ISSN 0891-4168, Molecular Genetics, Microbiology and Virology, 2015, Vol. 30, No. 1, pp. 15–23. © Allerton Press, Inc., 2015.
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EXPERIMENTAL WORKS

MLVA Typing of Clinical *Vibrio cholerae* Strains Isolated during Different Periods of the Current Cholera Pandemic

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Received March 6, 2014

Abstract—MLVA typing was performed for five variable loci of the 52 *Vibrio cholerae* El Tor biovar strains isolated before the onset and in different periods of the seventh cholera pandemic, as well as for eight strains of the classical *V. cholerae* biovar—the causative agent of the preceding Asiatic cholera pandemics. It has been shown that the studied strains, which differ in their molecular genetic properties and are characterized by 38 MLVA types, form seven clonal clusters. Clusters I and II were formed, respectively, by *V. cholerae* strains of the classical biovar and prepandemic *V. cholerae* strains, whereas clusters III–VII were formed by *V. cholerae* El Tor biovar strains isolated in different periods of the latest pandemic. It has been shown that the MLVA typing allows us to clearly differentiate *V. cholerae* El Tor biovar strains into typical and genetically altered strains. The genetically altered strains are characterized by higher virulence compared with the typical strains, as well as the presence of the *ctxB* gene of the classical cholera vibrio in the genome. The study showed the possibility of differentiating genovariants isolated in different periods and having different structures of pandemicity island VSP-II. Since the VSP-II structure directly correlates with the epidemic potential of the strains, the possibility of differentiating isolates with intact and deleted VSP-II using the MLVA method deserves further research. Based on the obtained results, we can propose a polyclonal origin of different strains of genovariants.

Keywords: *Vibrio cholera*, genovariants, allele of the *ctxB* gene, pandemicity island VSP-II, MLVA typing
DOI: 10.3103/S0891416815010085

INTRODUCTION

The genome of the causative agent of cholera with a significant part thereof represented by mobile genetic elements has undergone considerable changes over a relatively short period of evolution. As a result, this has led to the emergence of toxigenic *Vibrio cholerae* strains varying in genetic traits and epidemic potential. We especially note the *V. cholerae* O1-serogroup strains that belong to two different biovars—classical and El-Tor—which have caused seven recorded cholera pandemics. The classical *V. cholerae* biovar caused the sixth and, seemingly, the first five pandemics (1817–1923), and the El Tor cholera vibrios caused the current (seventh) pandemic (from 1961 to present). The biovar was replaced between 1910 and 1961; however, the exact causes for the replacement of classical strains are yet unknown [1, 2]. The search for differences between the genomes of the *V. cholerae* strains isolated in different periods has revealed chromosomal fragments undergoing significant changes in evolution. The highest variability was demonstrated by CTXφ prophage genome with the *ctxAB* operon encoding cholera toxin (CT), which is the key virulence factor. The classical and El Tor strains contained different types of the CTXφ prophage (CTXφ^{Class} and CTXφ^{Eltor}) differing in allelic variants of the *ctxB* gene encoding the B-subunit of CT. All classical strains contained the *ctxB1* allele, whereas the El Tor cholera vibrios carried the *ctxB3* allele [3, 4]. One of the biological consequences of these differences is a lower level of virulence in typical El Tor vibrios than in classical strains. Another significant difference between the two biovars is the presence of two additional fragments of DNA in the genome, the so-called pandemicity islands VSP-I and VSP-II (*Vibrio* seventh pandemic island), which probably determine the high level of resistance in this pathogen to different environmental stress exposures and are a genetic marker for the causative agent of the current pandemic [2]. It has comparatively recently (1990–1991) become apparent that we have encountered newly emerged genetically altered *V. cholerae* El Tor strains or genovariants characterized by higher virulence, compared with typical isolates [5–9]. These genovariants are thought to have resulted from the acquisition of the CTXφ^{Class} prophage of classical vibrios or its *ctxB1* gene by typical *V. cholerae* El Tor genome strains through a horizontal gene transfer.

When the first genovariants of the cholera El Tor pathogen were identified, the next question concerned the types of strains serving as a possible donor for the classical type *ctxB* gene. No clear answer has been received so far to this question. We can only suggest

that the donor of this gene, apart from the classical *V. cholerae* biovar, could also have been the *V. cholerae* El Tor biovar strains, which were isolated prior to the onset of the seventh cholera epidemic on Celebes Island in Indonesia (1937) and which carried the $CTX\varphi^{Class}$ prophage genome [1]. Nevertheless, we have very limited data on the genetic links between possible donors for the *ctxB1* gene and the genovariants that acquired this gene.

The circulation of typical and genetically altered strains of the *V. cholerae* El Tor biovar with varying levels of virulence and varying epidemic potential in the world's regions that are endemic for cholera also creates preconditions for their emergence in this country. Hence, studies aimed at differentiating between typical strains and genovariants carried by tourists to Russia and causing epidemic outbreaks or local cholera cases are important. The question of whether the origin of these genovariants is mono- or polyclonal is also still to be answered. Some authors suggest that a the wide range of genovariants is the result of expansion of a single clone that was a derivative of some single prototypic strain of the El Tor cholera agent and emerged in a particular territory. According to the second hypothesis, such an event had to be caused by the multiclonal emergence of genovariants in several endemic regions [10]. The latter implies that the genetic diversity of genovariants is quite significant.

To solve this problem through molecular typing, we chose multilocus variable number tandem repeat analysis (MLVA) based on comparative analysis of the number of variable tandem repeats in the loci located at chromosomes I and II of the cholera vibrio [11, 12]. The high resolution capacity of MLVA typing compared with other methods (based on a single nucleotide polymorphism, SNP, and multilocus sequence typing, MLST) was previously shown in the study of various strains of many pathogenic bacteria, including the cholera vibrio [13–16].

The aim of this work was to study the possibility of differentiating between typical and genetically modified strains of the El Tor cholera agent using the MLVA method, evaluate the genetic diversity in the genovariants, and elucidate their phylogenetic links with the classical *V. cholerae* biovar and prepandemic *V. cholerae* El Tor biovar strains.

MATERIALS AND METHODS

Bacterial strains. The study was based on 59 clinical *V. cholerae* strains and one strain isolated from the water. Among the investigated isolates, 8 strains belonged to the classical *V. cholerae* biovar, 52 isolates belonged to the *V. cholerae* El Tor biovar isolated before the onset (1937) and in different periods (1967–2012) of the seventh pandemic in Russia's regions, as well as in countries of the near and far abroad. The pandemic strains were represented by 20 typical strains of the agent and by 30 genovariants thereof. All the strains were provided by the State Collection of Pathogenic Bacteria of the Microbe Russian Research Anti-Plague Institute, where they were preserved in a lyophilized state. Luria Bertani (LB) broth and agar were used for culturing the bacteria.

Bacterial DNA was isolated with guanidine thiocyanate using licensed commercial kits for its isolation (DNA-sorb, AmpliSens, Russia) in accordance with the attached guidelines. The obtained samples containing total DNA of cholera vibrios were used for amplifying gene fragments.

MAMA PCR. Mismatch Amplification Mutation Assay (MAMA) PCR was used for differentiating the classical and El Tor alleles of the *ctxB* gene [17]. Determination of the corresponding gene allele was based on the detection of differences in its nucleotide sequence at position 203 using three primers: one common direct (5'-ACTATCTTCAGCATATGCACAGG-3') and two reverse primers specific, respectively, for the classical *ctxB* type (5'-CCTGGTACTTCTACTTGAAACG-3') or the El Tor type (5'-CCTGGTACTTCTACTTGAAACA-3') [18].

Sequencing the *ctxB* gene. Genomic DNA was sequenced on an ABI 3500×1 genetic analyzer. The obtained nucleotide sequence of the *ctxB* gene was analyzed using the Mega 5.0 software and compared with the sequence of the reference strains represented in the GenBank database. Cytosine (C) was present in the nucleotide sequence of the *ctxB1* at positions 115 and 203, whereas the *ctxB3* alleles had thymine (T) at the same positions [4].

Structural study of pandemicity island VSP-II. The structure of VSP-II was determined by the PCR method using 12 pairs of specific primers described previously in [19].

MLVA typing. The variable fragments of the *V. cholerae* genome were represented by five previously described loci VC0144, VC0436-7, VC01650, VCA0171, and VCA0283; the first three are localized at the first chromosome and the last two at the second chromosome [11, 14]. Using specific primers (Table 1) for the PCR, the scheme of which was described previously in [14], we obtained amplicons, 5 for each of 60 strains. DNA amplification was performed using an iCycler IQ5 computerized thermocycler with a heated lid (BioRad, United States). The obtained amplicons were sequenced on an ABI 3500×1 genetic analyzer.

Analysis of the results. The primary analysis of DNA sequences was performed using the Data Collection v. 1.0 and Sequencing Analysis Software v. 5.4. For the further analysis, we used the BioEdit 7.1.3 and Mega 5 software. To determine the number of tandem repeats, we used the Tandem Repeats Finder software v. 4.0 (G. Benson, 2004, http://tandem.bu.edu/trf/trf.download.html). The obtained data were imported into the BioNumerics 7.1 database (Applied Maths, Belgium). To construct the phylogenetic tree, we used

Table 1. VNTR loci and sequences for the primers used in the study

Chromosome	Locus	Nucleotide repeat sequence	Primers (5'–3')
I	VC0147 (protein FtsY)	AACAGA	CCAAACCACTGCAACGATAGCTGCTCGACCTGAGAGAGA
	VC0436-7 (intergenic region)	GACCCTA	CGTGGTACTAAGTTCCACGCCGTTTTTACCACGCTCCGCTTC
	VC1650 (collagenase)	GATAATCCA	CTACCAAGCGGCGGTTAAGCTGTGGGCAACCTGCTGGTAGC
II	VCA0171 (hypothetical protein)	TGCTGT	GCATCATCCACAGCGTTTGGGCTGAAGCCTTTCGCGATCC
	VCA0283 (hypothetical protein)	ACCAGA	CTTCATCGGCAAACAAGACATTGCGCACAATTCTCTTTGA

VNTR loci and the sequences of primers were cited from [10].

the Maximum parsimony tree method with the category coefficient. The variable number tandem repeats (VNTR) of the loci were determined using the Nei diversity index (DI) [20].

RESULTS AND DISCUSSION

Specific molecular genetic traits of the studied *V. cholerae* strains. To find genetic links between the recently emerged cholera vibrio El Tor genovariants and other virulent *V. cholerae* strains isolated in different periods and assess their possible differentiation using the MLVA method, we took three groups of isolates differing from one another in the structure of genomic fragments responsible for virulence and adaptation to the environmental stress exposures. The first group was represented by eight classical *V. cholerae* biovar strains isolated on the territory endemic for cholera (India and Pakistan) in 1937–1969. The MAMA PCR and sequencing results showed the presence of the CTXφ^{Class} prophage genome containing the *ctxB1* allele in their chromosomes. At the same time, these strains lacked pandemicity island VSP-II (Table 2). These results completely agree with the data reported by other authors [2].

The second group included two clinical *V. cholerae* El Tor biovar strains (MAK757 and MAK676) isolated in 1937 on Celebes Island (Indonesia) before the onset of the current cholera pandemic. Analysis of the molecular genetic traits of these prepandemic strains showed the presence of the CTXφ^{Class} prophage with the *ctxB1* allele in their genome and the absence of VSP-II, which was confirmed by other studies [1, 2].

The third group consisted of 49 clinical *V. cholerae* El Tor biovar strains isolated in different periods of the seventh cholera pandemic and one strain isolated from the sea water. Among them, 20 clinical strains were isolated in the initial period of the pandemic (1967–1990) on the territories of India and Russia and carried the *ctxB3* allele in the CTXφ^{Eltor} prophage and the intact VSP-II [19]. The other 30 strains, including one from seawater, have been imported into Russia and Ukraine from different countries endemic for cholera over the last two decades (1991–2012) and belonged to the above-mentioned genovariants containing a DNA fragment of the classical cholera vibrio biovar in their genome. The totality of our data show that the represented strains of the genovariants differed from one another in the structure of their genome [19, 21]. The *ctxB1* allele was present in the genome of 90% of the studied genovariants. At the same time, researchers found strains differing from other genovariants in the presence of another allele of the *ctxB* gene—namely, *ctxB7*—which had an additional mutation—a nonsynonymous substitution of C for A at position 58. Furthermore, the genovariants isolated over the last two decades (1991–2001 and 2002–2012) differed from one another in the structure of pandemicity island VSP-II. Whereas the genovariants isolated in 1991–2001 carried an intact VSP-II or VSP-II with a short deletion embracing four genes (VSP-IIΔvc0495-vc0498), all the genovariants imported to Russia over the last decade (2002–2012) had VSP-II with an extended deletion, or VSP-IIΔvc0495-vc0512 (see Table 2) [19, 21]. The cause for this variability of VSP-II in the genovariants is still unclear. However, the important issue is that the indicated restructuring in this genomic island may, perhaps, serve as a genetic marker for strains with a high pandemic potential, since strains containing VSP-II with an extended deletion have forced out the genovariants with the prototypic VSP-II in many regions endemic for cholera.

Thus, the studied cholera vibrio strains isolated from patients and outer media during periods of various epidemic complications are characterized by considerable genetic diversity, which was manifested by the presence of individual nucleotide substitutions, deletions, as well as by the acquired genetic informa-

Table 2. Molecular genetic characteristics for the studied *V. cholerae* strains of classical and El Tor biovars and their MLVA profiles

No.	Strain	Place and year of isolation	Allele of the *ctxB* gene	Pandemicity island VSP-II	Allelic profiles VC0147, VC0436-7, VC1650, VCA0171, VCA0283	MLVA type
Classical *V. cholerae* biovar						
1	1488"a"	India, Calcutta, 1937	*ctxB1*	–	8, 3, 3, 7, 8	1
2	B1307	Pakistan, Dhaka, 1964	*ctxB1*	–	8, 4, 3, 20, 26	2
3	325	India, 1946	*ctxB1*	–	8, 3, 3, 7, 20	3
4	J89	Pakistan, 1946	*ctxB1*	–	8,3,3,23,30	4
5	266	India, 1949	*ctxB1*	–	8, 3, 3, 16, 17	5
6	D3	Pakistan, 1958	*ctxB1*	–	8, 4, 3, 21, 26	6
7	569B	India, 1948	*ctxB1*	–	10, 4, 3, 16, 34	7
8	Dakka 35	Pakistan, Dhaka, 1958	*ctxB1*	–	8, 4, 3, 15, 27	8
Prepandemic strains of *V. cholerae* El Tor biovar						
9	MAK676	Indonesia, Celebes I., 1937	*ctxB1*	–	10, 6, 7, 14, 13	9
10	MAK757	Indonesia, Celebes I., 1937	*ctxB1*	–	10, 6, 7, 14, 16	10
Typical pandemic strains of *V. cholerae* El Tor biovar						
11	9/67	India, 1967	*ctxB3*	VSP-II	8, 6, 7, 15, 24	11
12	1/67	India, 1967	*ctxB3*	VSP-II	8, 6, 7, 15, 24	
13	M736	Russian Federation (RF), Perm, 1970	*ctxB3*	VSP-II	8, 6, 7, 18, 26	12
14	M738	RF, Perm, 1970	*ctxB3*	VSP-II	8, 6, 7, 18, 26	
15	M818	RF, Saratov, 1970	*ctxB3*	VSP-II	8, 6, 7, 18, 25	13
16	M887	RF, Astrakhan, 1970	*ctxB3*	VSP-II	8, 6, 7, 18, 25	
17	M963	RF, Astrakhan, 1972	*ctxB3*	VSP-II	8, 6, 7, 18, 25	
18	M888	RF, Astrakhan, 1970	*ctxB3*	VSP-II	8, 6, 7, 18, 24	14
19	M1011	RF, Bashkortostan, Ufa, 1972	*ctxB3*	VSP-II	8, 6, 7, 18, 24	
20	M1013	RF, Bashkortostan, Ufa, 1972	*ctxB3*	VSP-II	8, 6, 7, 18, 24	
21	P2938	Ukraine, Kerch, 1970	*ctxB3*	VSP-II	9, 6, 7, 14, 21	15
22	P3109	Ukraine, Odessa, 1970	*ctxB3*	VSP-II	9, 6, 7, 14, 27	16
23	M582	RF, Kalmykia, Elista, 1974	*ctxB3*	VSP-II	8, 6, 7, 19, 25	17
24	M568	RF, Mordovia, Saransk, 1974	*ctxB3*	VSP-II	8, 6, 7, 19, 23	18
25	M569	RF, Mordovia, Saransk, 1974	*ctxB3*	VSP-II	8, 6, 7, 19, 23	
26	M589	RF, Perm, 1974	*ctxB3*	VSP-II	8, 6, 7, 19, 23	
27	C-402	RF, Stavropol, 1990	*ctxB3*	VSP-II	9, 6, 8, 22, 20	19
28	C-447	RF, Stavropol, 1990	*ctxB3*	VSP-II	9, 6, 8, 22, 20	
29	M1261	RF, Perm, 1990	*ctxB3*	VSP-II	9, 6, 8, 23, 20	20
30	M1259	RF, Perm, 1990	*ctxB3*	VSP-II	9, 6, 8, 23, 20	
Genetically altered pandemic strains of *V. cholerae* El Tor biovar						
31	P15384	Ukraine, Vilkino, 1991	*ctxB1*	VSP-II	8, 7, 8, 16, 6	21
32	P15653	Ukraine, Nikolaev, 1991	*ctxB1*	VSP-II	8, 7, 8, 15, 14	22
33	M1297	RF, Dagestan, Makhachkala, 1993	*ctxB1*	VSP-II	8, 7, 8, 14, 20	23
34	M1275	RF, Dagestan, Makhachkala, 1993	*ctxB1*	VSP-II	8, 7, 8, 14, 20	
35	M1264	RF, Krasnodar, 1993	*ctxB1*	VSP-II	8, 7, 8, 11, 20	24
36	M1266	RF, Perm, 1993	*ctxB1*	VSP-II	8, 7, 8, 11, 20	
37	M1299	RF, Krasnodar, 1993	*ctxB1*	VSP-II	8, 7, 8, 11, 24	25
38	M1272	RF, Krasnodar, 1993	*ctxB1*	VSP-II	8, 7, 8, 11, 21	26

Table 2. (Contd.)

No.	Strain	Place and year of isolation	Allele of the *ctxB* gene	Pandemicity island VSP-II	Allelic profiles VC0147, VC0436-7, VC1650, VCA0171, VCA0283	MLVA type
39	M1271	RF, Tatarstan, Kazan, 1993	*ctxB1*	VSP-II	8, 7, 8, 11, 21	
40	M1270	RF, Tatarstan, Kazan, 1993	*ctxB1*	VSP-II	8, 7, 8, 11, 21	
41	M1293	RF, Dagestan, Sulina, 1994	*ctxB1*	VSP-II	8, 7, 8, 15, 20	27
42	M1294	RF, Dagestan, Aidi Kutan, 1994	*ctxB1*	VSP-II	8, 7, 8, 15, 20	
43	M1269	RF, Magnitogorsk, 1994	*ctxB1*	VSP-II	8, 7, 8, 15, 20	
44	M1268	RF, Magnitogorsk, 1994	*ctxB1*	VSP-II	8, 7, 8, 15, 20	
45	P17647	RF, Achinsk, 1997	*ctxB1*	VSP-IIΔvc0495-0498	9, 8, 6, 13, 24	28
46	P17644	RF, Achinsk, 1997	*ctxB1*	VSP-IIΔvc0495-0498	9, 8, 6, 13, 24	
47	M1326	RF, Dagestan, Rubas, 1998	*ctxB1*	VSP-II	9, 7, 8, 15, 22	29
48	M1327	RF, Dagestan, Khorezi, 1998	*ctxB1*	VSP-II	9, 7, 8, 15, 22	
49	M1328	RF, Dagestan, Khorezi, 1998	*ctxB1*	VSP-II	9, 7, 8, 15, 22	
50	M1344	RF, Tatarstan, Kazan, 2001	*ctxB1*	VSP-II	11, 7, 6, 9, 13	30
51	M1345	RF, Tatarstan, Kazan, 2001	*ctxB1*	VSP-II	11, 7, 6, 6, 13	
52	M1349	RF, Tatarstan, Kazan, 2001	*ctxB1*	VSP-II	11, 7, 6, 6, 13	
53	M1429	RF, Bashkortostan, Ufa, 2004	*ctxB1*	VSP-IIΔvc0495-0512	9, 3, 6, 20, 17	31
54	M1430	RF, Tver, 2005	*ctxB1*	VSP-IIΔvc0495-0512	9, 3, 6, 20, 18	32
55	P18899	RF, Murmansk, 2006	*ctxB1*	VSP-IIΔvc0495-0512	9, 3, 6, 10, 19	33
56	L-3225	RF, Moscow, 2010	*ctxB7*	VSP-IIΔvc0495-0512	9, 3, 6, 14, 19	34
57	L-3226	RF, Moscow, 2010	*ctxB7*	VSP-IIΔvc0495-0512	9, 3, 6, 15, 19	35
58	L-4150	RF, Moscow, 2010	*ctxB7*	VSP-IIΔvc0495-0512	9, 3, 6, 13, 20	36
59	301	RF, Taganrog, 2011	*ctxB1*	VSP-IIΔvc0495-0512	8, 3, 6, 19, 18	37
60	M1509	RF, Moscow, 2012	*ctxB1*	VSP-IIΔvc0495-0512	9, 3, 6, 14, 22	38

* Pandemicity island VSP-II is intact; VSP-IIΔvc0495-0498—carrying a short deletion of four genes; VSP-IIΔvc0495-0512—has an extended deletion of 21 genes.

tion affecting the expression of virulent traits and the adaptation to stress exposures.

MOLECULAR TYPING OF THE STUDIED *V. cholerae* STRAINS BY THE MLVA METHOD

To perform MLVA typing of the studied strains as variable fragments of *V. cholerae* genome, we used five loci with the following designations: VC0147, VC0436-7, VC1650, VCA0171, and VCA0283 [11, 14]. The loci VC0147, VC0436-7, and VC1650 are localized at chromosome I in the areas of 136806–137459, 466811–467459, and 1778210–1778872 bps, respectively, whereas loci VCA0171 and VCA0283 are localized at chromosome II in the areas of 187459–188196 and 303639–304322 bps, respectively. The nucleotide sequences of the loci and their specific primers applied are indicated in Table 1. It has been established based on the study of DNAs of 60 strains that the loci at chromosome I are characterized by the presence of three to five alleles, whereas the loci at chromosome II are represented by 14–15 alleles. Differences in the level of polymorphism among the compared loci are also confirmed by the Nei diversity index (DI) [20]. The DI for the loci of chromosome I and chromosome II accounted for 0.66–0.68 and 0.91–0.92, respectively. This means that there is a higher level of stability of the first three chromosomal loci, compared with the other two localized at chromosome II, which completely agree with many other reported data [13, 14, 22].

Analysis of the obtained results has shown that these 60 strains are referred to 38 different MLVA types. Eight *V. cholerae* classical biovar strains belonged to eight MLVA types while two prepandemic isolates belonged to two types. Such a genetic diversity of the strains isolated nearly 40–70 years ago was associated only with a high variability of the studied loci at chromosome II (see Table 2). The *V. cholerae* El Tor biovar strains have shown 10 MLVA types revealed among 20 typical strains versus 19 types among 30 genovariants. These data point to a pronounced variability in the genome of the studied strains, which may be caused by their time-related (1967–2012) and geographical separateness, as well as

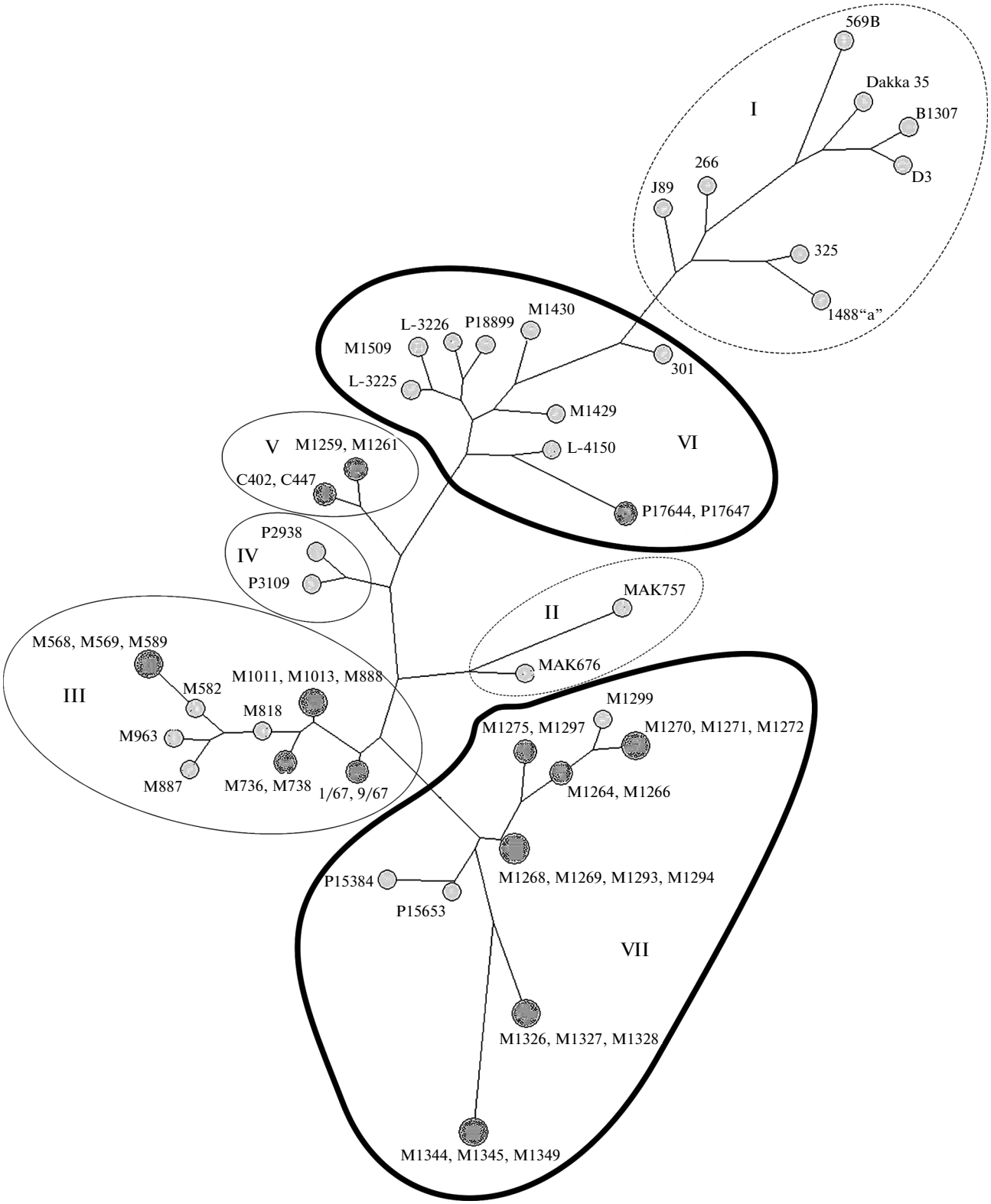
associated with evolutionary processes in the agent's wild populations.

The phylogenetic tree construction based on the MLVA typing for the above mentioned loci and the analysis of the tree allowed us to distinguish seven clusters (I–VII), with each containing closely related strains that had either identical or close genotypes (see figure). Cluster I was formed by the classical strains with allelic profiles clearly differing from those of other strains. All these strains carried the CTXφ^{Class} prophage with the *ctxB1* allele and had no pandemicity islands VSP-I and VSP-II. Cluster II was formed by the prepandemic *V. cholerae* El Tor biovar isolates with very similar allelic profiles 10, 6, 7, 14, 13/10, 6, 7, 14, and 16, which contained, in analogy with the classical strains, the CTXφ^{Class} prophage genome, but lacked VSP-I and VSP-II. Three separate clusters (III, IV, and V) were composed of typical *V. cholerae* El Tor biovar strains carrying the CTXφ^{Eltor} prophage with the *ctxB3* allele and the prototypic VSP-II. The distribution of typical strains across different clonal clusters depended on the time and place of isolating the cultures. Cluster III was represented by 14 isolates referred to 6 different MLVA types (11, 12, 13, 14, 17, and 18). Twelve strains of them were related to the first import of the El Tor cholera to the Volga Region and Central Russia from the Southeast Asian countries in 1970–1974 [23]. When compared, their allelic profiles show that they differed only in the number of repeats in the two most variable loci of chromosome II (VCA0171 and VCA0283), whereas the loci VC0147, VC0436-7, and VC1650 from chromosome I were identical. Their number of alleles in all the compared strains accounted for 8, 6, and 7, respectively. This pointed to their clonal origin. As for the other two minor clusters IV and V, each included two and four strains that were imported either in the same pandemic period (1970) to Ukraine or 20 years after these events to Stavropol and Perm (1990). These isolates equally differed from one another and from the strains of cluster III in the structure of all five loci (see Table 2). This may mean that the epidemic complications in Ukraine (1970), as well as in Perm and Stavropol (1990), were caused by the import of two different agent clones that, in turn, differed from the clones responsible for the epidemic outbreak of cholera in 1970–1974 in Russia. Nevertheless, the allelic polymorphism of the studied loci in all typical strains from three clusters was, in total, of a low level, which pointed to their close interrelatedness (see Table 2 and the figure).

The strains of the *V. cholerae* El Tor genovariants, which caused epidemic outbreaks or sporadic cases of cholera in Russia in the current period of the seventh pandemic (1993–2012), attracted our special interest. As was mentioned above, the presence of the *ctxB1* allele, characteristic of the classical *V. cholerae* biovar, in the genome of the new El Tor pathogen variants was responsible for their difference from typical El Tor strains. It has been found that the studied genovariants formed two separate clusters VI and VII on the phylogenetic tree (see the figure). Their allelic profiles were significantly different from those in the typical strains forming clusters III, IV, and V (see Table 2). Therefore, MLVA typing by five variable loci allows us to differentiate pandemic strains of the *V. cholerae* El Tor biovar into typical and genetically altered ones. Another important conclusion drawn from the analysis of our data allows us to suggest that, using this method, we can differentiate between the groups of genovariants imported to Russia in different periods, according to their genetic traits. The latter is confirmed by the fact that the studied genovariants formed two clearly separate clusters. One (VI) included 20 clinical strains having 10 different allelic profiles and isolated in 1991–2001 in periods of epidemic outbreaks (Dagestan, 1993, 1994, and 1998; Tatarstan, 1993 and 2001; and Ukraine, 1991) and sporadic imports of cholera. Excluding two hypervariable loci localized at chromosome II from the analysis, we obtained the following allelic profiles in the genovariants of this cluster: 8, 7, 8, X, X (70% of isolates); 9, 7, 8, X, X (15% of isolates); and 11, 7, 6, X, X (15%). The presented data demonstrate genetic heterogeneity in strains of this cluster, which may be associated with different sources of infection. The other (VII) cluster contained ten genovariants isolated on the territory of Russia in 1997 (Achinsk) and in 2004–2012 (Ufa, Tver, Murmansk, Moscow, and Taganrog). In contrast to the strains of cluster VI, these isolates were characterized by the presence of either short or extended deletion in their pandemicity island VSP-II. Their allelic profiles (9, 8, 6, X, X and 9, 3, 6, X, X) were noticeably different from those in cluster VI (see Table 2 and the figure). This may mean that the compared groups of genovariants imported to Russia from countries endemic for cholera do not originate from the same clone. Most likely, cholera epidemics recorded in the current period in different regions of the world were caused by genovariants having a polyclonal origin, which agrees with the same idea suggested by other authors [10]. Nevertheless, all these hypotheses require further studies.

As for genetic links between the *V. cholerae* classical biovar and pandemic strains of the *V. cholerae* El Tor biovar, according to our data, the genovariants mainly isolated over the most recent decade of the current pandemic were the closest to them (cluster VII). This hardly anticipated fact can likely be explained by the great degree of genetic similarity between this very group of genovariants and classical vibrios, since the former have undergone additional genomic alterations and have lost a significant portion of genes in VSP-II completely absent in the agent of Asiatic cholera.

The prepandemic strains of the *V. cholerae* El Tor biovar form on the phylogenetic tree a separate branch positioned between genovariants from two different clusters. These data show that prepandemic strains



Phylogenetic tree constructed using the “maximum parsimony tree” method and BioNumerics v. 7.1 software based on MLVA typing for five chromosomal VNTR loci of classical *V. cholerae* biovar and El Tor biovar strains. The ovals encircle clonal clusters (I–VII) including strains of classical *V. cholerae* biovar (I) and *V. cholerae* El Tor biovar strains: prepandemic (II), pandemic typical (III, IV, and V), and genetically altered (VI and VII). The studied strains are shown in the circles.

with a unique genomic structure seem to have an independent origin.

Our comparison between the allelic profiles of the studied genovariants and the profiles of the strains circulating in different countries endemic for cholera and obtained by different teams of researchers have allowed us to suggest that the strains that caused epidemic hardships due to cholera in Dagestan (1993 and 1994), Krasnodar (1993), Perm (1993), Tatarstan (1993), and Magnitogorsk (1994) may have been transmitted from Bangladesh. The grounds for this belief are based in the fact that the allelic profiles of the *V. cholerae* El Tor biovar genovariants (8, 7, 8, X, X) isolated in the aforementioned regions of Russia did not differ from those of strains MJ1236, ME116926, and e1271 circulating among patients and in the outer environment in Bangladesh in 1991 and 1994 [24]. As to the most recent decade, all imports of clinical strains into Russia are associated with Bangladesh or India, since the detected allelic profiles of the genovariants (9, 3, 6, X, X) were the same as in the strains isolated in these regions endemic for cholera in 2004 (Bangladesh) and in 2006–2007 (India) [14]. These data, in total, coincide with the reports of epidemiological investigations. The MLVA typing data cannot help only in finding the issue of the origin for strain 301 within this cluster. This strain was isolated from Azov seawater near Taganrog in 2011 and, according to our results, belonged to a group of virulent genovariants with a high epidemic potential [5]. At the same time, it has recently been shown by the comparative analysis of nucleotide sequences for the genome of 54 *V. cholerae* strains that this genome is linked to the isolates causing cholera in South Africa and Pakistan [25].

Thus, MLVA typing of 52 *V. cholerae* El Tor biovar strains isolated before the onset and in different periods of seventh cholera pandemic, as well as eight strains of the classical *V. cholerae* biovar, which was the causative agent of the preceding six major Asiatic cholera pandemics, has shown that the studied strains, which differ in their molecular genetic traits and belong to 38 MLVA types, form seven clonal clusters. Clusters I and II are formed by strains of the classical *V. cholerae* biovar and prepandemic strains of the *V. cholerae* El Tor biovar, while clusters III–VII are formed by *V. cholerae* El Tor biovar strains isolated during the seventh pandemic. It has been established that MLVA typing allows us to clearly typify *V. cholerae* El Tor biovar strains isolated in different periods of the current pandemic into typical and genetically altered. We have also found the possibility of differentiating between the genovariants isolated in different periods of time and varying in the structure of pandemicity island VSP-II. Since there is a direct link between the structure of VSP-II and the epidemic potential of strains, the possibility, using the MLVA method, of differentiating between isolates with intact and deleted VSP-II deserves much more attention. We should also note that the genetic diversity of genovariants reflects continued evolutionary alterations in the genome of new cholera vibrio variants as a result of accumulated mutations and manifests itself in different types of their MLVA affinity. The obtained data allow us to suggest that different genotypes have a polyclonal origin, which agrees with the reports of other researchers [8]. In addition, the MLVA typing has shown a real possibility for the identification of potential sources of infection, which is one of the major tasks of molecular epidemiological monitoring of cholera.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, grant no. 12-04-00285a.

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Translated by N. Tarasyuk