The Seventh Pandemic Vibrio cholerae O1 El Tor Isolate in China Has Undergone Genetic Shifts

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A total of 330 clinical Vibrio cholerae O1 serogroups from China dating between 1961 and 2010 were investigated. By phenotypic biotyping and genetic analysis, during the seventh pandemic of V. cholerae O1 in China, the isolates of hybrid biotype (mixed classical phenotypes) were present during the entire 1961–2010 period, while El Tor genetic shifts appeared in 1992 and replaced the prototype El Tor from 2002 to 2010.

Vibrio cholerae is the causative agent of the life-threatening diarrheal disease cholera (1). Seven distinct pandemics of cholera have been recorded since the first pandemic in 1817. The sixth pandemic, and presumably the earlier pandemics, were caused by V. cholerae O1 of the classical biotype. The current seventh pandemic, which originated in Indonesia in 1961, is the most extensive in geographic spread and duration, and the causative agent is V. cholerae O1 of the El Tor biotype. In 1992, an outbreak of O139 cholera emerged in the coastal areas of India and then spread to many countries in Asia (2, 3).

The classification of the classical and El Tor biotypes of V. cholerae O1 is based on several phenotypic and genetic traits. The phenotypic traits include chicken erythrocyte agglutination (CCA), Voges-Proskauer (VP) test results, susceptibility to polymyxin B (PB; 50 units), and biotype-specific phages (1). The genetic traits include the variants of the gene encoding the cholera toxin subunit B (ctxB). In addition, the repeat sequence transcriptional regulator (rstR) gene and the major toxin coregulated pilus gene (tcpA) possess classical and El Tor-specific alleles, while the repeat in the toxin gene (rtxC) is present in El Tor but absent in classical biotype isolates (4).

Several atypical or variant El Tor biotypes have recently been identified. The Matlab variant was the first atypical El Tor biotype. It was identified in Matlab, Bangladesh, between 1991 and 1994 (5). Another study (6) reported a hybrid CTXφ isolate carrying El Tor rstR and classical ctxB that has completely replaced the El Tor biotype in Kolkata, India, since 1995. Other atypical El Tor isolates have been reported in other countries in Asia (7, 8) and Africa (9, 10), as well as in Mexico (11). Previously, we identified three novel El Tor variants from China in which the ctxB genotype was different from known genotypes (12). These results suggested that there were variants in China; however, the traits have not been investigated.

In this study, 330 V. cholerae O1 El Tor biotype isolates were characterized and compared; these isolates were collected over nearly 50 years (1961 to 2010) and were obtained from different provinces in China from 1961 to 2010, either from outbreaks or sporadic cases. All of the bacterial isolates were screened for the oxidase reaction and were identified by a slide agglutination test using specific polyvalent antisera against V. cholerae O1 (Ogawa and Inaba; S&A Reagents Lab, Bangkok, Thailand). The serogroups of these isolates were reconfirmed by real-time PCR targeting the O1 rfb-specific O biosynthetic gene (13).

For phenotypic tests, polymyxin B (PB; 50 units) susceptibility test, CCA, and the VP reaction were performed using standard procedures (14) and a previous report (15). The V. cholerae reference classical isolate 569B and the reference El Tor isolate N16961 were included as controls. The phenotypic tests were repeated three times to ensure reliable results.

To complement the phenotypic characterization of the biotypes, PCR assays were carried out using conventional PCR amplification. The target genes included ctxB (16), El Tor and classical variants of rstR (17, 18), and tcpA (19), and the repeat in the toxin gene (rtxC) (4). Table 1 shows the sequences used for primer design and their origins. A commercial company (TaKaRa, Dalian, China) performed the sequencing of the PCR products of ctxB. Comparative analyses of the ctxB sequences were conducted using BioEdit. ClustalW was used to obtain multiple alignments of the nucleotide and predicted amino acid sequences of ctxB. The ctxB sequences of isolates N16961 and 569B were used as El Tor and classical references, respectively.

For classifying the biotypes of the variant V. cholerae O1 isolates, we referred to the literature (20). We designated “atypical El Tor” as all isolates with mixed classical and El Tor traits. Isolates having conventional phenotypic properties of both classical and El Tor biotypes were designated as having a “hybrid biotype,” and isolates similar to the El Tor biotype in conventional phenotypic traits but with the classical ctxB and/or rstR genotype were designated as being “El Tor variants.”

As shown in Table 1 in the supplemental material, among the 330 V. cholerae O1 isolates, 110 were identified as having the Inaba
serotype and 220 were identified as having the Ogawa serotype. Phenotypic tests revealed that 250 isolates (75.8%) were typical El Tor prototypes (CCA\(^+\), VP\(^+\), and PB resistant [PB\(^-\)]) identical to the El Tor reference isolate N16961. The other 80 isolates (24.2%) belonged to the classical phenotypes: classic phenotypes of CCA\(^-\), VP\(^-\), and PB susceptible [PB\(^+\)] accounted for 30, 51, and 14 isolates, respectively. Specifically, the most common phenotype combinations were CCA\(^+\) VP\(^+\) PB\(^-\) (40 isolates) and then CCA\(^-\) VP\(^+\) PB\(^+\) (20 isolates), followed by CCA\(^+\) VP\(^-\) PB\(^+\) (9 isolates), CCA\(^-\) VP\(^-\) PB\(^+\) (6 isolates), CCA\(^-\) VP\(^-\) PB\(^-\) (4 isolates), and CCA\(^+\) VP\(^-\) PB\(^-\) (1 isolate). No isolate had a phenotype combination of CCA\(^+\) VP\(^-\) PB\(^-\). The classical phenotype isolates were present in the period from 1961 to 2010 (Fig. 1).

Genetic analysis showed that 278 isolates (84.2%) were positive for thectxB gene. All isolates except four were positive for the rtSC gene, which verified that genetically, the majority of the isolates belonged to the El Tor biotype, with toxin-producing capacity and epidemic potential. The rstR PCR results showed that 304 (92.1%) isolates were positive for El Tor (rstR\(^{\text{ET}}\)), classical (rstR\(^{\text{C}}\)), or El Tor and classical rstR genes (rstR\(^{\text{ET/C}}\)). In a detailed analysis of rstR-positive isolates, 254 (83.6%, 254/304) were positive for El Tor rstR only, 27 (8.9%) were positive for classical rstR only, and 23 (7.6%) were positive for both El Tor and classical. During the period 1961 to 1991, all rstR-positive isolates were rstR\(^{\text{ET}}\), except for two isolates from 1986 that were rstR\(^{\text{C}}\) and rstR\(^{\text{ET/C}}\), respectively. Between 1992 and 2010, the isolates carried either rstR\(^{\text{ET}}\) or rstR\(^{\text{C}}\) or both rstR\(^{\text{ET}}\) and rstR\(^{\text{C}}\). Of the isolates in this study, 302 (91.5%) carried tcpA\(^{\text{ET}}\), but tcpA\(^{\text{C}}\) was not found (see Table S1 in the supplemental material).

Based on amino acid residue substitutions at positions 39, 46, and 68, three ctxB genotypes have been identified among O1 V. cholerae isolates, genotypes 1, 2, and 3 (21). In our study, of all 330 V. cholerae O1 isolates, 278 (84.2%) were positive for ctxB, and 212 (64.2%) isolates were classified as genotype 3 on the basis of multiple sequence alignments with ctxB from the typical El Tor reference isolate N16961. The other 66 (20%) isolates were classified as genotype 1, carrying the classical trait of ctxB, typical of the classical reference isolate 569B (see Table S1 in the supplemental material).

From 1961 to 1991, only the El Tor allele of ctxB was present. The first classical biotype ctxB clinical isolate emerged in 1992, while the others were El Tor biotype ctxB. In 1993, isolates carrying classical ctxB were found in two other provinces, and classical ctxB isolates coexisted with the El Tor biotype of ctxB but gradually increased and became predominant by 2001. During the period 2002 to 2010, clinical isolates carrying classical biotype ctxB were completely replaced with the El Tor biotype ctxB allele (Fig. 2).

This is the first study that describes the phenotypic traits of clinical V. cholerae O1 isolates in China over a long period of time (1961 to 2010). Although 75.8% of the isolates were typical El Tor, 24.2% were the classic phenotypes. It is noteworthy that the hybrid biotype isolates were present in all years from 1961 to 2010 (Fig. 1), whereas all hybrid biotype isolates except four harbored the rtSC gene, an El Tor biotype-specific genetic marker (22). This result indicates that the phenotypic changes in El Tor isolates occurred throughout the seventh pandemic in China. Although there is no other continual report of phenotypic changes in isolates from the first stage of the seventh pandemic, this hybrid biotype in the first stage of the seventh pandemic may be a universal phenomenon. We propose that there was a “phenotypic shifting period” before 1961, when classical and El Tor phenotypes coexisted among isolates, similar to the “genetic shifting period” of isolates between 1991 and 1994 in Matlab, Bangladesh (5), be-

![FIG 1 Distribution of V. cholerae O1 El Tor isolates based on deduced biotypes, by year.](http://jcm.asm.org/Downloaded_from/September_23%2C_2017_by_guest)
between 1990 and 1994 in Kolkata, India (6), and between 1992 and 2002 in China, although the mechanisms involved in the emergence of the hybrid biotype are not clear.

In addition to reports of atypical El Tor biotypes in Bangladesh and India and Mozambique variants in Africa, studies have also identified an altered variant that completely replaced the progenitor El Tor isolates in Thailand (7), Vietnam (8), and Angola (9), all around 1991. Taken together with our study in China, it is clear that the genetic shift in El Tor V. cholerae O1 occurred around 1991 or even before, becoming the predominant isolate or replacing the progenitor El Tor isolate in many Asian and African countries.

In the present study, the biotypes of CTXΦ in China underwent the following shifts: a period of typical CTXΦET (rstRC \( \rightarrow \) ctxBET) (1961 to 1992); a period of coexistence of CTXΦET and CTXΦEC (rstRC \( \rightarrow \) ctxBEC) (1993 to 2001); and a period in which CTXΦEC replaced CTXΦET (2002 to 2010). This process suggests that during the genetic shifting of El Tor V. cholerae O1, horizontal gene transfer of virulence genes, as well as genetic recombination and mutation, might have occurred (22).

In conclusion, a retrospective assay of the phenotypic and genotypic characteristics of clinical isolates from the seventh pandemic V. cholerae O1 in China was undertaken. The El Tor variants have replaced the prototype seventh pandemic El Tor variant in China, which is consistent with the shift in most countries in Asia and Africa. Recently, V. cholerae O1 El Tor isolates producing Haitian variant cholera toxin (HCT) and showing reduced susceptibility to ciprofloxacin caused a cholera outbreak associated with a high case fatality rate in India (23). HCT-secreting strains have been responsible for severe cholera epidemics in western Africa (24) and Haiti (25). The role of new variants in cholera epidemics and pathogenicity should be noted, and additional surveillance is required to understand the epidemiology and the pathogenic and molecular evolution of atypical El Tor isolates.

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