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Molecular Analyses of *Vibrio cholerae* O1 Clinical Strains, Including New Nontoxigenic Variants Isolated in Mexico during the Cholera Epidemic Years between 1991 and 2000[▽]

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We studied the evolution of *Vibrio cholerae* O1 during the 1991 to 2000 cholera epidemic in Mexico by biochemical, serological, and molecular characterization of strains collected during this period. Strains were divided into toxigenic and nontoxigenic groups according to the presence or absence of genes encoding cholera toxin. As previously reported, we characterized two populations among toxigenic strains, which were present from the first year of the epidemic. BglI rRNA analysis revealed that these strains had ribotype profiles, denoted M5 and M6 in our study, that were identical to those previously designated Koblavi B5 or Popovic 5 and Popovic 6a or Tamayo B21a, respectively. Ribotype M5 was isolated between 1991 and 1993. This ribotype had a low level of genetic variation as detected by pulsed-field gel electrophoresis (PFGE). Ribotype M6 persisted from 1991 to 2000. However, PFGE profiles suggested that two epidemiologically unrelated strains coexisted within this single ribotype from 1995 until the end of the epidemic. We identified three new BglI ribotypes, Mx1, Mx2, and Mx3, from nontoxigenic *V. cholerae* O1 strains isolated between 1998 and 2000; one of them grouped strains positive for the toxin-coregulated pilus island. They differed from nontoxigenic clones isolated in Latin America and on the U.S. Gulf Coast and are probably autochthonous Mexican *V. cholerae* O1 variants. Most of these new variants were isolated from states surrounding the Gulf of Mexico, where the highest incidence of cholera in the country was recorded. Thus, the Mexican Gulf Coast, like the U.S. Gulf Coast, may act as an environmental reservoir of *V. cholerae* O1.

The seventh cholera pandemic, characterized by *Vibrio cholerae* O1 biotype El Tor, is still present around the world. Moreover, the number of reported cholera cases has continuously increased since 2004. The World Health Organization (WHO) (http://www.who.int/wer) reported a 30% increase in cases of cholera worldwide between 2004 (101,383 cases) and 2005 (131,943 cases) and a further 79% increase between 2005 and 2006 (236,860 cases), whereas the number of countries reporting cases has remained constant. At the same time, the global case-fatality rate rose from 1.72% in 2005 to 2.66% in 2006. However, the actual numbers of cholera cases globally are estimated to be much higher than officially reported, due to underreporting and other limitations of surveillance systems. In 2006, the total number of cases reported in Africa accounted for 99% of the global total; Africa has been the continent with the highest number of officially reported cholera cases since 1996. However, in America the number of cases has greatly decreased since 1999, with only 10 cases reported from Canada and the United States (http://www.who.int/wer/2007/wer8231/en/index.html), 4 of which were indigenous to the United States.

Two basic facts distinguish the seventh cholera pandemic from the other six, proposed by Pollitzer (26), occurring before 1961: (i) the epidemic clone shifted from the classic to the El Tor biotype, with an origin in Indonesia rather than the Indian subcontinent, and (ii) the seventh pandemic evolved in two waves, with the first one spreading throughout Asia between 1961 and 1966 and the second spreading to Asia, Africa, and Latin America around 1970 (20).

Before the 1991 epidemic, cholera was mostly absent for a century in South, Central, and North America; a few sporadic cases were reported between 1973 and 1992 on the U.S. Gulf Coast (2), and one case was reported in 1983 in Cancun, Mexico, in a tourist from the United States (1). The epidemic began in 1991 in the coastal regions of Peru and spread rapidly to the eastern, northern, and southern countries of the continent, forming a branch of the seventh pandemic. The appearance of the disease in coastal areas of Peru remains a mystery; some studies suggest maritime transport as the source, whereas others suggest a local source (36).

Following the first report in Peru in January 1991, cholera was reported in Mexico in June 1991. The number of reported cases in Mexico increased from 1991 until 1993, decreased substantially in 1994, but rose to its highest level ever recorded in 1995 (15,526 cases). Thereafter, cholera cases consistently decreased, with no cases of cholera reported by the Mexican Instituto Nacional de Diagnóstico y Referencia Epidemiológica (INDRE) (National Diagnostics and Epidemiological Reference Institute) since 2002 (32). Therefore, two epidemic cycles have been identified: between 1991 and 1994 and between 1995 and 2001.

Strains responsible for the cholera epidemic in Latin America have been extensively characterized using various molecular methods such as restriction endonuclease digestion of plasmids or chromosomal DNA, ribotyping, multilocus enzyme

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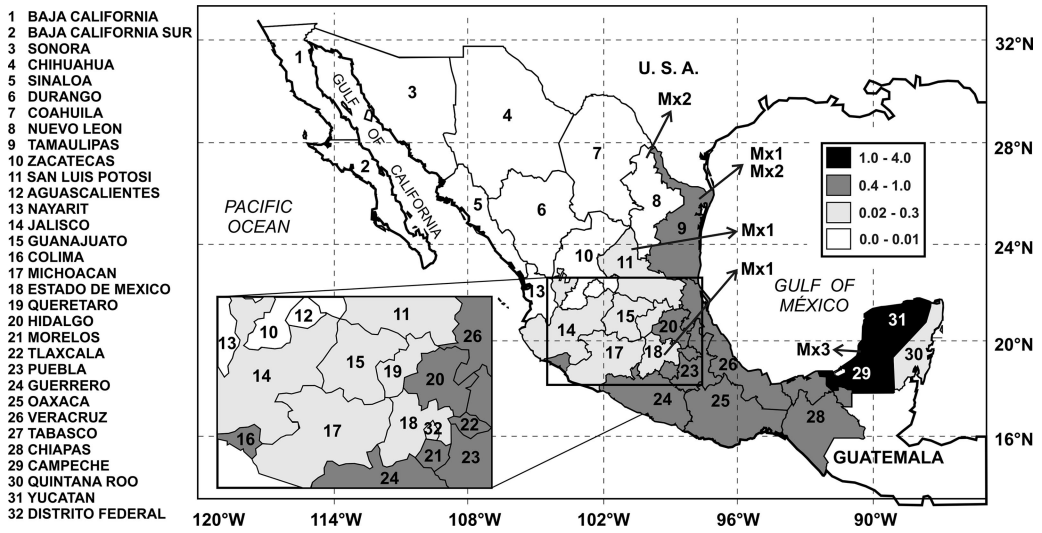

FIG. 1. Average number of cholera cases per 10,000 inhabitants of Mexican states over 10 years (1991 to 2000) and states where Mx ribotypes were isolated.

electrophoresis, and pulsed-field gel electrophoresis (PFGE). These molecular approaches first associated the Latin American epidemic with a single strain, *V. cholerae* O1 biotype El Tor serotype Inaba, which was related to seventh-pandemic isolates from other parts of the world and described as ribotype B5 or 5 according to ribotype schemes reported by Koblavi et al. (21) and Popovic et al. (27) and as a distinct electrophoretic type, electrophoretic type 4, as defined by multilocus enzyme electrophoresis studies (37). A second and distinct epidemic strain, *V. cholerae* O1 biotype El Tor serotype Ogawa, was later associated with the Mexican epidemic. This strain, reported by Popovic et al. (27) to be ribotype 6a, identical to ribotype B21a described by Tamayo et al. (35), belonged to electrophoretic type 3 (37) and was initially isolated in 1991 in Latin America from cholera cases occurring in Mexico and Central America. It may have been responsible for a second epidemic wave (14). The emergence of the cholera epidemic in Latin America led to a closer surveillance of diarrhea diseases and to the consequent isolation of groups of *V. cholerae* O1 strains lacking the pathogenicity factor cholera toxin (CT) in several countries. These nontoxigenic *V. cholerae* O1 clusters, normally found among epidemic *V. cholerae* O1 strains, were reported as *V. cholerae* O1 "variants"; one, called Amazonia, was identified by Coelho et al. (9) in the northern region of Brazil, and a "Tucumán variant" was identified by Pichel et al. (25) in Argentina. An increasing number of these nontoxigenic *V. cholerae* O1 strains, as well as some nontoxigenic *V. cholerae* non-O1/non-O139 strains, have been reported to be associated with diarrhea disease in a number of different countries, causing mild to severe cholera-like diarrhea (31, 33). Such variants were not described in Mexico during the Latin American epidemic. The emergence of these new variants, also detected by *V. cholerae* repeat sequence PCR and repetitive sequence-based PCR systems (13), emphasizes the need for an accurate epidemiological tool to monitor their spread and evolution.

Previous studies of Mexican *V. cholerae* O1 strains subtyped only isolates collected between 1991 and 1995. Consequently, as they do not cover the whole epidemic period, those findings may not be a complete representation. In this study, we selected clinical *V. cholerae* O1 strains collected by INDRE and isolated in different states of Mexico between 1991 and 2000 from patients with severe diarrhea associated with cholera on the basis of clinical symptoms; we examined the evolution of these strains over this period using two DNA-related typing methods, ribotyping and PFGE analysis, selected because of their extensive and successful use for molecular epidemiology surveillance of *V. cholerae* O1 clones.

MATERIALS AND METHODS

Bacterial strains. We studied 125 clinical strains of *V. cholerae* O1, of which 124 were isolated in different states of Mexico between 1991 and 2000, donated by INDRE (Fig. 1; Table 1); the *V. cholerae* O1 strain isolated in Cancun in 1983 (1) (a kind gift from A. Cravioto, Mexican UNAM, Faculty of Medicine) (FMU 87295/0) and responsible for the last cholera case in Mexico before the arrival of the seventh pandemic was included in the study. Strain phenotypes were characterized using biochemical, culturing, and serotyping methods. As references for ribotyping analysis, we used strains representing the 27 ribotype patterns reported by Popovic et al. (27) (provided to the National Reference Center for Vibrios and Cholera by the Centers for Disease Control and Prevention [CDC], Atlanta, GA).

DNA preparation. DNA was extracted by a miniprep procedure adapted from the method of Brenner et al. (4); samples were microdialyzed for 2 hours using VS nitrocellulose filters with 0.025-μm pores (Millipore) against distilled water. The amount and purity of the DNA were measured by spectrophotometry (230, 260, and 280 nm) using the double-stranded DNA programmed method with the Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany).

PCR analysis. The pathogenicity factor genes, *ctxA*, *ctxB*, and *tcpA*, encoding the CT A and B subunits and the toxin-coregulated pilus (TCP) pilin subunit, respectively, were amplified in PCR assays as described elsewhere; all the strains were tested for the presence of classical and El Tor biotype-specific *tcpA* genes (16, 24, 29). Primers were synthesized by Proligo (Paris, France).

Ribotype analysis. We adapted the methods described by Popovic et al. (27) for ribotype analysis. Briefly, DNA (5 μg) was digested overnight with 10 units/μg of BglI restriction endonuclease (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C according to the manufacturer's specifications. Restriction fragments were separated on a 0.8% (wt/vol) agarose gel (type II; Sigma, St. Louis MO) in 1× Tris-borate-EDTA buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3) (Sigma), alkali blotted onto a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany), and immobilized by incubation for 1 hour at 80°C. Digoxigenin-cDNA generated from 16 rRNA plus 23S rRNA from *Escherichia coli* (Roche Diagnostics) was used as a probe. MluI

TABLE 1. Mexican epidemic strains of *V. cholerae* O1 isolated during the seventh cholera pandemic

Isolation yr	Mexican state	No. of strains	Serotype	Ribotype	Pulsotype	*ctxA*	*ctxB*	*tcpA*
1991	Campeche	2	Inaba	M5	PM5.1	+	+	+
	Edo. Mexico	3	Inaba	M5	PM5.1	+	+	+
	Hidalgo	1	Inaba	M5	PM5.2	+	+	+
		1	Ogawa	M6	PM6.1	+	+	+
		2	Ogawa	M6	PM6.2	+	+	+
	Tamaulipas	1	Inaba	M5	PM5.1	+	+	+
	Veracruz	2	Inaba	M5	PM5.1	+	+	+
	Yucatan	1	Inaba	M5	PM5.1	+	+	+
1992	Campeche	1	Inaba	M5	PM5.3	+	+	+
	Colima	1	Ogawa	M6	PM6.1	+	+	+
	Oaxaca	1	Inaba	M5	PM5.1	+	+	+
	Tabasco	1	Inaba	M5	PM5.2	+	+	+
	Veracruz	3	Inaba	M5	PM5.1	+	+	+
		1	Ogawa	M6	PM6.1	+	+	+
	Yucatan	1	Inaba	M5	PM5.1	+	+	+
		1	Ogawa	M6	PM6.1	+	+	+
1993	Oaxaca	1	Ogawa	M6	PM6.3	+	+	+
	Tabasco	1	Ogawa	M5	PM5.1	+	+	+
		1	Ogawa	M6	PM6.1	+	+	+
	Tamaulipas	1	Ogawa	M6	PM6.1	+	+	+
		1	Ogawa		PM6.3	+	+	+
	Veracruz	1	Ogawa	M6	PM6.1	+	+	+
	Yucatan	1	Inaba	M5	PM5.1	+	+	+
1994	Oaxaca	1	Ogawa	M6	PM6.1	+	+	+
	Tamaulipas	2	Ogawa	M6	PM6.1	+	+	+
		1	Ogawa		PM6.3	+	+	+
	Veracruz	1	Inaba	M6	PM6.1	+	+	+
		3	Ogawa			+	+	+
1995	Campeche	1	Ogawa	M6	PM6.1	+	+	+
	Chiapas	1	Ogawa	M6	PM6.1	+	+	+
	Distrito Federal	1	Ogawa	M6	PM6.1	+	+	+
	Nayarit	1	Ogawa	M6	PM6.3	+	+	+
	Oaxaca	1	Ogawa	M6	PM6.1	+	+	+
	Tabasco	1	Inaba	M6	PM6.1	+	+	+
		1	Ogawa			+	+	+
	Tamaulipas	5	Ogawa	M6	PM6.1	+	+	+

Continued on following page

TABLE 1—*Continued*

Isolation yr	Mexican state	No. of strains	Serotype	Ribotype	Pulsotype	*ctxA*	*ctxB*	*tcpA*
	Veracruz	5	Ogawa	M6	PM6.1	+	+	+
		1	Ogawa		PM6.2	+	+	+
	Yucatan	4	Ogawa	M6	PM6.1	+	+	+
1996	Chiapas	1	Ogawa	M6	PM6.1	+	+	+
	Tabasco	3	Ogawa	M6	PM6.1	+	+	+
	Veracruz	1	Ogawa	M6	PM6.1	+	+	+
		1	Ogawa		PM6.3			
	Yucatan	10	Ogawa	M6	PM6.1	+	+	+
1997	Hidalgo	1	Inaba	M6	PM6.3	+	+	+
	Quintana Roo	1	Ogawa	M6	PM6.1	+	+	+
	Tamaulipas	4	Ogawa	M6	PM6.1	+	+	+
		1	Ogawa		PM6.2	+	+	+
		1	Ogawa		PM6.3	+	+	+
	Veracruz	5	Ogawa	M6	PM6.1	+	+	+
1998	Chiapas	1	Ogawa	M6	PM6.1	+	+	+
		1	Ogawa		PM6.2	+	+	+
	Edo. de Mexico	1	Ogawa	Mx1	PMx1.1	−	−	−-
	Guanajuato	3	Ogawa	M6	PM6.2	+	+	+
	Guerrero	1	Ogawa	M6	PM6.1	+	+	+
	Hidalgo	1	Ogawa	M6	PM6.2	+	+	+
	San Luis Pot.	1	Ogawa	Mx1	PMx1.1	−	−	−
		1	Ogawa		PMx1.2			
	Sonora	1	Inaba	M6	PM6.1	+	+	+
	Tamaulipas	1	Inaba	Mx1	PMx1.1	−	−	−
1999	Edo. de Mexico	1	Ogawa	Mx1	PMx1.1	−	−	−
	Morelos	1	Ogawa	M6	PM6.1	+	+	+
	Nuevo Leon	1	Ogawa	Mx2	PMx2	−	−	+
	Tabasco	2	Ogawa	M6	PM6.2	+	+	+
	Tamaulipas	1	Ogawa	Mx1	PMx1.1	−	−	−
		2	Ogawa	Mx2	PMx2	−	−	+
2000	Campeche	3	Ogawa	Mx3	PMx3	−	−	−
	Chiapas	1	Ogawa	M6	PM6.1	+	+	+
		9	Ogawa		PM6.2	+	+	+
		1	Owaga		PM6.4	+	+	+
	Tamaulipas	1	Ogawa	Mx2	PMx2	−	−	+

(Roche Diagnostics) restriction fragments of *Citrobacter koseri* strain 32 (Collection de l'Institut Pasteur CIP 1066-70) were used as molecular weight markers. Hybridization was performed at 65°C. Hybridized fragments were detected by antidigoxigenin alkaline phosphatase-labeled antibodies and the appropriate colorimetric reagents (Roche Diagnostics).

Ribotype patterns were digitalized and interpreted with Pasteur Taxotron programs (Taxolab; Pasteur Institute, Paris, France), using the global reciprocal method as the interpolation algorithm; strains were grouped using the single-linkage method. Schematic dendrograms were produced using the program Dendrograf.

PFGE analysis. DNA for PFGE was prepared as previously described (5). Total DNA was digested with NotI endonuclease (Roche Diagnostics, Mannheim, Germany), which is commonly used for *V. cholerae* PFGE studies (see, e.g., references 7, 17, 19, 28, and 30). Restriction fragments were separated by electrophoresis in a 1.2% pulsed-field certified agarose gel (Bio-Rad Laboratories, Richmond, CA) in 0.5× Tris-borate-EDTA in a Bio-Rad CHEF-DR III system. Conditions used for electrophoresis were 14°C, 6 V/cm (200 V), a field angle of 120°, and a 3- to 4-s linear ramp time for 12 h followed by a 5- to 50-s linear ramp time for a further 22 h. Phage lambda DNA ladders (Bio-Rad Laboratories) were used as molecular size markers. Gels were stained with ethidium bromide (0.5 μg/ml; Eurobio, France) and visualized with a Bio-Rad Gel Doc (EQ 170-8066). PFGE patterns were analyzed with Pasteur Taxotron programs using the Spline method.

RESULTS

The average number of cholera cases per 10,000 inhabitants in Mexico over a 10-year period (1991 to 2000) was calculated using INDRE epidemiological data (www.ssa.gob.mx) (Fig. 1). Overall, high frequencies of disease (0.4 to 4) were observed in the southeast zone of the country and low rates (0.00 to 0.01) in a northwest zone. The states surrounding the Gulf of Mexico all had high rates, with the highest rates in Yucatan and Campeche. Borroto and Martinez-Piedra (3) described a similar distribution of cholera cases in Mexico for the period between 1991 and 1996, suggesting that a high level of poverty, a low level of urbanization, and a southern location favor the development of cholera in Mexico. Many strains collected for this study were isolated in these states.

Bacterial strains. The year and state of isolation, serotype, ribotype, pulsotype, and presence of *ctxA*, *ctxB*, and *tcpA* genes for Mexican epidemic strains are listed in Table 1. All the studied strains belonged to the O1 serogroup of the species *V. cholerae*. With the exception of the Cancun strain, belonging to the classical biotype, all the strains had the El Tor biotype; 22 strains had the Inaba serotype, and 102 had the Ogawa serotype.

Genetic diversity. Strains were characterized according to the presence or absence of genes encoding pathogenicity factors; 111/124 epidemic strains were positive for the presence of *ctxA* and *ctxB* genes, encoding the CT A and B subunits. All these toxigenic *V. cholerae* O1 strains were positive for the El Tor-specific *tcpA* gene encoding the TCP factor. Among the 13 nontoxigenic *V. cholerae* O1 strains, 4 were positive for the El Tor-specific *tcpA* gene. The other *V. cholerae* O1 nontoxigenic strains were negative for the *tcpA* gene, using El Tor or classical biotype-specific primer pairs. The *V. cholerae* O1 strain isolated in Cancun in 1983 was positive for the *ctxA* and *ctxB* genes and for the classical biotype-specific *tcpA* gene.

Ribotypes. We found five closely related BglI ribotype patterns among the *V. cholerae* O1 strains studied (Fig. 2). All toxigenic isolates belonged to ribotypes denoted M5 and M6 in this study. Ribotype M5 was identical to BglI ribotype B5 of Koblavi et al. or 5 of Popovic et al., and ribotype M6 was identical to ribotype 6a or B21a of Popovic et al. and Tamayo et al., respectively (21, 27, 35). Three new ribotypes, Mx1, Mx2, and Mx3, were identified among nontoxigenic strains. The ribotype M5 cluster included 19 strains isolated between 1991 and 1993, 18 of which had the Inaba serotype and 1 of which had the Owaga serotype. The ribotype M6 cluster contained 92 strains isolated between 1991 and 2000, including 88 Ogawa serotype and 4 Inaba serotype strains. Ribotypes Mx1, Mx2, and Mx3 were isolated between 1998 and 2000. Ribotype Mx1 strains included five Owaga strains and one Inaba strain isolated in 1998 and 1999 from Tamaulipas, San Luis Potosi, and Estado de México.

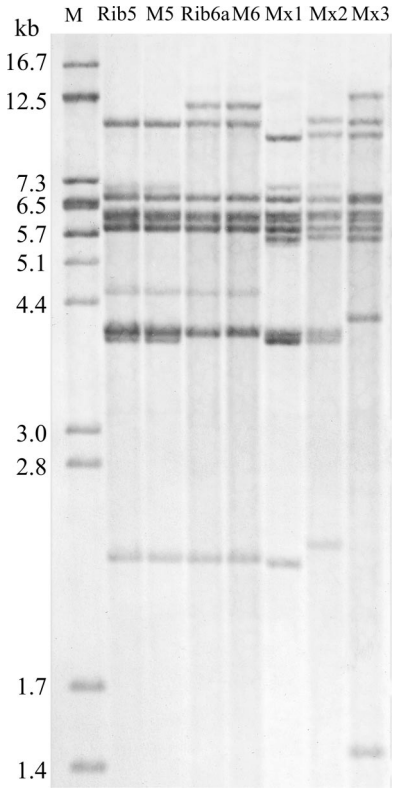

FIG. 2. Representative BglI ribotype patterns. Each lane is designated by the ribotype number used in the study. Rib5a refers to profiles B5 and 5 of Koblavi et al. (21) and Popovic et al. (27), and Rib6 refers to profiles 6a and B21a of Popovic et al. (27) and Tamayo et al. (35).

Ribotype Mx2 included four strains with the Ogawa serotype, isolated in 1999 and 2000 from Tamaulipas and Nuevo León, and ribotype Mx3 included three Ogawa strains isolated in 2000 in Campeche. The Mx2 ribotype included exclusively all the nontoxigenic strains positive for the *tcpA* gene.

Analysis of ribotype patterns with Pasteur Taxotron programs showed that the Mx1, Mx2, and Mx3 ribotypes did not match any profile from the Pasteur Institute digital ribotype database (data not shown) or nontoxigenic *V. cholerae* O1 ribotype patterns 11 to 19 reported by Popovic et al. (27) (Fig. 3). The ribotype pattern of *V. cholerae* O1 with the classical biotype (Cancún strain) had the highest level of dissimilarity compared to strains of the El Tor biotype (data non shown).

Pulsotypes. Our PFGE analysis (Fig. 4) demonstrated three slightly different patterns (PM5.1, PM5.2, and PM5.3), differ-

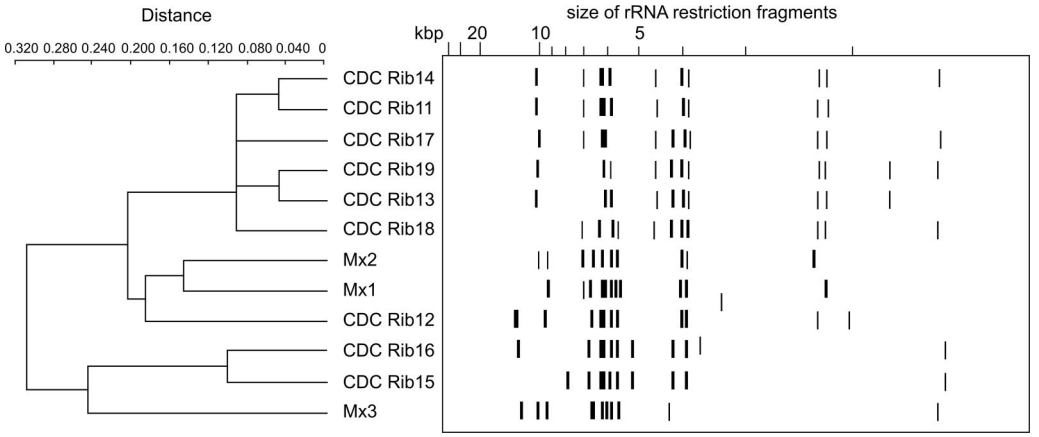

FIG. 3. Dendrogram of Mexican and CDC nontoxigenic *V. cholerae* O1 representative BglI ribotypes. The Mexican ribotypes are designed Mx1, Mx2, and Mx3. CDC strains are designed by the ribotype number in the ribotype scheme of Popovic et al. (27).

ing by no more than two bands, among strains related to ribotype M5. Four patterns (PM6.1, PM6.2, PM6.3, and PM6.4) were identified among strains of the M6 ribotype, with one of them, pattern PM6.4, consisting of a single strain; pulsotypes PM6.3 and PM6.4 had six bands differing from the original profile, PM6.1.Two PFGE patterns (PMx1.1 and PMx1.2) were identified for ribotype Mx1 and one pattern each for ribotypes Mx2 and Mx3. The PFGE patterns of ribotype Mx nontoxigenic *V. cholerae* O1 strains differed from previously published PFGE patterns of nontoxigenic *V. cholerae* O1 Amazonia and Tucumán variants (13, 25).

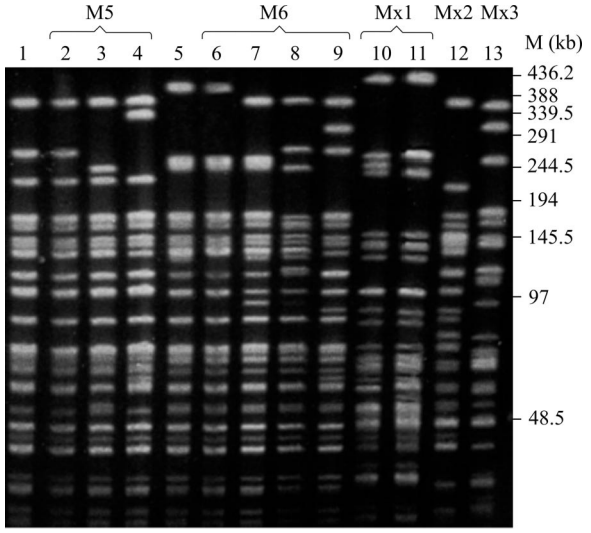

FIG. 4. Representative PFGE NotI patterns. Lane 1, strain representative of ribotype 5 or B5, isolated in Peru in 1991. Lanes 2 to 4, ribotype M5 PFGE patterns PM5.1, PM5.2, and PM5.3, respectively. Lane 5, strain representative of ribotype 6a, isolated in Romania in 1991. Lanes 6 to 9: ribotype M6 PFGE patterns PM6.1, PM6.2, PM6.3, and PM6.4, respectively. Lanes 10 and 11, ribotype Mx1 PFGE patterns PMx1.1 and PMx1.2, respectively; lane 12, PMx2; lane 13, PMx3.

DISCUSSION

We identified two ribotypes, designated M5 and M6, among the studied toxigenic *V. cholerae* O1 strains. These ribotypes were identical to patterns previously reported in BglI ribotype schemes of *V. cholerae* O1 strains, designed B5 or CDC ribotype 5 and CDC ribotype 6a or ribotype B21a, respectively (21, 27, 35). The presence of two strains in Mexico, each corresponding to one of these two ribotypes, was reported by other authors who studied the strains isolated in that country during the 1991 to 1995 epidemic period. Those authors associated these two different strains with the Inaba and the Ogawa serotypes, respectively, as the main serotype isolated in 1991 was Inaba whereas Ogawa seemed to be predominant in 1993 (18). Molecular studies of strains clearly demonstrate that the serotype change observed during this period of time was really due to the progressive replacement (or substitution) of the first strain (mainly the Inaba serotype) by another distinct strain (mainly the Ogawa serotype). However, it is important to remember that the change of serotype during an epidemic is not systematically linked to the emergence of a new strain. Conversion between the Inaba and Ogawa serotypes is frequently observed during the course of an epidemic within the epidemic strain. This switching phenomenon is linked to a mutation in the *wbeT* gene (34) and could possibly be due to selective pressure at the level of the host immune response (23). However, such a subtle genetic event is unlikely to be detectable using typing methods designed for the study of diversity within conserved genes, such as ribotyping. During the course of this Mexican epidemic, serotype conversions were observed within each of the two toxigenic *V. cholerae* O1 clonal populations: one strain expressing the Ogawa serotype, isolated in 1993, belonged to ribotype M5; similarly, both Inaba and Ogawa serotypes were observed among the M6 group. Within each group, the different serotypes were indistinguishable by PFGE. This confirms that serotypes seem to be of very limited value as an epidemiological marker. Furthermore, their use could be very confusing if the mechanisms controlling the temporal change within the epidemic serotypes are not clearly explained.

Thus, two distinct epidemic strains, belonging to clonal

groups with different ribotypes, coexisted in Mexico from 1991 until the end of 1993, when one clonal population gradually disappeared. INDRE records reveal that these two distinct populations did not appear in "waves" but were both present at the very start of the epidemic. Indeed, both M5 and M6 ribotype strains were isolated within the first days of the epidemic in neighboring states in central Mexico, Estado de México (M5) and Hidalgo (M6). M5 was the predominant ribotype in 1991, but a progressive replacement by strains with the M6 ribotype was then observed, such that all toxigenic strains isolated between 1994 and 2000 were M6 strains. There was no geographical trend in ribotype distribution: M5 and M6 strains were isolated in different Mexican states, with no differences observed between northern and southern localities or between Pacific and Gulf of Mexico coastal towns (Table 1).

The reasons why these strains suddenly appeared on the American continent remain unclear. Ribotype M5 was found to be similar to seventh-pandemic isolates from other parts of the world (38). Although Mexico is the source of ribotype M6 in Latin America, this ribotype, identical to ribotype 6a of Popovic et al. (27) and to ribotype B21a of Tamayo et al. (35), was identified previously in other parts of the world. In the 1990s, ribotype B21a was associated with strains isolated in Romania (12) and India and Southeast Asia (14). Damian et al. (12) reported its isolation since 1980 in Turkey; between 1991 and 1994 in various countries in Europe, Africa, and Asia; and in Colombia in America. Koo et al. (22) and Dalsgaard et al. (10) reported the presence of a new epidemic El Tor strain in Guatemala, Vietnam, Thailand, and India that shared common phenotypic traits (serotype and antibiotic susceptibility patterns) with the ribotype M6 Mexican isolates. This indicates a high circulation of strains sharing this ribotype; nevertheless, there is no available epidemiological data tracing its origin in Mexico from other countries. Given that strains of ribotypes M5 and M6 appeared at the same time in 1991 in Mexico and that ribotype 6a (M6) was not found among *V. cholerae* O1 Ogawa strains isolated in Peru between 1991 and 1995 (11), it seems unlikely that strains belonging to ribotype M6 were derived from ribotype M5 strains in the Latin America epidemic context. These findings therefore support the presence of a previously unknown independent source of cholera. Furthermore, our findings from PFGE analysis suggested that strains with various genotypes were associated with cholera in Mexico, possibly due to genetic changes during the course of the epidemic or to the emergence of strains newly introduced from elsewhere or emerging locally from the environment. Our results can support these two hypotheses. There was less genetic variation within ribotype M5 than within ribotype M6; we observed three slightly different PFGE patterns within ribotype 5, appearing in a relatively short period of time and differing by only two bands which correspond to closely related strains. In contrast, four different pulsotypes were found for ribotype 6a strains, two of which had six bands differing from the original profile; these strains could be possibly related, or even different, and we can suppose that a distinct, new toxigenic *V. cholerae* O1 was identified. Thus, at least three strains of toxigenic *V. cholerae* O1 could be the source of the cholera epidemic in Mexico. Nontoxigenic *V. cholerae* O1 strains are isolated from the environment and are regularly reported as being involved in cases of diarrheal diseases. The clinical significance of nontoxigenic *V. cholerae* O1 strains in causing symptomatic disease is not clearly understood. Nontoxigenic strains with Mx1, Mx2, and Mx3 ribotypes were isolated between 1998 and 2000 from patients with severe diarrhea associated with cholera on the basis of clinical symptoms. Previous studies have isolated nontoxigenic *V. cholerae* O1 El Tor Inaba and Ogawa strains from the U.S. Gulf of Mexico coast, as well as Peru, Brazil, and Argentina (8, 9, 25), both from the environment and from clinical cases. However, such a phenomenon had not been described previously in Mexico. Our PFGE results for these strains differ from PFGE patterns of the nontoxigenic Latin America and U.S. Gulf Coast strains and from PFGE patterns previously obtained for Amazonia and Tucumán nontoxigenic clinical variants (13, 25), so strains belonging to these three ribotypes seem to represent a "Mexican" variant subgroup. Interestingly, Mx genotypes seemed to cluster geographically, being isolated mainly in states surrounding the Gulf of Mexico and more specifically at ports on the Gulf of Mexico; some were found in other places during 1998 to 2000 and thus seemed to have the capacity, despite the absence of CT genes, to persist in and spread to zones other than coastal areas (Table 1; Fig. 1). The Mx3 group was an exception, however, containing only three strains, all of which were isolated in 2000 at the port of Campeche. A significant and unexpected finding was the presence of *tcpA* genes in Mx2 ribotype strains. Faruque and colleagues isolated O1 and non-O1/non-O139 strains that were positive for the TCP island but negative for the CTX prophage (15). These strains seem to be intermediate strains, likely to be competent for toxigenic conversion by CTXΦ, and are generally isolated from the environment. Their presence and impact on human health enhance the interest in developing projects including environmental surveillance.

The extensive and unexpected variability of strains involved in the Mexican epidemic, in such a short period of time, and the isolation of all these variants from states on the Gulf of Mexico suggest that the Gulf of Mexico acts as an environmental reservoir; this was previously demonstrated for the U.S. northern Gulf Coast (2). The relative high rate of genetic changes observed within *V. cholerae* O1 strains may reflect changes of previously nonpathogenic local strains, following the introduction of toxigenic *V. cholerae* O1 into an epidemic context, and the presence of nontoxigenic *V. cholerae* O1 strains with the *tcp* genes may be the first step in an evolution to future toxigenic and epidemic *V. cholerae* O1. This is of great concern for human health, particularly in areas where consumption of shellfish and seafood is important. Commercial oysters are harvested for local and national consumption from coastal lagoons in the southern Gulf of Mexico. Toxigenic and nontoxigenic *V. cholerae* O1 strains were isolated in oysters and water samples in the state of Veracruz (Gulf of Mexico) in July, August, and September 2002 (6). Paradoxically, however, no cholera cases were reported in these areas that year; furthermore, none of the *V. cholerae* O1 nontoxigenic strains present in Latin America before the arrival of the cholera pandemic persisted or evolved to epidemic clones during the epidemic events. Despite the absence of new reported cases in Mexico since 2002, cholera is still present in America: between 2003 and 2006, 103 imported and domestic cases were reported to WHO. Therefore, this disease has the potential to cause a new epidemic event in Mexico.

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