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Emergence and genetic diversity of El Tor *Vibrio cholerae* O1 that possess classical biotype *ctxB* among travel-associated cases of cholera in Japan

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Vibrio cholerae O1 are classified into two biotypes, classical and El Tor, each encoding a biotype-specific cholera toxin. However, El Tor strains have recently emerged with a classical cholera-toxin genotype (El Tor variant). We characterized El Tor strains of *V. cholerae* O1 from travel-associated cases of cholera in Japan isolated from 1991 to 2006 by cholera toxin B subunit gene (*ctxB*) typing and by molecular epidemiological methods. *ctxB* in the biotype El Tor shifted from the El Tor-specific type to the classical-specific type around 1993, and this type fully dominated the later half of the 1990s. Based on the results of PFGE and multilocus variable-number tandem repeat analysis, strains of the classical biotype remained diverse from those of El Tor biotype. The El Tor biotype strains formed multiple minor clusters and intermingled with each other irrespective of their origins and toxin types. El Tor variant strains are widespread in Asian countries and show significant genetic diversity, indicating that their spread is a result of multiclonal expansion rather than spread from a single clone.

INTRODUCTION

Vibrio cholerae O1, the causative agent of cholera, has two biotypes, classical and El Tor, which can be differentiated by various phenotypic traits, such as the ability to haemolyse sheep red blood cells and to agglutinate chicken red blood cells, the Voges–Proskauer reaction, and susceptibility to polymyxin B and biotype-specific phages (Kaper *et al.*, 1995). Classical biotype strains were responsible for the fifth and sixth cholera pandemics while the ongoing seventh pandemic was caused by El Tor biotype strains, which have now replaced the classical biotype globally (Sack *et al.*, 2004). Cholera toxin, the principal virulence factor of cholera, is composed of two functional units, an enzymic A subunit of 27 kDa and an intestinal receptor-binding B subunit consisting of five identical 11.6 kDa peptides (Van Heyningen, 1976). Although sequences of the *ctxA* 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 (Olsvik *et al.*, 1993; Popovic *et al.*, 1994). Recently, a new variant called 'El Tor variant' has emerged, where *V. cholerae* O1 shows the typical El Tor biotype but produces cholera toxin of the classical type (Raychoudhuri *et al.*, 2008). In Bangladesh the El Tor variants have completely replaced the prototype seventh pandemic strain of the El Tor biotype that produced cholera toxin of the El Tor type (Nair *et al.*, 2006), and have spread to several other countries in Asia and Africa (Nguyen *et al.*, 2009; Raychoudhuri *et al.*, 2009; Safa *et al.*, 2008). As far as we know, however, there are no reports of a detailed investigation of the emergence of El Tor variant strains and their molecular epidemiology. In this study, we examined El Tor strains from travel-associated cholera cases in Japan during 1991–2006 with *ctxB* typing by a mismatch amplification mutation assay PCR (MAMA-PCR) developed in our previous study (Morita *et al.*, 2008), and further characterized the strains by the use of multiple molecular typing techniques. Consequently, El Tor strains with the classical type of *ctxB* were detected in samples from the early nineties onwards, and they seemed

Abbreviations: MAMA-PCR, mismatch amplification mutation assay PCR; MLVA, multilocus variable-number tandem repeat analysis.

to replace the typical El Tor strains in Asian endemic areas. El Tor variant strains showed a variety of genotypes for molecular epidemiology, which suggests that they originated from multiple geographical areas.

METHODS

Bacterial strains. We collected 67 clinical strains of *V. cholerae* O1 from travel-associated cholera cases in Japan between 1991 and 2006. Sixty-four were from patients with a history of travel to Asian countries, two were from patients who had travelled to Latin America (Bolivia and Peru) in 1992 and one was from Africa (Madagascar) in 2000. The fully genome sequenced El Tor strain N16961 and ten typical classical strains were also subjected to molecular epidemiological studies for use as reference strains. The biotypes of the strains were confirmed by testing chicken erythrocyte agglutination, and sensitivity to polymyxin B, Mukerjee classical phage IV and Basu and Mukerjee El Tor phage V.

***ctxB* typing by MAMA-PCR.** MAMA-PCR was employed to detect sequence polymorphism between classical and El Tor *ctxB* genes at nucleotide position 203 for toxin typing (Morita *et al.*, 2008). Briefly, amplification of the *ctxB* gene was accomplished using primer 5′-ACTATCTTCAGCATATGCACATGG-3′ (Fw-con), with 5′-CCTGG-TACTTCTACTTGAAACG-3′(Rv-cla, specific for the classical *ctxB*) or 5′-CCTGGTACTTCTACTTGAAACA-3′(Rv-elt, specific for the El Tor *ctxB*), in PCR mixture (10 µl) containing 1 µl 10 × *Ex Taq* buffer (Takara Bio), 100 µM dNTPs and 0.5 µl overnight strain culture with 0.25 U *Ex Taq* (Takara Bio). PCR conditions were as follows: initial denaturation at 96 °C for 2 min; 25 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 30 s; and final extension at 72 °C for 2 min. The amplified fragments were detected after 3 % agarose gel electrophoresis and staining with ethidium bromide (0.1 µg ml^{-1}).

PFGE. PFGE was performed as previously described using the *Salmonella enterica* serovar Braenderup H9812 as a standard strain (Cooper *et al.*, 2006). The DNA in agarose plugs was digested with *Not*I (Promega). Digested DNA was separated through a 1 % SeaKem Gold agarose gel (Cambrex Bio Science) in 0.5 × TBE buffer [50 mM Tris, 45 mM boric acid, 0.5 mM EDTA (pH 8.4)] at 14 °C in a CHEF DR-III (Bio-Rad Laboratory) under the following electrophoresis conditions: switch time of 2–10 s for 13 h and 20–25 s for 6 h, 6 V cm^{-1}, at an angle of 120°. The resulting profiles were scanned and saved in TIFF format to be analysed using BioNumerics software (Applied Math). Similarity was determined using the Dice coefficient, and clustering was based on UPGMA with a band position tolerance of 1.2 %.

Multilocus variable-number tandem repeat analysis (MLVA). MLVA of *V. cholerae* was performed using the loci described by Danin-Poleg *et al.* (2007) with modifications. Seven loci were selected and applied in this study. The five loci (locus names 1, 2, 3, 5 and 6) were on the large chromosome and the two loci (locus names 7 and 8) were on the small chromosome. The details of the loci and primers are listed in Table 2. *V. cholerae* O1 strains were cultivated on nutrient agar with 1 % sodium chloride. DNA was extracted from fresh colonies using QuickGene-810 with the QuickGene DNA tissue kit S (Fujifilm). The seven selected variable number of tandem repeats loci were amplified in a single multiplex PCR. The PCR mixture contained 0.2 µM each primer, 1 µl DNA template and 1 × multiplex PCR mixture (Qiagen). The PCR conditions were as follows: initial denaturation at 95 °C for 15 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 90 s and extension at 72 °C for 60 s; and final extension at 72 °C for 10 min.

Table 1. Type of *ctxB* encoded in El Tor *V. cholerae* strains from 1991 to 2006

Year	*ctxB* type*		Region† (no. of strains)
	El Tor	Classical	
1991	6		South-East Asia (5), South Asia (1)
1992	5		South-East Asia (3), Latin America (2)
1993	2	3	South-East Asia (5)
1994	1	2	South-East Asia (2‡), East Asia (1)
1995		10	South-East Asia (10)
1996		1	South-East Asia (1)
1997		14	South-East Asia (11), East Asia (3)
1998		2	South-East Asia (1), East Asia (1)
1999		4	South-East Asia (1), South Asia (3)
2000		1	Africa (1)
2001		2	South-East Asia (2)
2002		1	South-East Asia (1)
2003		0	
2004		8	South-East Asia (3), South Asia (5)
2005		3	South-East Asia (2), South Asia (1)
2006		2	South-East Asia (1), South Asia (1)
Total	14	53	

**ctxB* typing based on nucleotide 203 of the *ctxB* gene was performed using MAMA-PCR.
†Africa – Madagascar; East Asia – China, Korea, North Korea; South Asia – India; South-East Asia – Indonesia, the Philippines, Singapore, Thailand; Latin America – Bolivia, Peru.
‡One strain harboured El Tor *ctxB* and the other harboured classical *ctxB*.

The resulting PCR products were diluted 1 : 10 with Hi-Di formamide. A 2 µl aliquot of each diluent was mixed with 10 µl Hi-Di formamide containing Gene Scan LIZ 600 size standard (Applied Biosystems). After denaturing by heating, PCR products were separated using ABI 3130 (Applied Biosystems). Sizes were determined using GeneMapper software and converted to the repeat copy number; that of the null allele was designated −2 (Hyytiä-Trees *et al.*, 2006). The repeat copy numbers were used with BioNumerics software and analysed using a categorical multi-state coefficient and UPGMA clustering.

RESULTS AND DISCUSSION

In Bangladesh, El Tor variant strains of *V. cholerae* O1 that belong to the El Tor biotype but produce cholera toxin of the classical type have completely replaced the prototype seventh pandemic El Tor strain (Nair *et al.*, 2006). Such strains have also been identified in several other countries in Asia and Africa (Nguyen *et al.*, 2009; Raychoudhuri *et al.*, 2009; Safa *et al.*, 2008). This suggests that the El Tor variants of *V. cholerae* O1 have now prevailed and become the epidemic strains. Cholera has become a predominantly travel-associated disease in Japan, where most cases are associated with travel to Asian countries. Accordingly, we examined *V. cholerae* O1 strains from travel-associated cholera cases in Japan as an indicator of the epidemiology

Table 2. Primers used for MLVA and repeat variations of *V. cholerae* O1

Locus name	Locus tag in N16961	Primer pair (5′→3′)		No. of alleles	Index of diversity
		Forward primer	Reverse primer		
1	VC0147	CTGCAACGGATACTCAAACGCAGGA	GTTCAACAATACGTGCAGGTTC	7	0.782
2	VC0436-7	GAGGTTAAAGGTCCTAACAACCGT	CCGCCATCACCAGCTTGAACTT	5	0.472
3	VC0500	CGTATCTCAATTTGCTCTGGTGTCAT	CATGACAGGTGAAACTATGACCA	4	0.337
5	VC1457-8	CGATAAAAACAGAAAATGATAAAAAAGGAC	CATTTGAGTACCTCGGTCAAAGTACTC	6	0.467
6	VC1650	GGCTGGATACATTGCAACGCGAT	CGAATTGGCCGCTAACTGAGTGA	8	0.807
7	VCA0171	CGACACAATTTAGACGTGGTCA	CTGTATTTGAGGGATTTGCTGATGAG	19	0.877
8	VCA0283	AAATATCTGTAGCCTCCTCAG	CTCTCTGAACTGGTTTTTGTGTACC	20	0.929

in Asian endemic areas. All the 67 strains in this study were confirmed to be El Tor biotype. We typed the *ctxB* gene of all the biotype El Tor strains by MAMA-PCR to identify the El Tor variants of *V. cholerae* O1. As shown in Table 1, El Tor variants were first identified in isolates from 1993. Of three El Tor strains of 1994, two possessed the classical-type *ctxB* gene, namely, they were El Tor variants; and all the El Tor strains of *V. cholerae* O1 after 1994 were El Tor variants. These results include a bias associated with the relatively small number of strains and reflect that the strains were only from patients not from environmental samples. But they suggest that the emergence and spread of El Tor variants took place around 1993, and that the toxin-type shift might have been completed in the latter half of the 1990s. Namely, the toxin type of the El Tor strains shifted around 1993, and El Tor variants have now completely replaced typical El Tor strains in endemic areas of Asia. This is consistent with other recent findings, indicating that the El Tor variant has spread ubiquitously (Nair *et al.*, 2006; Nguyen *et al.*, 2009; Raychoudhuri *et al.*, 2009; Safa *et al.*, 2008).

There are two hypotheses for the marked spread of El Tor variant; the first is that it might have resulted from clonal expansion of the single ancestral El Tor variant that first acquired the classical *ctxB* gene. The second is that it might have developed from multiclonal emergence of the El Tor variant in each region; therefore, we performed two different molecular genotyping methods, PFGE and MLVA, to examine the clonality of the El Tor variants. Both methods are accepted as those with discriminatory power and reproducibility, but their results make no obvious correlations with each other (Danin-Poleg *et al.*, 2007). A dendrogram based on PFGE profiles with *Not*I digestion is shown in Fig. 1(a). Typical classical and El Tor strains were clearly divided into different clusters with similarity of less than 75 %. And the El Tor variants were assigned into the cluster of the typical El Tor strains. The biotype El Tor strains formed multiple minor clusters, some of which were related to their geographical area and/or time, but overall they intermingled with each other. MLVA is a newly developed technique for genotyping bacterial micro-organisms based on the combination of various short tandem repeats at multiple loci. In this study, we selected seven loci that displayed a relatively high resolution power in a previous study (Danin-Poleg *et al.*, 2007). The number of variations and Simpson's diversity index of each locus are shown in Table 2. After combining all the loci, 70 types were identified for 78 strains and the overall diversity index was 0.98, indicating that these seven loci are appropriate for molecular genotyping. Fig. 1(b) shows the dendrogram generated from the results of MLVA. As observed in Fig. 1(a), the biotype El Tor strains were not divided into obvious clusters by their toxin type.

We used two methods, PFGE and MLVA, for molecular typing to examine the bacterial genome by different criteria and to reinforce the results from each individual assay. Similar results were observed not only in PFGE analysis but also in MLVA, though the components of some of the minor clusters were different between the methods. Furthermore, even the oldest El Tor variants in this study had already showed some genetic diversity and were divided into different minor clusters. These results suggest that El Tor variants are related to various types of typical El Tor strains rather than classical type strains, and that the El Tor variant epidemic was likely to be caused by simultaneous or sequential emergence and expansion of multiclones, and not by the prevalence of a certain single clone.

In 1992, a new serogroup, defined as O139, caused a severe outbreak of cholera in South-East India. During the following 10 months, the O139 serogroup spread throughout the Indian subcontinent and soon thereafter spread to neighbouring countries, resulting in temporary displacement of the O1 serogroup (Sack *et al.*, 2004; Bhattacharya *et al.*, 1993). This period coincides with the emergence and spread of the El Tor variant strains. The seventh pandemic of *V. cholerae* O1 is ongoing after a transient epidemic due to *V. cholerae* O139. The re-emerged *V. cholerae* O1 biotype El Tor strains, however, have been reported to differ in genetic characteristics from the *V. cholerae* O1 biotype El Tor strains of the pre-O139 period, and identification of strains that could not be biotyped as El Tor or classical has also been reported (Nair *et al.*, 2006; Faruque *et al.*, 1997;

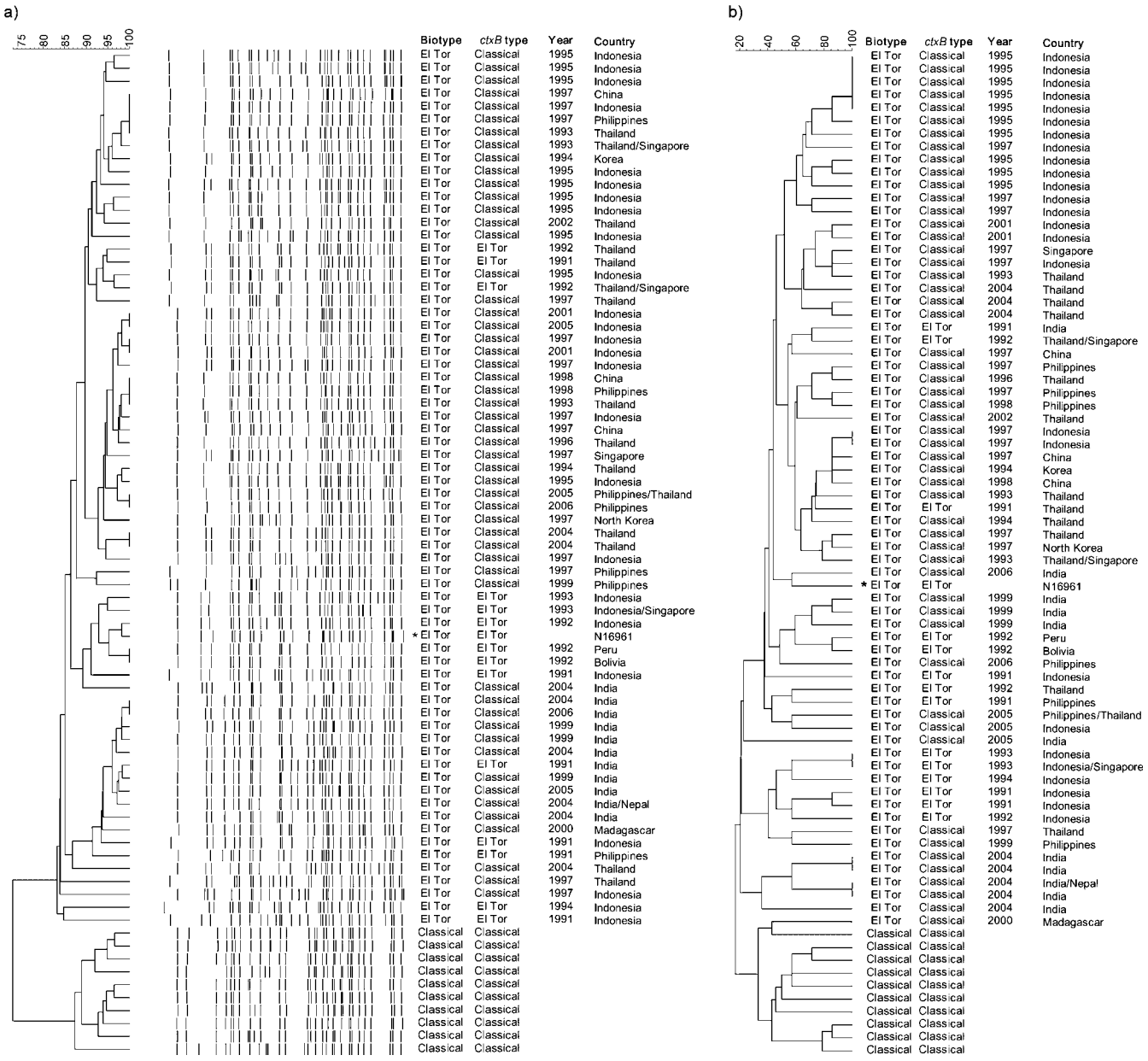

Fig. 1. Dendrograms based on (a) *Not*I-digested DNA PFGE analysis and (b) MLVA. Biotype, *ctxB* type, year isolated and country visited by the patient are indicated for each strain. The genome strain, N16961, is indicated by an asterisk.

Yamasaki *et al.*, 1997). The mechanism of emergence and the evolutionary process of the El Tor variant remain to be elucidated. More broad and extensive surveillance of clinical and environmental strains should be carried out to clarify the evolutionary process of the newly emerging type of *V. cholerae* O1. However, in this process, phage-mediated lateral gene transfer could also be involved in the acquisition of the *ctx* gene by ancestor El Tor strains from putative phage sources such as non-O1 non-O139 *V. cholerae* as suggested by Udden *et al.* (2008). We again emphasize that while statistically significant correlations suggest that our genotype clusters align themselves with different environmental conditions, further characterization of the sampling sites and the relative fitness of genotypes is needed to assess the importance of our observed genomic diversity.

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