneo Journal of Medical Sciences (2016) volume 10, issue 1, pp: 36-46

Atypical El Tor: new *Vibrio cholerae* O1 biotype causing epidemic cholera

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(Received: May 26, 2015; Accepted: June 15, 2015)

ABSTRACT

Toxigenic strains of *Vibrio cholerae* serogroup O1 and O139 are causative agents of deadly diarrheal disease named cholera. *Vibrio cholerae* O1 is traditionally divided into two biotypes, classical and El Tor, which are different in phenotypic as well as genotypic traits. Since 1961, classical strains of *Vibrio cholerae* O1 serogroup has become obsolete as the cause of epidemic and pandemic cholera and replaced by El Tor strains. Since 2002, atypical O1 El Tor strains possessing the traits of classical strains have been increasingly recognized as the cause of cholera in many countries across the world. This article focuses on the genetic traits of O1 classical and El Tor strains. Furthermore, an overview of emergence of atypical O1 El Tor strains and their genetic traits is presented.

Keywords: Cholera, *Vibrio cholerae*, biotype, atypical O1 El Tor

INTRODUCTION

Cholera is a life-threatening acute diarrhoeal disease characterized by watery diarrhea and capable of causing death within hours. According to World Health Organization, there are estimated 3-5 million cases of cholera every year resulting in 100000-120000 deaths. *Vibrio cholerae* (*V. cholerae*) is the Gram-negative comma-shaped motile bacterium responsible for this deadly diarrheal disease[1]. Based on somatic O antigens, *V. cholerae* can be divided into more than 200 serogroups. However, only the toxigenic strains of serogroups O1 and O139 are capable of producing cholera toxin and causing cholera[1]. *V. cholerae* O1 strains can be divided into two biotypes - classical and El Tor, and three serotypes - Ogawa, Inaba and Hikojima[1].

Cholera toxin (CT) is the primary virulence factor responsible for induction of watery diarrhoea, which is the main manifestation of this deadly disease. CT is a A-B type toxin composed of one A subunit and five identical B subunits[2]. The B subunit is responsible for binding to GM1 ganglioside receptor on intestinal cells and translocation of CT into the cell

where A subunit exerts its action leading to hypersecretion of water and electrolyte and watery diarrhea[2]. Toxin coregulated pilus (TCP) is a major colonization factor proven to be important in cholera pathogenesis in animal models as well as human volunteers[3]. In addition, TCP functions as the receptor for CTXϕ phage which harbor the *ctxAB* gene encoding CT and therefore important in the evolution of the pathogenic *V. cholerae*[3].

Epidemiologically, cholera is characterized by its tendency to cause explosive outbreaks and its potential to cause pandemics. Of the seven major pandemics of cholera that have occured since the begining of 19th century, *V. cholerae* O1 serogroup classical biotype was thought to be responsible for the first six pandemics originating in Indian subcontinent[1]. El Tor strains caused the seventh cholera pandemic beginning from Sulawesi Island, Indonesia in 1961[1]. During this pandemic, El Tor strains become well established around the world while its predecessor, classical biotype become extinct. The year 1992 has seen the emergence of new toxigenic serogroups that caused cholera outbreaks in India and Banglasesh and spread across Asia[4]. This pathogenic O139 strains are thought to arise from O1 El Tor strains by acquiring changes in genes for synthesis of somatic O antigens and even called El Tor in different robe. However O1 El Tor strains later replace the O139 strains and regained their dominance in the region[4].

As mentioned earlier, biotyping is the epidemiologically important classification method for *V. cholerae* O1 strains and *V. cholerae* O1 strains can be divided into classical and El Tor biotypes. The strain of *V. cholerae* serogroup O1 referred to as biotype El Tor today was first isolated in 1905 from six pilgrims in a quarantine camp located in the city of El Tor, Sinai Pennisula, Egypt and initially considered to be a separate species, *V. Eltor*[5]. However, based on similarities with *V. cholerae*, it was later reclassified as the El Tor biotype of *V. cholerae* O1 strains[5]. El Tor strains has been associated with sporadic cholera and localized epidemics before emerging as the agent responsible for the seventh pandemic in 1961.

PHENOTYPIC TRAITS OF *V. CHOLERAE* O1 BIOTYPES

Classical and El Tor biotypes can be identified based on a battery of phenotypic tests[5]. Conventionally, El Tor strains were differentiated from classical strains by hemolysis of sheep erythrocytes, agglutination of chicken erythrocytes, positive Voges-Proskauer test, resistance to polymyxin B, resistance to classical bacteriophage IV and susceptibility to El Tor-specific bacteriophage V[5].

GENOTYPIC DIFFERENCES BETWEEN CLASSICAL AND EL TOR BIOTYPES

Polymorphism in ctxB gene

Genetic analyses have revealed many genotypic differences between two biotypes. One of the most important differences between classical and El Tor strains is in the sequence of *ctxB*gene

encoding subunit B of CT. Three genotypes of *ctxB* gene has been described based on the presence or absence of single nucleotide polymorphisms (SNP) (Table 1)[6].

Table 1: Genotypes of *ctxB* gene in *V. cholerae* O1 strains

ctxB genotype	Nucleotide positions (Amino acid positions)				References
	58(20)	115(39)	138(46)	203(68)	
1	C(His)	C(His)	T(Phe)	T(Thr)	6
2	C(His)	C(His)	G(Leu)	T(Thr)	6
3	C(His)	T(Tyr)	T(Phe)	T(Ile)	6
7	A(Asn)	C(His)	T(Phe)	T(Tr)	29, 32, 34

Genotype 1 is associated with classical strains. Majority of El Tor strains possess genotype 3 except for US Gulf Coast El Tor strains haboring genotype 1 *ctxB* gene and Australian El Tor strains harboring genotype 2 *ctxB* gene. Apparently, *ctxB* gene is conserved in classical strains whereas it is heterogenous in El Tor strains. Furthermore, based on the variation in B subunit of CT, CT can be divided into two immunologically related but not identical subtypes – CT1 produced by classical strains and CT2 produced by El Tor strains[7].

Classical and El Tor alleles of tcpA

The genes for TCP biosynthesis is located on a DNA element termed as *Vibrio* pathogenicity island (VPI) which is frequently found in pathogenic strains. One of these genes, *tcpA*, codes for major pilin subunit of TCP and has sequence variations between classical and El Tor biotype which was used to develop a PCR assay for distinguishing two biotypes[8]. The classical and El Tor variants of *tcpA* are 83% identical at amino acid level (Figure 1).

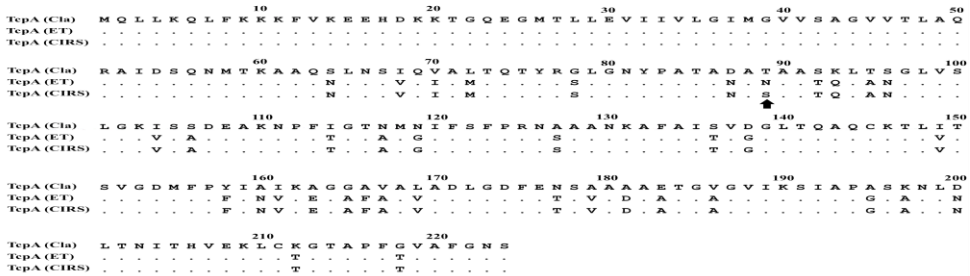

Figure 1: Alignment of amino acid sequence of TcpA, major pilin subunit of toxin-coregulated pilus, encoded by classical (Cla), El Tor (ET) and CIRS alleles of *tcpA* gene. Identical residues are indicated by dot. Arrow indicates the position of amino acid replacement resulting from SNP in CIRS allele of *tcpA* gene.

Variations in CTXϕ prophages

The *ctxA* and *ctxB* gene encoding subunit A and subunit B of cholera toxin is part of the integrated genome of filamentous lysogenic CTXϕ prophage[9]. The CTXϕ prophage genome has two regions: 4.6 kb core region and 2.7 kb regulatory RS2 element[10]. Core region contains *ctxAB* together with many other genes encoding Psh, core-encoded pilin, accessary cholera toxin, and zonula occludens, which are required for phage morphogenesis. RS2 element contains three open reading frames: *rstR* that regulate the phage DNA expression; *rstA* involved in phage DNA replication and *rstB* involved in integration of phage genome[10]. Apart from the biotype-specific difference in *ctxB* gene, CTXϕ phage genome shows significant diversity between two biotypes in *rstR* gene - one of three open reading frames on RS2 element that codes for phage repressor protein[10,11]. Accordingly *rstR* gene has two alleles – classical allele in classical strains and El Tor allele in El Tor strains, which shows 29% identity at amino acid level[11]. In addition to classical and El Tor alleles of *rstR* gene, two other variants has been detected in *V. cholerae* non-O1 strains – Calcutta *rstR* allele in O139 strain from Calcutta and environmental *rstR* allele in environmental non-O1/O139 strains[12,13]. Based on the *rstR* allele they harbor, CTXϕ prophages can be classified accordingly as classical, El Tor, Calcutta or environmental phage.

Classical and El Tor strains also differ in the number of copy, distribution and arrangement of CTXϕ prophage DNA. *V. cholerae*, classical strains as well as El Tor strains, contain two chromosomes of different sizes – a large one referred to as chromosome I and a small one referred to as chromosome II. The classical strain O395 isolated in India in 1964 has the integrated prophage genome at two different loci – one in large chromosome and one in small chromosome[14]. However, El Tor strains contain either one or two copies of phage genome. The reference El Tor strain, N16961, has only one copy of CTXϕ prophage integrated in large chromosome[14]. When El Tor strains have two copies, both are integrated in tandem in one of the two chromosomes. In addition, the two prophages can be of different types. For example, O1 El Tor strains isolated in India just before emergence of pathogenic O139 strains in 1992 have two different CTXϕ prophages – El Tor CTXϕ and Calcutta CTXϕ integrated in tandem in small chromosome[15].

RS1 element of El Tor strains

An attribute of El Tor CTXϕ prophage that is distinct from its classical counterpart is its association with RS1 element[10]. RS1 element is found adjacent to the El Tor CTXϕ prophage. RS1 element is similar but not identical to RS2 element of CTXϕ prophage. RS1 contain an additional gene, *rstC*, which encodes antirepressor protein promoting the expression of CTXϕ

gene. Therefore, *rstC* gene is unique to El Tor strains and used as one of the specific markers of El Tor lineage[10].

El Tor-specific rtxC gene

Another El Tor-specific marker would be *rtxC* gene which is part of the *rtxABCD* gene cluster encoding the cytotoxic activity. The *rtxC* gene is specific to El Tor strains because classical strains have an internal deletion in this gene cluster disrupting the *rtxC* gene[16].

hlyA gene of classical and El Tor strains

One of the genotypic differences between classical and El Tor biotypes is in the structural gene for hemolysin *hlyA*. Sequencing analyses revealed the presence of 11bp deletion in *hlyA* gene of classical biotype,[17,18] which explained lack of production of functional hemolysin with the classical strains and was exploited to develop a 19 bp probe for differentiating the two biotypes[19].

Vibrio seventh pandemic island-1 and 2

Comparative genomic analysis has identified the genes unique to El Tor strains causing seventh pandemic. In 2002, Diziejman *et al*. [20] developed a microarray for comparing gene content of the reference El Tor strain N16961 with that of classical strains as well as other El Tor strains. Using this tool, they discovered two blocks of DNA which were found exclusively in El Tor strains causing seventh cholera pandemic and termed as *Vibrio* seventh pandemic island-1 (VSP-1) and *Vibrio* seventh pandemic island-2 (VSP-2). VSP-1 is 16 kb in size and contain 11 ORFs wheaes VSP-2 is 7.5 kb genomic element composed of 8 ORFs[20]. The lower GC content of VSP-I and VSP-II implies that these genomic regions are acquired by horizontal transfer[20].

Genotyping of *tcpA*, *rstR* and *ctxB* genes, and detection of El Tor specific genes, which include *rtxC*, VSP-1 and 2, are commonly used to differentiate two biotypes.

EMERGENCE OF ATYPICAL VARIANTS OF *V. CHOLERAE* O1

Biotyping *V. cholerae* O1 strains has become more complicated due to the emergence of atypical O1 strains haboring classical allele of *ctxB* gene. These strains have varying combination of phenotypic and genotypic traits of the two biotypes. Some strains having the mixture of classical and El Tor phenotypic and genotypic traits cannot be biotyped and are referred to as hybrid biotype. Strains having phenotypic and genotypic traits of El Tor biotype but classical allele of *ctxB* gene are called atypical El Tor biotype. A consistent finding among these atypical strains is their possession of classical *ctxB* allele. Starting from 2002, these atypical *V. cholerae* O1 strains has been identified as the causative agents of cholera in many different geographical areas.

In 2002, Nair *et al*.[21] carried out the characterization of *V. cholerae* O1 strains isolated from cases of acute diarrhea in Matlab, Bangladesh between 1991 and 1994. These Matlab strains were found to possess mixed phenotypic traits and were classified as hybrid biotype[21]. In addition, genetic analysis revealed that these strains harbored the classical allele of *tcpA* and *ctxB* gene as well as El Tor-specific genetic elements – *rtxC* gene, VSP-1 and VSP-2, supporting the hybrid nomenclature of these strains[22]. However, ribotyping analysis showed that these strains shared ribotypes with previous El Tor strains,[21] suggesting that these strains are more related to El Tor strains despite having some of genotypic and phenotypic characteristics of classical strains and should be referred to as atypical El Tor. The existence of such atypical El Tor strains harboring classical *ctxB* allele in Bangladesh is further supported by CT subtyping analysis which showed that *V. cholerae* O1 strains isolated in Bangladesh before 2001 produced CT of El Tor biotype whereas those isolated thereafter produced CT of classical biotype[23].

In 2004, Ansaruzzaman *et al*. reported the presence of classical type CTXϕ prophage in El Tor strains isolated in cholera treatment center in Beira, Mozambique[24]. These Mozambique El Tor strains are classified as atypical El Tor as they harbor the classical *ctxB* allele despite their otherwise El Tor characteristics - El Tor-specific phenotypic traits, El Tor-specific *rtxC* and El Tor *tcpA* allele[24].

According to the report on Matlab strains in 2002, it appears that atypical El Tor strains first emerged in Bangladesh in 1991[21]. Analysis of historical collection of previously isolated strains and review of old literature have revealed that such strains have existed in places other than Bangladesh long before they appear in Bangladesh in 1991. When Safa *et al.* analysed 41 *V. cholerae* O1 strains isolated from a number of countries in Asia and Africa - Japan, Hong Kong, China, Vietnam, Sri Lanka and Zambia during 13 year period from 1991 to 2004, 30 of these strains were found to be atypical El Tor strains harboring classical allele of *ctxB* gene. It can be implied from this study that atypical El Tor strains were circulating in these countries[25]. Similarly, when a collection of *V. cholerae* O1 strains isolated in Calcutta over 17 years period from 1989 to 2005 were characterized with regard to *ctxB* allele they possesses, it was found that El Tor allele of *ctxB* had been replaced by classical allele since 1990 in Calcutta[26]. Furthermore, classical allele of *ctxB* gene was detected in El Tor strains isolated from US Gulf Coast in late 1970s and early 1980s, indicating that atypical El Tor strains have existed as early as 1970s[6].

Atypical El Tor strains have been identified as the causative agents of cholera outbreaks in Thailand, Vietnam, Nigeria, Angola, Tanzania and Haiti over the last 10 years[27-32]. Almost all of *V. cholerae* strains isolated during these outbreaks were positive for classical *ctxB* gene. In other word, these atypical El Tor strains have replaced the prototype El Tor strain in these countries.

CTXΦ PROPHAGE IN ATYPICAL O1 EL TOR STRAINS

The typical El Tor strains harbor the El Tor CTXϕ prophage which contain El Tor *ctxB* allele and El Tor *rstR* allele; however, the CTXϕ prophage in the atypical O1 El Tor strains, depending on *rstR* allele, can be categorized into two types: classical CTXϕ containing classical *ctxB* and classical *rstR* allele, and hybrid CTXϕ containing classical *ctxB* and El Tor *rstR* allele. For instance, Matlab strains contain classical *ctxB* gene but as for *rstR* gene, these strains contain either of classical and El Tor allele. Therefore it can be implied that CTXϕ prophage in these strains could be of either classical or hybrid type. In contrast, the CTXϕ phage in Mozambique strains positive for classical *rstR* allele is the classical type. The schematic diagram of classical CTXϕ prophages in the Mozambique strain B33[33] is shown in Figure 2.

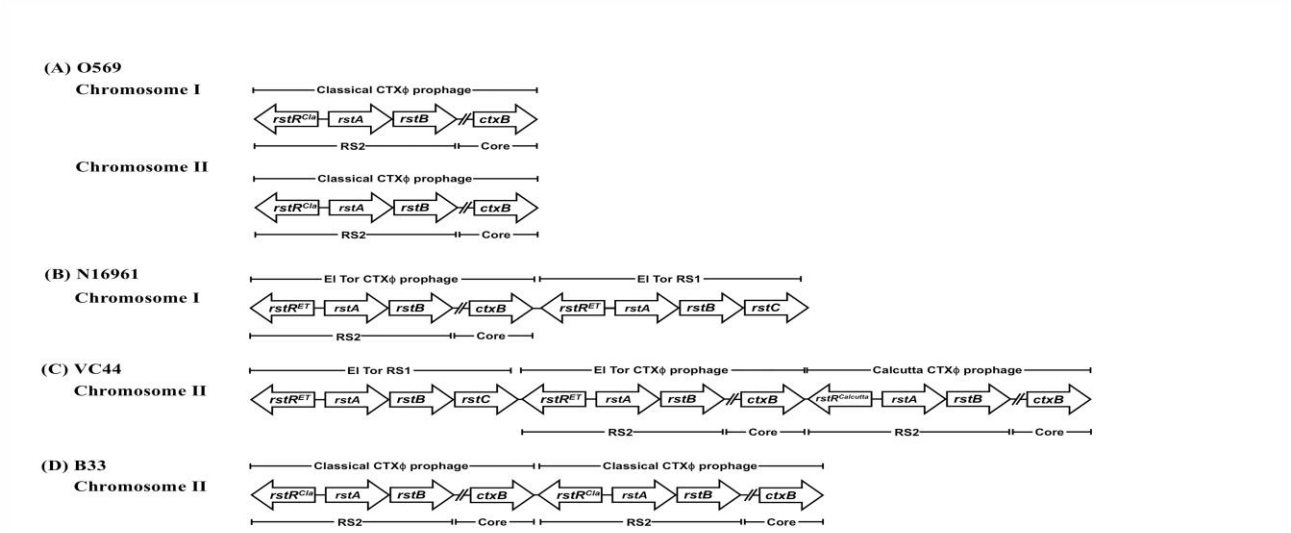

Figure 2: Copy number, distribution and arrangement of CTXϕ prophage(s) in classical strain O569 (A), El Tor strain N16961 (B), El Tor strainVC44 (C), and atypical El Tor strain B33 (D). Arrows indicate the genes in CTXϕ prophage. Direction of arrows indicates the direction of transcription but length of arrows does not represent the length of genes. Genes other than *ctxB* in core regions of CTXϕ phage such as *psh*, *cep*, *ace*, *zot*, are omitted. *V. cholerae* has two chromosomes but the chromosome(s) harboring CTXϕ prophage in each strain is(are) illustrated in the figure. Classical strain O569 has two copies of classical CTXϕ propage, one on each chromoseome. El Tor strain N16961 has only one copy of El Tor CTXϕ prophage in chromosome I. El Tor strain VC44 contains two different CTXϕ prophages – El Tor CTXϕ and Calcutta CTXϕ in chromosome II. Atypical El Tor strain B33 harbors two copies of classical CTXϕ prophages in chromosome II.

ONGOING EVOLUTION OF ATYPICAL EL TOR STRAINS

New genotypic variants of virulence genes were identified in atypical El Tor strains suggesting the presence of ongoing evolution among these atypical strains. A new allele of *tcpA* gene referred to as CIRS allele was first identified in atypical El Tor strains CIRS101 from the acute diarrheal patients in Bangladesh. As indicated in Figure 1, this new allele has a SNP (A→G) at nucleotide position 266 resulting in replacement of asparagine with serine at position 89 but is otherwise identical to El Tor *tcpA* allele[14,23]. In addition to the previously described genotype 1, 2 and 3 of O1 strains, a new genotype of *ctxB* emerged during cholera outbreak caused by atypical El Tor strains in Orissa, Eastern India in 2007, which contained a SNP (C→A) at position 58 compared to classical *ctxB*[34]. The amino acid sequence of CT subunit B encoded by this new allele is similar to that of reference classical strain 569 except for the replacement of histidine at position 20 with asparagine. This new *ctxB* allele was referred to as genotype 7 because genotype 4, 5 and 6 were already assigned to the *ctxB* alleles found in *V. cholerae* O139 strains. Both of these variants, genotype 7 of *ctxB* and CIRS allele of *tcpA* gene, were later detected in atypical El Tor strains causing cholera outbreaks in Haiti and Nigeria in 2010[29,32].

GENETIC CHARACTERIZATION AS ESSENTIAL PART OF BIOTYPING *VIBRIO CHOLERAE* O1

Given the spread of atypical O1 strains, monitoring of these strains has become important. These atypical strains, despite their variability in genotypic and phenotypic traits, are more similar to El Tor strains but harbor classical *ctxB* gene. Characterization of future *V. cholerae* O1 isolates should include both phenotypic and genotypic tests for biotyping. Phenotypic tests should be followed by genotyping *ctxB* and *rstR* gene, and detection of El Tor-specific traits – El Tor *tcpA* allele and *rtxC* gene, which will allow the identification of the atypical El Tor strains and the determination of the type of integrated CTXϕ prophage.

CONCLUSION AND RECOMMENDATION

Classical strains are known to be more toxigenic, producing clinically more severe disease[1]. In contrast, El Tor strains are more adapted to environment and able to survive in the environment better[1]. It has been reported that atypical El Tor strains produce a higher amount of CT than prototype El Tor strains[31,35] and their CT production is more or less equal to that of classical strains[35]. In toxin-mediated disease, bacteria serves as the vehicle that delivers the toxin gene to the specific site of toxin action where toxin is produced resulting in disease. O1 classical strains producing classical CT have become extinct and replaced by El Tor strains during seventh cholera pandemic. However, classical *ctxB* gene encoding classical subtype of CT has come back using different vehicle that could survive the environment better, namely

atypical El Tor strain. It can be speculated that there must be some kind of selective pressure that favors this classical *ctxB* to persist so that atypical El Tor strains harboring *ctxB* have emerged. It is recommended that a rapid identification method to detect the genotypic characteristics of those atypical El Tor strains should be developed prior to the emergence of uncontrollable epidemics in the near future.

CONFLICT OF INTEREST: None

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