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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 entire1961-2010 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 1961to 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 S1 in the supplemental material, among the 330 *V. cholerae* O1 isolates, 110 were identified as having the Inaba

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TABLE 1 PCR primers used in this study

Primer	Nucleotide sequence (5′ to 3′)[a]	Amplicon size (bp)	Use	Reference
O1-*rfb*	GCGTAAATATCTAAACGATTGCATTG, AAACTCAGTTTCGAAGCGATCAA	83	Real-time PCR	13
ctxB	GCCGGGTTGTGGGAATGCTCCAAG, CATGCGATTGCCGCAATTAGTATGGC	536	PCR, sequencing	16
rstR^{ET}	GAGCTAAAATACAGCAACCAATGC, ACTCACCTTGTATTCG	487	PCR	17
rstR^{C}	TATTGGGATTGTAAACAGCTGTCC, ACTCACCTTGTATTCG	480	PCR	18
tcpA 72F	CACGATAAGAAAACCGGTCAAGAG			19
tcpA 477R	CGAAAGCACCTTCTTTCACGTTG	451 (El Tor)	PCR	19
tcpA 647R	TTACCAAATGCAACGCCGAATG	620 (classical)	PCR	19
rtxC	CGACGAAGATCATTGACGAC, CATCGTCGTTATGTGGTTGC	265	PCR	4

[a] In entries with two sequences, the sequence for the reverse primer follows that for the forward primer.

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^r]), identical to the El Tor reference isolate N16961. The other 80 isolates (24.2%) belonged to the classic phenotypes: classic phenotypes of CCA^-, VP^-, and PB susceptible [PB^s] accounted for 30, 51, and 14 isolates, respectively. Specifically, the most common phenotype combinations were CCA^+ VP^- PB^r (40 isolates) and then CCA^- VP^+ PB^r (20 isolates), followed by CCA^+ VP^+ PB^s (9 isolates), CCA^- VP^- PB^r (6 isolates), CCA^- VP^- PB^s (4 isolates), and CCA^+ VP^- PB^s (1 isolate); no isolate had a phenotype combination of CCA^- VP^+ PB^s. 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 the *ctxB* gene. All isolates except four were positive for the *rtxC* 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*^{ET}), classical (*rstR*^{C}), or El Tor and classical *rstR* genes (*rstR*$^{ET/C}$). 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 El Tor and classical. During the period 1961 to 1991, all *rstR*-positive isolates were *rstR*^{ET}, except for two isolates from 1986 that were *rstR*^{C} and *rstR*$^{ET/C}$, respectively. Between 1992 and 2010, the isolates carried either *rstR*^{ET} or *rstR*^{C} or both *rstR*^{ET} and *rstR*^{C}. Of the isolates in this study, 302 (91.5%) carried *tcpA*^{ET}, but *tcpA*^{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 1961to 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 *rtxC* 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-

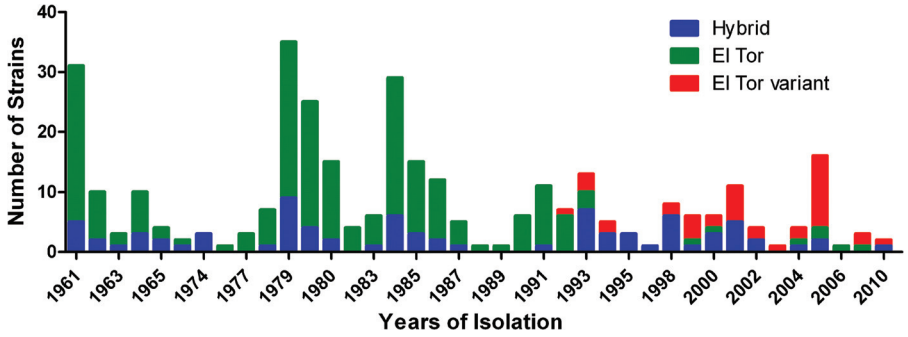

FIG 1 Distribution of *V. cholerae* O1 El Tor isolates based on deduced biotypes, by year.

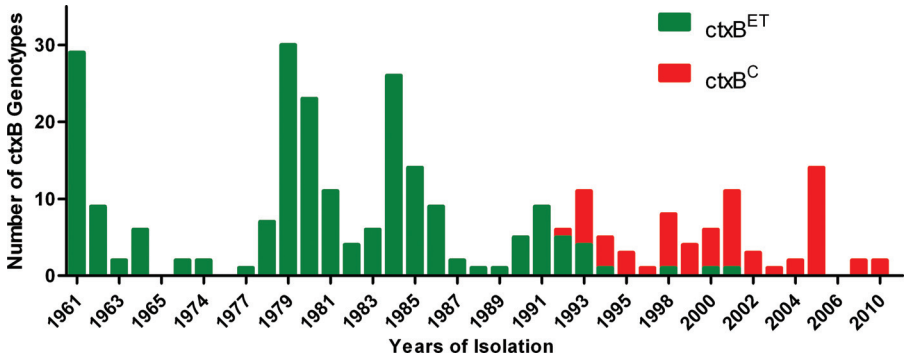

FIG 2 Distribution of *V. cholerae* O1 El Tor isolates based on the *ctxB* subtypes (amino acid sequence alignment), by year.

tween 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* O1occurred 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 CTXΦ^{ET}(*rstR*^{ET} *ctxB*^{ET}) (1961 to 1992); a period of coexistence of CTXΦ^{ET} and CTXΦ^{C} (*rstR*^{C} *ctxB*^{C}) (1993 to 2001); and a period in which CTXΦ^{C} replaced CTXΦ^{ET} (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 genetic 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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