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Multilocus variable-number tandem repeat analysis of *Vibrio cholerae* O1 El Tor strains harbouring classical toxin B

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Atypical *Vibrio cholerae* O1 strains – hybrid strains (strains that cannot be classified either as El Tor or classical biotype) and altered strains (El Tor biotype strains that produce classical cholera toxin) – are currently prevalent in Asia and Africa. A total of 74 hybrid and altered strains that harboured classical cholera toxin were investigated by multilocus variable-number tandem repeat analysis (MLVA). The results showed that the hybrid/altered strains could be categorized into three groups and that they were distant from the El Tor strain responsible for the seventh cholera pandemic. Hybrid/altered strains with a tandem repeat of the classical CTX prophage on the small chromosome were divided into two MLVA groups (group I: Mozambique/Bangladesh group; group III: Vietnam group), and altered strains with the RS1–CTX prophage containing the El Tor type *rstR* and classical *ctxB* on the large chromosome were placed in two MLVA groups (group II: India/Bangladesh group; group III: India/Vietnam group).

INTRODUCTION

Cholera is a severe diarrhoeal disease caused by *Vibrio cholerae* (Kaper *et al.*, 1995; Sack *et al.*, 2004). Among the more than 200 serogroups of *V. cholerae*, only O1 and O139 cause epidemic cholera. The O1 serogroup has been divided into three serotypes, Ogawa, Inaba and Hikojima (Kaper *et al.*, 1995), and into two biotypes, classical and El Tor. Classical biotype strains are considered to be responsible for the first six cholera pandemics since the early 19th century, whilst El Tor biotype strains are responsible for the current (seventh) cholera pandemic, which began in 1961 (Kaper *et al.*, 1995; Sack *et al.*, 2004).

Since the early 1990s, new variants of the *V. cholerae* O1 serogroup – hybrid and altered strains – have emerged and have entirely replaced prototype El Tor strains in Asian countries and Mozambique (Lee *et al.*, 2006; Nair *et al.*, 2002, 2006; Nguyen *et al.*, 2009). Hybrid strains were initially defined as atypical strains that could not be biotyped as classical or El Tor and alternatively named the Matlab variants (Nair *et al.*, 2002). Hybrid strains are reported to harbour the El Tor type cholera toxin (CT) B subunit gene (*ctxB*) and/or classical *ctxB*, whilst altered strains contain classical *ctxB* (Safa *et al.*, 2006). The El Tor biotype strains that harbour classical *ctxB* have been designated altered strains (Nair *et al.*, 2006). Recently, an alternative nomenclature for the hybrid and altered strains has been suggested, with 'El Tor variants' as well as a new biotyping scheme for *V. cholerae* O1 (Raychoudhuri *et al.*, 2008; Safa *et al.*, 2010). Two different genetic structures of the CTX prophage and RS1 element on each chromosome have been reported among the hybrid and altered strains (Lee *et al.*, 2006, 2009; Nguyen *et al.*, 2009). As there are different CTX prophage arrays on different chromosomes among these, we postulated that these strains could have differences in their genomes. However, when we applied multilocus sequence typing analysis to the altered strains, most contained the same sequence type as the prototype El Tor strain, N16961 (Lee *et al.*, 2006). A number of

Abbreviations: CT, cholera toxin; MLVA, multilocus variable-number tandem repeat analysis.

multilocus variable-number tandem repeat analysis (MLVA) studies of *V. cholerae* strains collected mainly in Bangladesh and India have been reported (Danin-Poleg *et al.*, 2007; Ghosh *et al.*, 2008; Stine *et al.*, 2008). We therefore employed MLVA to find relevant genetic differences among the hybrid/altered strains, which were collected mainly in India and Bangladesh. We found that hybrid/altered strains could be categorized into three groups. Strains collected in Mozambique and some strains from Bangladesh, which had a tandem repeat of the CTX prophage on the small chromosome, could be grouped together, whilst all analysed Asian strains containing the RS1–CTX prophage array on the large chromosome were subdivided into two groups.

METHODS

***V. cholerae* strains.** A total of 77 *V. cholerae* O1 isolates, including strain N16961 (biotype El Tor), O395 and 569B (biotype classical) for reference, were analysed in this study. B33, which has a tandem repeat of the classical CTX prophage on the small chromosome, and four isolates collected together with B33 in Mozambique in 2004 were included (Faruque *et al.*, 2007; Lee *et al.*, 2006). Nineteen *V. cholerae* isolates collected from northern Vietnam (ten isolates collected from 1995 to 2004 and nine collected during cholera outbreaks in 2007–2008), 16 isolates from the International Centre for Diarrhoeal Disease Research, Bangladesh, and 34 randomly selected isolates from a collection of 424 clinical isolates from Kolkata, India (collected from 2003 to 2007) were analysed (Lee *et al.*, 2006; Nguyen *et al.*, 2009; Roychowdhury *et al.*, 2008).

The CTX prophage and RS1 array and the *ctxB* sequence of the *V. cholerae* strains were determined as described previously (Lee *et al.*, 2009; Nguyen *et al.*, 2009).

Genetic analysis. Genomic DNA was prepared from agar-grown cultures using a Prepman Ultra kit (Applied Biosystems). Five loci for MLVA were amplified using primers and PCR conditions as described in previous studies (Ghosh *et al.*, 2008; Stine *et al.*, 2008) and in Table 1. The purified PCR products were sequenced in both directions using a Big Dye Cycle Sequencing kit (Applied Biosystems) and sequencing was performed on an ABI 3770 automatic sequencer according to the manufacturer's instructions. Sequence data for each isolate were added to a group of known sequences, which were aligned simultaneously and edited through jPHYDIT (Jeon *et al.*, 2005). MLVA types were assigned by combining numbers of repeat units of each locus in the order described above. Minimum spanning trees were generated using BioNumerics (Applied Maths) based on the categorical coefficient.

RESULTS AND DISCUSSION

V. cholerae strains and their CTX prophage

Two strains, MJ1236 and MG116926, are known as hybrid strains or Matlab variants I and III, respectively, and contain a tandem repeat of the classical CTX prophage on the small chromosome (Lee *et al.*, 2009; Safa *et al.*, 2006). All other strains, except for N16961 (El Tor biotype), O395 and 569B (classical biotype), were altered strains containing either a tandem repeat of the classical CTX prophage on the small chromosome (ten isolates collected in Vietnam from 1995 to 2004, E1781 collected in Bangladesh and five Mozambican isolates) or harbouring an El Tor type CTX prophage with classical *ctxB* on the large chromosome (Nguyen *et al.*, 2009). Detailed information on the isolates, including the year and country of isolation and the CTX prophage and RS1 element array on each chromosome, is listed in Table 2.

MLVA analysis of *V. cholerae* strains

The MLVA scheme that was applied previously to *V. cholerae* strains from Bangladesh and India was used in this study (Ghosh *et al.*, 2008; Stine *et al.*, 2008). Instead of using arbitrary allele type numbers, we used the number of repeats of each locus directly in this study. In the previous studies, a new allele type number was assigned to represent the number of repeats of each locus; however, the number of repeats of each locus is shown directly in this study. The MLVA profile was assigned by combining the number of repeats of five loci. Compared with the three loci on the large chromosome (VC0147, VC0436-7 and VC1650), on which five, four and five different allele types were identified, respectively, two loci on the small chromosome

Table 1. MLVA loci characteristics and the number of alleles identified in this study

Chromosome	Locus	Repeat unit	Primers (5′→3′)	No. of alleles
1	VC0147 [cell division protein (*ftsY*)]	AACAGA	CCAAACCACTGCAACGGATA*	5
			GCTGCTCGACCTGAGAGAGA*	
	VC0436-7 [non-coding (intergenic)]	GACCCTA	CGTGGTACTAAGTTCCACGC	4
			CGTTTTTACCACGCTCCGCTTC	
	VC1650 (collagenase)	GATAATCCA	CTACCAAGCGGCGGTTAAGCTG	5
			TGGGCAACCTGCTGGTAGC	
2	VCA0171 (hypothetical protein)	TGCTGT	GCATCATCCACAGCGTTTGG	15
			GCTGAAGCCTTTCGCGATCC	
	VCA0283 (hypothetical protein)	ACCAGA	CTTCATCGGCAAACAAGACA*	20
			TTGCGCACAATTCTCTTTGA*	

*Primers designed in this study based on previous reports. Other primers have been described previously (Ghosh *et al.*, 2008; Stine *et al.*, 2008).

Table 2. *V. cholerae* strains and isolates analysed in this study

The country and year in which each isolate was collected, CTX prophage and RS1 element array on each chromosome, and MLVA profile are indicated. Allele numbers represent the total number of repeats of each locus. CTX^{ET}, CTX prophage containing El Tor *rstR* and El Tor *ctxB*; CTX^{cl}, CTX prophage containing classical *rstR* and classical *ctxB*; CTX^{ec}, CTX prophage containing El Tor *rstR* and classical *ctxB*; TLC, toxin-linked cryptic element.

Original ID	Country, year	Chromosome 1	Chromosome 2	VC0147	VC0436-7	VC1650	VCA0171	VCA0283	MLVA group
O395	Reference strain, classical	TLC-truncated CTX^{cl}–CTX^{cl}	CTX^{cl}	7	4	3	24	14	–
569B	Reference strain, classical	TLC-truncated CTX^{cl}–CTX^{cl}	CTX^{cl}	10	4	3	15	39	–
N16961	Reference strain, El Tor	TLC–CTX^{ET}–RS1	–	9	7	7	23	14	–
B-33	Mozambique, 2004	–	CTX^{cl}–CTX^{cl}	8	7	8	11	20	I
B-81	Mozambique, 2004	–	CTX^{cl}–CTX^{cl}	8	7	8	11	20	I
042-3	Mozambique, 2004	–	CTX^{cl}–CTX^{cl}	8	7	8	11	22	I
127-1	Mozambique, 2004	–	CTX^{cl}–CTX^{cl}	7	7	8	11	20	I
308-1	Mozambique, 2004	–	CTX^{cl}–CTX^{cl}	8	7	8	11	21	I
07.95/Vc.P	Vietnam, 1995	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	26	III
32.02/Vc.P	Vietnam, 2002	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	26	III
272.03/Vc.P	Vietnam, 2003	–	CTX^{cl}–CTX^{cl}	10	6	8	17	28	III
307.03/Vc.P	Vietnam, 2003	–	CTX^{cl}–CTX^{cl}	10	6	8	17	28	III
43.04/Vc.P	Vietnam, 2004	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	29	III
55.04/Vc.P	Vietnam, 2004	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	29	III
62.04/Vc.P	Vietnam, 2004	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	29	III
71.04/Vc.P	Vietnam, 2004	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	29	III
73.04/Vc.P	Vietnam, 2004	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	29	III
84.04/Vc.P	Vietnam, 2004	CTX^{cl}	CTX^{cl}–CTX^{cl}	10	6	8	16	26	III
01.07/Vc.P	Vietnam, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	16	16	III
34.07/Vc.P	Vietnam, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	17	III
69.07/Vc.C*	Vietnam, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	17	III
81.07/Vc.V	Vietnam, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	16	16	III
629.07/Vc.W*	Vietnam, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	16	16	III
226.08/Vc.P	Vietnam, 2008	TLC–RS1–CTX^{ec}	–	10	6	7	16	16	III
491.08/Vc.V*	Vietnam, 2008	TLC–RS1–CTX^{ec}	–	10	6	7	17	19	III
1056.08/Vc.C*	Vietnam, 2008	TLC–RS1–CTX^{ec}	–	10	6	7	17	19	III
2061.08/Vc.F*	Vietnam, 2008	TLC–RS1–CTX^{ec}	–	10	6	7	16	16	III
VC073	Bangladesh, 1994	TLC–RS1–CTX^{ec}	–	8	7	6	18	18	–
MQ4	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	10	7	6	17	11	II
MQ1194	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	3	6	10	10	II
MQ1273	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	3	6	10	10	II
MQ1356	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	3	6	21	12	II
MQ1379	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	3	4	10	10	II
MQ1795	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	3	6	16	11	II
MQ2200	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	10	3	6	15	10	II
CRS101	Bangladesh, 2002	TLC–RS1–CTX^{ec}	–	9	3	6	16	11	II
MJ1236	Bangladesh, 1994	–	CTX^{cl}–CTX^{cl}	8	7	8	12	19	I
MG116926	Bangladesh, 1991	TLC–RS1–RS1	CTX^{cl}–CTX^{cl}	8	7	8	14	23	I
E1781*	Bangladesh, 2000	RS1–RS1	CTX^{cl}–CTX^{cl}	8	7	8	9	24	I
E1978*	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	7	6	16	8	II
E1979*	Bangladesh, 2001	TLC–RS1–CTX^{ec}	–	9	7	6	16	8	II
EC1293*	Bangladesh, 1999	TLC–RS1–CTX^{ec}	–	9	7	6	15	11	II
AR32732	Bangladesh, 2004	TLC–RS1–CTX^{ec}	–	9	3	6	22	12	II
7	India, 2004	TLC–RS1–CTX^{ec}	–	9	7	6	19	16	II
26	India, 2005	TLC–RS1–CTX^{ec}	–	9	7	6	19	16	II
87	India, 2004	TLC–RS1–CTX^{ec}	–	11	7	7	14	16	III
104	India, 2006	TLC–RS1–CTX^{ec}	–	11	7	7	14	19	III

Table 2. cont.

Original ID	Country, year	Chromosome 1	Chromosome 2	VC0147	VC0436-7	VC1650	VCA0171	VCA0283	MLVA group
178	India, 2006	TLC–RS1–CTX^{ec}	–	9	3	6	19	17	II
200	India, 2006	TLC–RS1–CTX^{ec}	–	9	3	6	22	19	II
214	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	19	16	II
236	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	17	16	II
301	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	6	14	18	III
302	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	6	14	18	III
303	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	19	16	II
304	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	19	16	II
305	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	14	14	III
315	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	14	14	III
316	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	15	III
317	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	16	14	III
324	India, 2007	TLC–RS1–CTX^{ec}	–	10	7	7	14	19	III
340	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	20	18	II
345	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	15	III
350	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	19	III
360	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	15	III
365	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	14	20	III
370	India, 2007	TLC–RS1–CTX^{ec}	–	10	6	7	15	15	III
380	India, 2006	TLC–RS1–CTX^{ec}	–	10	6	7	14	21	III
386	India, 2006	TLC–RS1–CTX^{ec}	–	10	6	7	14	21	III
406	India, 2006	TLC–RS1–CTX^{ec}	–	11	6	7	14	18	III
407	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	17	16	II
410	India, 2007	TLC–RS1–CTX^{ec}	–	9	3	6	19	17	II
411	India, 2005	TLC–RS1–CTX^{ec}	–	11	6	7	14	18	III
412	India, 2006	TLC–RS1–CTX^{ec}	–	11	6	7	14	18	III
419	India, 2006	TLC–RS1–CTX^{ec}	–	9	3	6	20	25	II
420	India, 2007	TLC–RS1–CTX^{ec}	–	11	6	7	14	18	III
421	India, 2006	TLC–RS1–CTX^{ec}	–	11	6	7	14	18	III
423	India, 2007	TLC–RS1–CTX^{ec}	–	11	6	7	14	18	III

*Environmental isolates. All others are clinical isolates.

(VCA0171 and VCA0283) were shown to be more variable, as the allele numbers of these loci were 15 and 20, respectively (Table 1).

Overall, the *V. cholerae* O1 strains analysed in this study could largely be divided into three MLVA profile groups: group I, strains containing MLVA profile 8,7,8,X,X; group II, strains with the MLVA profile 9,7,6,X,X, or 9,3,6,X,X; and group III, strains with the MLVA profile 10,6,7,X,X (Fig. 1 and Table 2). Based on the finding that the last two loci on the small chromosome were not the determinant factor of this grouping, we denoted these two loci as X.

The Mozambican isolates and Bangladeshi strains MJ1236, MG116926 and E1781 had a tandem repeat of the CTX prophage on the small chromosome and had the MLVA profile 8,7,8,X,X (one variant with the profile 7,7,8,X,X was found in Mozambican strain 127-1). This implied that these strains were more closely related than other altered strains with respect not only to their CTX prophage array but also to their genomic variations. The other 13 Bangladeshi isolates contained the RS1–CTX prophage (El Tor *rstR* and classical *ctxB*) array on the large chromosome and belonged to MLVA profile group II with a number of variations (Table 2).

The Indian isolates belonged to one of two MLVA profiles: group II (9,3,6,X,X or 9,7,6,X,X) and group III (10,6,7,X,X). All had an RS1–CTX prophage array on the large chromosome. The CTX prophage on the large chromosome contained the El Tor type *rstR* and the classical type *ctxB*. A number of Indian isolates had an additional amino acid change on the classical *ctxB* background (CT genotype 6) (Goel *et al.*, 2008). This variant *ctxB* was found mainly in group II (9,3,6,X,X); however, a few isolates were also identified in group III (11,6,7,X,X). This suggested that the changes in *ctxB* were independent events from changes on the genome. None of the Indian isolates fitted into group I; however, we cannot exclude the possibility of the presence of strains belonging to group I in India. As the Indian isolates analysed in this study were collected recently (2004–2007) and only from Kolkata, more diverse groups might be found among

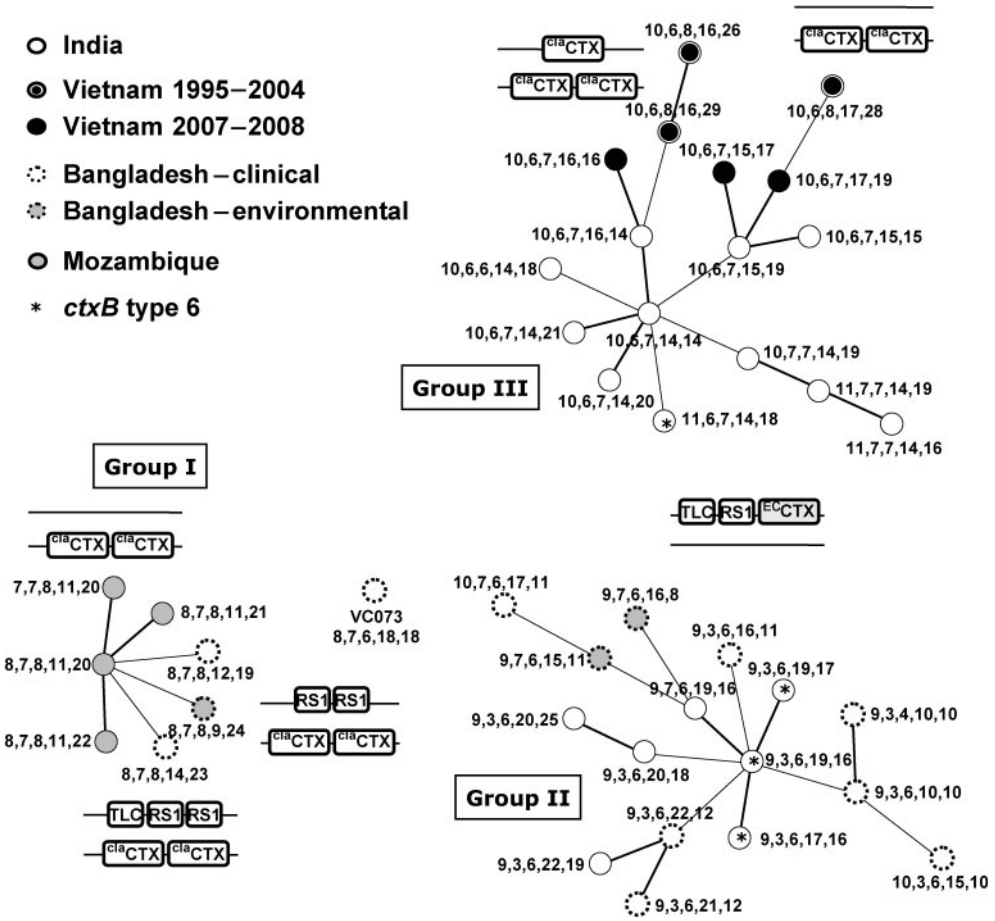

Fig. 1. Minimum spanning tree (based on the categorical coefficient and generated by BioNumerics software; Applied Maths) of *V. cholerae* strains based on MLVA. Countries in which the isolates were collected are indicated by different types of circle. Strains belonging to group I and Vietnamese isolates collected between 1995 and 2004 in group III contained a tandem repeat of the classical CTX prophage on the small chromosome (simple diagrams of the CTX prophage and RS1 element array on both chromosomes are indicated). All others contained the RS1–CTX prophage array with El Tor *rstR* (ET) and classical *ctxB* (cla) on the large chromosome. Isolates that had an additional amino acid change on the classical *ctxB* background are marked with an asterisk. The MLVA profiles of reference strains N16961 (9,7,7,23,14), O395 (7,4,3,24,14) and 569B (10,4,3,15,39) are distant from those of the hybrid/altered strains and are not shown on the tree. TLC, Toxin-linked cryptic element.

isolates collected earlier and from other regions. Chatterjee *et al.* (2009) suggested that some of the Indian isolates collected in 1992 could be considered the progenitors of the Mozambican strains based on CTX prophage array and ribotyping results.

All the Vietnamese isolates belonged to group III (10,6,7,X,X), but we found considerable changes in the CTX prophage array with accompanying changes in the MLVA profile in the span of just a few years (Fig. 1). Among the isolates collected from 1995 to 2004, isolates having a tandem repeat of the classical CTX prophage on the small chromosome had an MLVA profile 10,6,8,**17**,28, whilst isolates with an additional classical CTX prophage on the large chromosome had MLVA profile 10,6,8,**16**,26 or 10,6,8,**16**,29 (Table 2). The isolates collected during cholera outbreaks in 2007 and 2008 had an RS1–CTX prophage array with El Tor *rstR* and classical *ctxB* on the large chromosome, which was a distinguishable characteristic compared with earlier isolates (Nguyen *et al.*, 2009). The MLVA profile of these isolates was 10,6,**7**,X,X, which was also different from the earlier isolates. Although strains with the 10,6,7,X,X profile and RS1–CTX prophage array on the large chromosome were also found in India, strains with an MLVA profile of 10,6,8,X,X and a tandem repeat of the classical CTX prophage on the small chromosome were not found in India or in Bangladesh (Table 2).

In previous reports, O1 strains belonging to group II (9,3,6,X,X profile) and O139 strains belonging to group I (8,7,8,22,23, 8,7,8,21,23 and 8,7,8,21,16) were identified in Bangladesh (Stine *et al.*, 2008). The Bangladeshi *V. cholerae* O1 serogroup strains analysed in our study belonged to group I or group II. These O139 strains and O1 strains

belonging to group I should be analysed further to identify the origin of altered strains and O139 strains. Group II (9,3,6,X,X) and group III (11,7,7,14,X) strains were found throughout India as described previously (Ghosh *et al.*, 2008).

Hybrid and altered strains have been isolated since the early 1990s, as has the O139 serogroup (Chatterjee *et al.*, 2009). An extensive study analysing O1 El Tor and O139 serogroup strains collected during that period is necessary to investigate the generation of the O139 serogroup and O1 hybrid/altered strains of *V. cholerae*. The results in this report suggest that a group of El Tor strains (group I strains and earlier Vietnamese strains) obtained the classical CTX prophage, which integrated into the small chromosome. The El Tor type *ctxB* on the El Tor CTX prophage of other groups of strains (groups II and III) was replaced by the classical *ctxB*. We should not rule out the possibility that the group II and III strains could have merged as they have the same RS1–CTX prophage array.

A possible generation mechanism of the Mozambican strain from El Tor strains has been suggested (Faruque *et al.*, 2007); however, further genetic analyses including whole-genome sequencing of diverse strains, prototype El Tor strains and hybrid/altered strains are required to elucidate the origin of hybrid/altered strains. Among the strains analysed in this study, we found that B33 and MJ1236 produced significantly more CT than other strains under *in vitro* AKI conditions (Iwanaga *et al.*, 1986). Detailed analyses of the amount of toxin they produce, the possible mechanism of this difference and a link between the degree of virulence of these strains and their toxin production abilities is under way.

In conclusion, three MLVA profile groups were identified among the hybrid and altered strains of *V. cholerae* O1 serogroup analysed in this study. We found that strains with a tandem repeat of the classical CTX prophage on the small chromosome could be categorized into two MLVA profile groups (group I with MLVA profile 8,7,8,X,X, collected in Mozambique and Bangladesh, and group III with MLVA profile 10,6,8,X,X collected in Vietnam). Strains with the RS1–CTX prophage (El Tor type *rstR* and classical *ctxB*) array on the large chromosome could be classified into two groups: group II strains with an MLVA profile of 9,3,6,X,X/9,7,6,X,X were found in India and Bangladesh, and group III strains containing the MLVA profile 10,6,7,X,X were found in India and Vietnam.

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