APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 2898–2904 Vol. 61, No. 8
0099-2240/95/$04.00+0

Enterobacterial Repetitive Intergenic Consensus Sequences and the PCR To Generate Fingerprints of Genomic DNAs from *Vibrio cholerae* O1, O139, and Non-O1 Strains

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Received 24 February 1995/Accepted 15 May 1995

Enterobacterial repetitive intergenic consensus (ERIC) sequence polymorphism was studied in *Vibrio cholerae* strains isolated before and after the cholera epidemic in Brazil (in 1991), along with epidemic strains from Peru, Mexico, and India, by PCR. A total of 17 fingerprint patterns (FPs) were detected in the *V. cholerae* strains examined; 96.7% of the toxigenic *V. cholerae* O1 strains and 100% of the O139 serogroup strains were found to belong to the same FP group comprising four fragments (FP1). The nontoxigenic *V. cholerae* O1 also yielded four fragments but constituted a different FP group (FP2). A total of 15 different patterns were observed among the *V. cholerae* non-O1 strains. Two patterns were observed most frequently for *V. cholerae* non-O1 strains, 25% of which have FP3, with five fragments, and 16.7% of which have FP4, with two fragments. Three fragments, 1.75, 0.79, and 0.5 kb, were found to be common to both toxigenic and nontoxigenic *V. cholerae* O1 strains as well as to group FP3, containing *V. cholerae* non-O1 strains. Two fragments of group FP3, 1.3 and 1.0 kb, were present in FP1 and FP2, respectively. The 0.5-kb fragment was common to all strains and serogroups of *V. cholerae* analyzed. It is concluded from the results of this study, based on DNA FPs of environmental isolates, that it is possible to detect an emerging virulent strain in a cholera-endemic region. ERIC-PCR constitutes a powerful tool for determination of the virulence potential of *V. cholerae* O1 strains isolated in surveillance programs and for molecular epidemiological investigations.

Vibrio cholerae serotype O1 is the causative agent of a severe secretory diarrhea and has historically been considered the cause of cholera epidemics. *V. cholerae* non-O1 serovars (serogroups which do not agglutinate with O1 *V. cholerae* antiserum) have been reported to cause gastroenteritis (31, 32, 34), extraintestinal infections (20), septicemia (18), and meningitis (39). At the end of 1992, a new serogroup emerged as an epidemic agent, *V. cholerae* O139, that was found to be the causative agent of outbreaks occurring in October 1992 in Madras, India (5).

The hypothesis is that *V. cholerae* is an autochthonous bacterial species in brackish water and that an environmental source can explain the seasonality (8, 9, 12) and distribution of the organism in regions of the world where cholera is endemic (9).

Laboratory diagnosis of cholera has traditionally been based on phenotypic characteristics of *V. cholerae* O1, expressed as morphological, physiological, and biochemical properties, including antigenic composition. Specific assays have been developed for detection of such antigens, enzymes, toxins, and related products (36). An empirical method currently employed in bacterial population genetics research is multilocus enzyme electrophoresis (MEE), which, by indexing allelic variation in sets of randomly selected structural genes of the chromosomal genome, provides a basis for estimating overall levels of single-locus and multilocus genotypic variation in populations and species (46). Several methods of indexing nucleotide sequence variation, other than protein electrophoresis and DNA hybridization, recently have been applied in epidemiological research, and genetic variation can be documented by a variety of molecular biology techniques. Genotyping of *V. cholerae* O1, i.e., construction of molecular genetic maps by analyzing the segregation of restriction fragment length polymorphisms (RFLPs) among the progeny of strains with different restriction enzymes, can be highly informative (25, 48, 52, 53). A standardized ribotyping has been proposed for *V. cholerae* O1, with a total of 17 rRNA gene restriction patterns observed after *Bgl*I cleavage (26, 36).

Detailed analysis can be performed by direct nucleotide sequence analysis of specific regions in the genome (15), but this technique is not applied in all microbiological laboratories. However, PCR can be performed with only one instrument for rapid detection of specific sequences of nucleic acid (27, 40).

Williams et al. (51) described a new DNA polymorphism assay based on amplification of random DNA segments with single primers of arbitrary nucleotide sequence, i.e., random amplified polymorphic DNA-PCR, arbitrary primer PCR, and PCR DNA fingerprinting. Dispersed repetitive DNA sequences have been described recently for eubacteria. Oligonucleotides matching enterobacterial repetitive intergenic consensus (ERIC) sequences (21) were synthesized, tested, and compared with ERIC-PCR primers for the amplification of eubacterial genomic DNA (47). In 1992, de Bruijn examined the distribution of dispersed repetitive DNA sequences in the genomes of a number of gram-negative soil bacteria using conserved primers corresponding to ERIC sequences by PCR (14).

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TABLE 1. *V. cholerae* O1, O139, and non-O1 strains used in this study

Serogroup	Source[a] and yr	Obtained from[b]:	Strain(s) (total no.)
O1	BS, 1978	M. T. Martins	TM16457, TM207831, TM207832 (3)
	BS, 1980	M. T. Martins	TM11079 (1)
	BS, 1991	CETESB	CT25016, CT25017 (2)
	BSRSt, 1992	CETESB	CT24082, CTMARMI1, CTMARMI2, CTMACMI1 (5)
	BS, 1993	CETESB	CT5369, CT7606, CT9995, CT15861, CT15989, CT16125, CT22317, CT31644, CT31802 (9)
	BSR, 1994	CETESB	CT1546, CT2578, CT2846, CT4252, CT7022, CT7237, CT7649, CT8511, CT8514, CT20093, CT20094 (12)
	PSt, 1993	IAL	IAL1941, IAL1943 (2)
	MSt, 1993	J. A. K. Hasan	JH332 (1)
O139	IC, 1993	G. B. Nair	CO391, CO396, CO403, CO404, CO411, CO415, B04, VO6, SO19, NPO390, MDO84, MDO-90 (12)
Non-O1	BS, 1977	M. T. Martins	TM35123, TM41338, TM34162 (3)
	BS, 1978	M. T. Martins	TM3634, TM52479, TM3233 (3)
	BS, 1979	M. T. Martins	TM19225, TM23256, TM31152, TM37869, TM9024 (5)
	BSw, 1981	M. T. Martins	TM50022, TM46720, TM45535 (3)
	BS, 1982	M. T. Martins	TM17019, TM48733, TM17015 (3)
	BSSw, 1983	M. T. Martins	TM1187, TM16589, TM5455 (3)
	BO, 1990	G. R. Matte (30, 31)	GM30, GM31, GM32, GM33, GM34, GM35, GM36, GM37, GM38, GM39 (10)
	BSR, 1992	CETESB	CT16291, CT10738, CT3481, CT3477, CT10834, CT12009 (6)

[a] Geographic: B, Brazil; I, India; P, Peru; M, Mexico. Isolation: S, sewage; St, stool; R, river; C, clinical; Sw, sea water; O, oysters and mussels.
[b] CETESB, Companhia de Tecnologia de Saneamento Ambiental; IAL, Adolfo Lutz Institute.

Molecular markers are necessary for monitoring the presence of toxigenic strains in environmental samples in order to understand the epidemiology of cholera. In this study, ERIC-PCR was used to analyze toxigenic and nontoxigenic strains of *V. cholerae* O1 and non-O1 isolated since 1977 from sewage and seawater in Säo Paulo State, Brazil, as well as *V. cholerae* O139 from India and epidemic strains from Peru and Mexico.

MATERIALS AND METHODS

Bacterial strains. A total of 83 *V. cholerae* strains, collected in Brazil since 1977, were subcultured onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco). A total of 32 *V. cholerae* O1 El Tor and 36 *V. cholerae* non-O1 strains isolated in the state of Säo Paulo, Brazil, 2 *V. cholerae* O1 El Tor strains from Peru, 1 *V. cholerae* O1 El Tor strain from Mexico, and 12 *V. cholerae* O139 strains from India were used in this study and are listed in Table 1 with their sources. *V. cholerae* O1 strains ATCC 25870 (569B), 14033, and 14035 and *V. cholerae* non-O1 strain J3 from an aquatic source in Japan (8) were used to determine fingerprint patterns (FPs) in known strains from sources other than Latin America. All strains were preserved in Luria-Bertani (LB) broth (43) amended with 30% glycerol and stored at −70°C. The identities of the *V. cholerae* O1 strains were confirmed by coagglutination with monoclonal antibody (11). *V. cholerae* non-O1 strains were characterized by the Biolog identification system (Biolog Inc., Hayward, Calif.).

All strains were previously tested for the presence of virulence factors, i.e., *ctx* and *zot* genes, by PCR techniques described elsewhere (7, 38).

Genomic DNA extraction. DNA was extracted by a modification of the plant DNA extraction method published by Murray and Thompson (35) and as described previously (38). DNA extracts were resuspended in Tris-EDTA (10 mM Tris-HCl, 0.10 mM EDTA [pH 8.0]) buffer and stored at 4°C for further analysis.

PCR primers and amplification conditions. The oligonucleotide ERIC primers ERIC1R (5′ATGTAAGCTCCTGGGGATTCAC3′) and ERIC2 (5′AA GTAAGTGACTGGGGTGAGCG3′) were designed as described elsewhere (47).

The following was added to each 100 μl PCR mixture: 10 μl of Mg-free 10× amplification buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 0.1% gelatin); 6 μl of 25 mM $MgCl_2$; 4 μl each of 1.25 mM dATP, dCTP, dGTP, and dTTP; forward and reverse ERIC primers (100 pmol of each); and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer-Cetus). PCR was carried out in 0.5-ml microcentrifuge tubes, with 19 μl of the PCR mixture described above and 1 μl (ca. 0.10 μg) of template DNA. The solution was overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (Perkin-Elmer DNA thermal cycler).

The DNA in the reaction mix was denatured at 95°C for 5 min. PCR amplification was done as follows: denaturation at 92°C for 45 s, annealing at 52°C for 1 min, and extension at 70°C for 10 min, with a final extension at 70°C for 20 min at the end of 35 cycles. The samples were maintained at 4°C.

The PCR products were separated by agarose gel electrophoresis in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]).

Analysis of FPs. The sizes of bands generated by electrophoresis of the PCR amplifications were calculated by comparison with DNA molecular size markers (*Hin*dIII-digested λ DNA and pGEM [Promega Corp., Madison, Wis.]). The migration positions of fragments of the marker were plotted on semilog paper according to molecular size, and the migration distances of bands in the samples were measured to establish the molecular size for each band.

Computer analysis. Data for the different sizes of fragments generated by ERIC-PCR for each FP group were analyzed by using SAS-Graph (22, 44, 45) for cluster analysis and employing the simple matching (*Sm*) and Jaccard (*Sj*) coefficients, with unweighted average linkage.

RESULTS

ERIC-PCR of genomic DNA from various *V. cholerae* strains resulted in amplification of multiple fragments of DNA in sizes ranging between 0.18 and 4.0 kb. A total of 17 FPs were identified for the strains of *V. cholerae* included in the study. The data on FPs associated with *V. cholerae* serogroups, their frequency of distribution, and the presence of toxin genes in each group are summarized in Table 2. Approximately 96.7% (29 of 30) of the *V. cholerae* serogroup O1 toxigenic (ctx^+/zot^+) strains isolated from clinical and environmental samples from Brazil, Peru, and Mexico and 100% (12 of 12) of the *V. cholerae* serogroup O139 strains isolated from clinical samples in India fell within the same FP group (FP1) (Table 2 and Fig. 1). All nontoxigenic *V. cholerae* O1 strains isolated before the cholera epidemic which occurred in 1991 in São Paulo State, Brazil, exhibited a different pattern and belonged to the FP2 group (Table 2 and Fig. 1). Isolates of *V. cholerae* non-O1 had a total of 15 different FPs (Table 2). Among the *V. cholerae* non-O1 FPs, FP3 (25%, 9 of 36) and FP4 (16.7%, 6 of 36) appeared more frequently than the other groups.

Within the FP groups, FP1 (comprising the toxigenic *V. cholerae* O1 and O139 serogroups), FP2 (nontoxigenic *V. cholerae* O1), and FP3 (nontoxigenic *V. cholerae* non-O1) showed the presence of three analogous fragments of 1.75, 0.79, and 0.5 kb (Table 2). FP3 included two fragment sizes, one coinciding with FP1 (1.3 kb) and the other with FP2 (1.0 kb).

One common band, 0.50 kb, was found in all strains of *V.*

TABLE 2. FPs obtained by ERIC-PCR for the *V. cholerae* strains included in the study

FP	*V. cholerae* serogroup	Strain(s)	Frequency[a] (%)	Fragment size(s) (kb)	Toxin genes[b]	
					ctx	*zot*
1	O1	CT25017, CT24082, CTMARMI1, CTMARMI2, CTMARCMI1, CTMARCMI4, CT5369, CT7606, CT9995, CT15861, CT15989, CT16125, CT22317, CT31644, CT31802, CT1546, CT2578, CT2846, CT4252, CT7022, CT7237, CT7649, CT8511, CT8514, CT20093, CT20096, JH332, IAL1941, IAL1943	96.7	1.75, 1.3, 0.79, 0.5	+	+
	O139	CO391, CO396, CO403, CO404, CO411, CO415, B04, VO6, SO19, NPO390, MDO84, MDO90	100	1.75, 1.3, 0.79, 0.5	+	+
2	O1	TM16457, TM207832, TM207832, TM11079, CT25016	100	1.75, 1.0, 0.79, 0.5	−	−
3	Non-O1	TM35123, TM19225, TM23256, TM31152, GM33, GM35, GM36, GM37, CT16291	25	1.75, 1.3, 1.0, 0.79, 0.5	−	−
4	Non-O1	TM41338, TM3634, TM17019, TM48733, TM1187, CT10738	16.7	1.0, 0.5	−	−
5	Non-O1	TM52479, TM16589, CT3481	8.3	1.75, 0.5	−	−
6	O1	CT7021	3.3	0.5	+	+
	Non-O1	TM50022, TM46720, CT3477	8.3	0.5	−	−
7	Non-O1	GM32, GM34	5.6	4.0, 1.7, 1.15, 1.0, 0.85, 0.78, 0.5	−	−
8	Non-O1	GM38, GM39	5.6	2.7, 1.4, 1.2, 1.1, 0.98, 0.88, 0.5, 0.4	−	−
9	Non-O1	TM17015, TM5455	5.6	1.2, 0.96, 0.5	−	−
10	Non-O1	TM37869, GM30	5.6	1.25, 0.76, 0.5	−	−
11	Non-O1	TM9024	2.8	1.4, 1.0, 0.73, 0.5, 0.19	−	−
12	Non-O1	CT10834	2.7	1.7, 1.25, 0.95, 0.5	−	−
13	Non-O1	GM31	2.8	1.2, 1.0, 0.78, 0.5	−	−
14	Non-O1	TM34162	2.7	1.0, 0.54, 0.5	−	−
15	Non-O1	TM3233	2.8	1.0, 0.5, 0.18	−	−
16	Non-O1	CT12009	2.7	1.7, 0.85, 0.5	−	−
17	Non-O1	TM45535	2.8	1.2, 0.5	−	−

[a] Frequency, percentage of strains in the serogroup showing that FP.
[b] Data on toxin genes are from reference 38. +, present; −, absent.

cholerae examined (Table 2). The FP group which contained the largest number of fragments was FP8, with eight fragments, followed by group FP7, with seven fragments. Both of these groups included only *V. cholerae* non-O1 strains (Fig. 2).

Among the known strains, *V. cholerae* non-O1 strain J3 revealed five bands, with three fragments of the same sizes as in the *V. cholerae* non-O1 group FP3. *V. cholerae* O1 strain ATCC 25870 (569B) exhibited only two fragments, 0.68 and 0.50 kb, one of which (0.68 kb) was not found in any other strain used in this study. The only fragment common to all strains was the 0.5-kb one (Tables 2 and 3).

The distribution of FPs of *V. cholerae* non-O1 strains during the period 1977 to 1992 is shown in Table 4. Again, the FP1 pattern, representative of the epidemic strains, was not observed in any of the environmental *V. cholerae* non-O1 isolates collected during the pre-epidemic period (Table 4).

The 17 FPs obtained for the 83 strains of *V. cholerae* examined in this study clustered into two major and three single-member groups in 0.80 simple-match similarity groups when the *Sm* coefficient and unweighted average linkage were used (Fig. 2). Further analysis with the *Sj* coefficient produced similar clusters (data not shown). Most of the *V. cholerae* O1 strains (toxigenic and nontoxigenic), all of the O139 strains tested, and a few strains of the non-O1 serogroups fell within the same cluster group (Fig. 2).

DISCUSSION

ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. ERIC se-

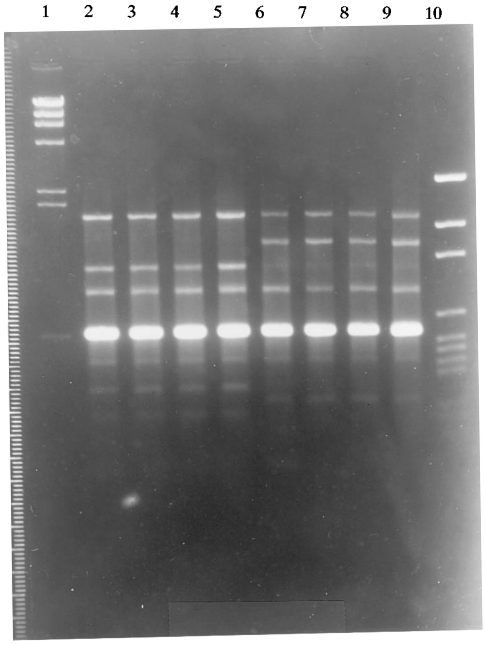

FIG. 1. DNA fingerprints of toxigenic and nontoxigenic strains of *V. cholerae* O1 generated by ERIC-PCR amplification. Lanes: 1, molecular size markers (*Hin*dIII-digested λ DNA [Promega]); 2 to 5, *V. cholerae* O1 strains lacking *ctx* and *zot* genes (FP2); 6 to 9, *V. cholerae* O1 strains with *ctx* and *zot* genes (FP1); 10, molecular size markers (pGEM [Promega]).

quences are novel and highly conserved at the nucleotide sequence level, but their chromosomal locations differ between species (21). The ERIC sequence in *V. cholerae* has been identified and is located near the hemolysin gene, apparently "hitchhiking" with the hemolysin gene (21). The hemolytic property of biotype El Tor has been shown to be less strong in *V. cholerae* strains isolated during the course of an epidemic (1), i.e., as the epidemic progresses, and is interconvertable within hemolytic and nonhemolytic variants (17).

It is possible, by ERIC-PCR, to generate a characteristic genomic fingerprint for given bacterial species, including *Vibrio mimicus*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio campbelli*, *Vibrio mediterranei*, *Vibrio alginolyticus*, *Escherichia coli*, *Shigella sonnei*, *Shigella dysenteriae*, *Shigella boydii*, *Shigella flexneri*, and *Bacillus subtilis*, which can be used to distinguish patterns of particular strains (37, 47). ERIC-PCR also allows clear distinction among bacterial species and strains containing these repetitive elements (47).

Both toxigenic and nontoxigenic strains of *V. cholerae* O1 demonstrated three identical fragments of 1.75, 0.79, and 0.5 kb (Table 2). About 96.7% of the toxigenic *V. cholerae* O1 El Tor and 100% of the *V. cholerae* O139 isolates demonstrated a common fingerprint (FP1). We also obtained the same fingerprint for epidemic *V. cholerae* O1 toxigenic strains from Brazil, Peru, and Mexico, as well as from *V. cholerae* O139 Bengal strains isolated in India. Similarity between strains of the epidemic serogroups O1 and O139 was also revealed by MEE, both O1 and O139 serogroups belonging to zymovar 14 (23, 42), and by Southern blot and hybridization with a probe for the iron-regulated gene (2). ERIC-PCR is relatively easy and rapid to perform compared with restriction analysis, probing, or MEE.

The nontoxigenic *V. cholerae* O1 strains showed a different FP (FP2), readily differentiated (visually) from toxigenic strains, in which the amplified 1.0-kb fragment was replaced by another fragment of 1.3 kb (Fig. 1). The relationship between toxigenic and nontoxigenic *V. cholerae* O1 isolates has been examined by MEE, and identical patterns were reported for 100% of the toxigenic strains and 30.4% of the nontoxigenic strains (42). By using ERIC-PCR, as described in this study, it is possible to differentiate toxigenic (FP1) from nontoxigenic (FP2) strains of *V. cholerae* O1.

Two of the nontoxigenic *V. cholerae* O1 strains included in this study, originally isolated from sewage samples collected in Brazil, were classified previously as ET-10 by MEE (4), belonging to pattern 13 of *Bgl*I-restricted RFLP of rRNA and electrophoretic type 6 by ribotyping (48). The molecular epidemiology of cholera in Latin America was studied by genomic analysis, rRNA RFLP, and MEE, all of which gave apparently conflicting results. The RFLP profiles of the Latin American isolates were identical to those of some isolates from the seventh pandemic, whereas the results of MEE indicated that the Latin American isolates were distinct from seventh-pandemic isolates (4, 48).

In this study, *V. cholerae* non-O1 strains gave 15 different FPs, indicating the existence of a heterogeneous group of organisms in the environment. FP3 (25%) was the most common, with five bands of 1.75, 1.3, 1.0, 0.79, and 0.5 kb (Table 2). The distribution of FPs among *V. cholerae* non-O1 strains was diverse and independent of sample type, as can be seen from data presented in Table 4. Since non-O1 isolates represented *V. cholerae* serogroups O2 through O138, it is expected that a diverse set of FPs would result from the strains used in this study.

Analysis of the data with the *Sm* coefficient resulted in the determination of five cluster groups, representing the 17 FPs. *V. cholerae* non-O1 group FP3 fell into the same cluster as the toxigenic *V. cholerae* O1 and O139 (Fig. 2), indicating molecular similarity of *V. cholerae* non-O1 strains to epidemic strains. *V. cholerae* non-O1 strains may have acquired genes in the environment from epidemic strains, a hypothesis that remains to be fully tested. Nevertheless, it is possible to distinguish toxigenic *V. cholerae* O1 from nontoxigenic O1 strains by ERIC-PCR followed by gel electrophoresis (Fig. 1).

It is important to determine the toxigenic potential of non-O1 isolates since, on occasion, they can cause gastroenteritis and extraintestinal infections (24). Furthermore, the so-called eight pandemic, caused by the new serogroup, *V. cholerae* O139, suggests that strains other than *V. cholerae* O1 can acquire epidemic potential (5). Therefore, the absence of *V. cholerae* O1 and presence of *V. cholerae* non-O1 strains in any environment should not be ignored. It is interesting to note here that, in this study, ERIC-PCR generated an identical FP for the toxigenic O1 and O139 serogroups of *V. cholerae* examined.

Epidemiologic surveillance of cholera has been limited in the past by rather insensitive laboratory systems, mainly culture and physiological and serological grouping of strains. Traditionally, toxigenic *V. cholerae* serogroup O1 strains, which are the major cause of epidemic cholera, are differentiated by serology into two serotypes, Inaba and Ogawa, and by bacteriological tests into two biotypes, classical and El Tor (49). Cook et al. (13) used plasmid profiles and Southern blot analysis with *ctx*A and *VcA*1 probes to show that 42 classical biotype strains, isolated as early as 1916 and 1921 (sixth pandemic), were indistinguishable from the 1982 isolates. Unlike toxigenic El Tor isolates, which showed no plasmid DNA, most classical isolates possess 4.8- and 33.6-kb plasmids. Furthermore, nontoxigenic O1 strains revealed no plasmid or up to three plasmids. No significant similarities between ERICs and plasmid, phage, or eukaryotic sequences were found in this

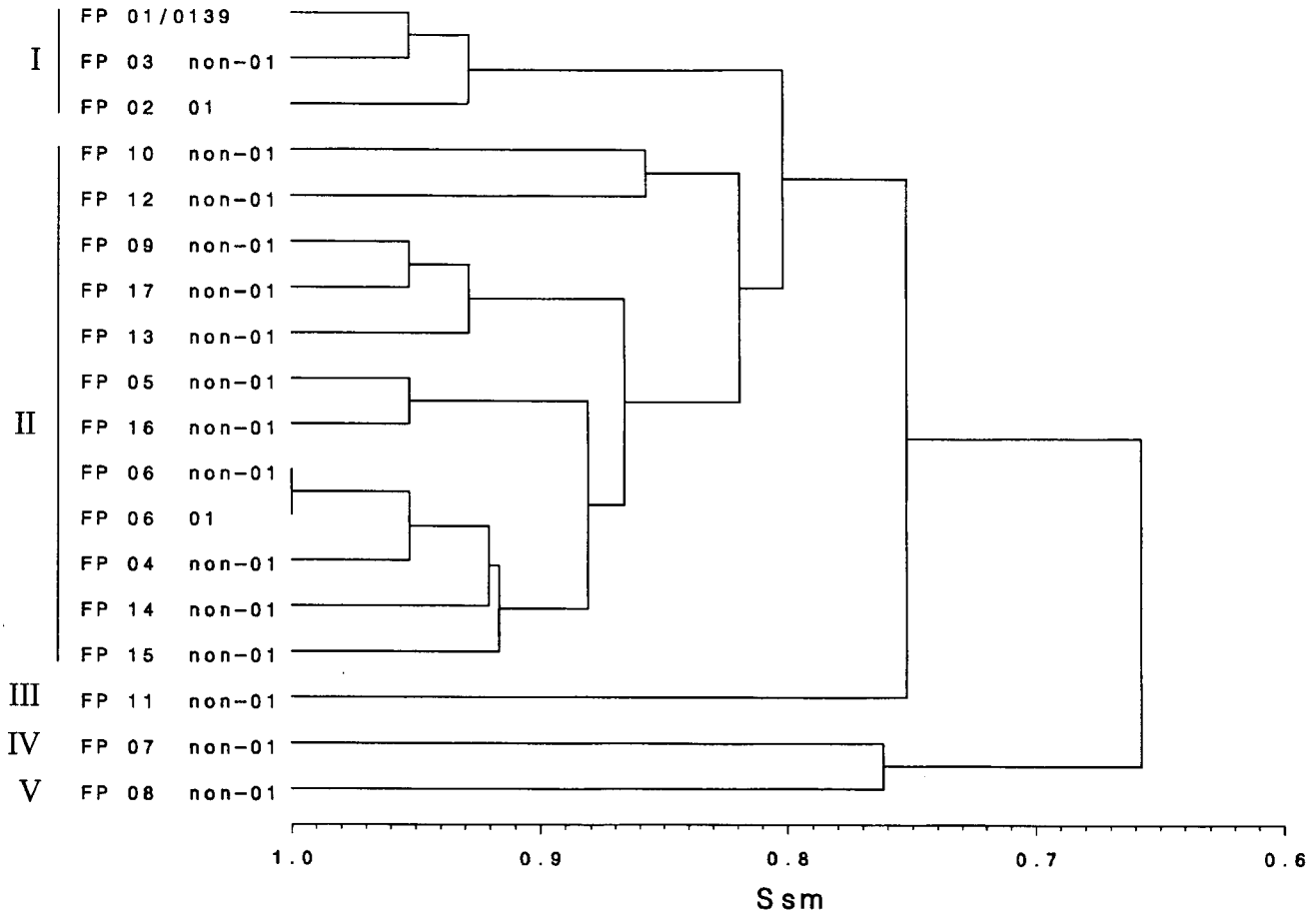

FIG. 2. Dendrogram of simple-match similarity matrix clustered by the unweighted pair-group method with arithmetic means.

study, and the ERICs did not resemble any known insertion sequence or transposable element (21).

According to Goldberg and Murphy (16), epidemiology based solely on hybridization of the cloned *E. coli* heat-labile toxin gene or on data obtained with cholera toxin gene probes is not sufficient to determine relationships among tox^+ and wild-type *tox* isolates. The conclusion was that there is a mechanism for the addition, deletion, or both of the cholera enterotoxin structural genes in *V. cholerae.*

The most discriminative typing schemes may be those that analyze several loci evenly scattered in the chromosome because they are most likely to reflect overall genomic DNA polymorphism. MEE was used to examine the relationships between *V. cholerae* strains; they were grouped into 73 zymovars (strain or group of strains with the same alleles) by using 13 structural loci (41) and into 10 electrophoretic types by using 16 enzyme loci (4).

Koblavi et al. (26) developed an rRNA RFLP, or ribotyping, assay based on *Bgl*I cleavage of whole-cell DNA to study a collection of 89 *V. cholerae* O1 isolates. A total of 17 rRNA gene restriction patterns were observed. No correlation between serotype and rRNA gene restriction pattern was obtained, however.

The molecular epidemiology of *V. cholerae* isolates in Latin America has been studied by using RFLP of rRNA, *ctx* genes, ribotyping, MEE, etc. It was established that there are at least four distinct toxigenic El Tor *V. cholerae* O1 clones: seventh pandemic (Eastern Hemisphere); U.S. Gulf Coast; Australia; and Latin America (48). Other methods were also used to distinguish different serovars and biotypes, such as RFLP-

TABLE 3. FPs obtained for known strains of *V. cholerae* used in the study

V. cholerae strain	*V. cholerae* serogroup	Fragment size(s) (kb)	Toxin genes[a]	
			ctx	*zot*
J3 (8)	Non-O1	4.3, 1.35, 1.3, 0.79, 0.5	−	−
ATCC 25870 (569B)	O1	0.68, 0.5	+	+
ATCC 14033	O1	0.5	NT	NT
ATCC 14035	O1	0.5	+	+

[a] See Table 2, footnote *b*. NT, not tested.

TABLE 4. Distribution of FPs for *V. cholerae* non-O1 strains isolated from environmental samples in São Paulo, Brazil, during the period 1977 to 1992[a]

Source	FP group distribution (no. of isolates)							
	1977	1978	1979	1981	1982	1983	1990	1992
Sewage	FP3	FP4	FP3 (3)	—[b]	FP4 (2)	FP4	—	FP3
	FP4	FP5	FP10	—	FP9	FP9	—	FP5
	FP14	FP15	FP11	—	—	—	—	FP6
	—	—	—	—	—	—	—	FP16
Seawater	—	—	—	FP6 (2)	—	FP5	—	—
	—	—	—	FP17	—	—	—	—
Oyster	—	—	—	—	—	—	FP10	—
	—	—	—	—	—	—	FP13	
Mussel	—	—	—	—	—	—	FP3 (4)	—
	—	—	—	—	—	—	FP7 (2)	—
	—	—	—	—	—	—	FP8 (2)	—
River	—	—	—	—	—	—	—	FP4
	—	—	—	—	—	—	—	FP12
Total	3	3	5	3	3	3	10	6

[a] See Tables 1 and 2 for corresponding strain designations.
[b] —, no isolates.

PFGE (pulsed-field gel electrophoresis) (6), but the method used was complicated. PFGE was found to be more discriminating than the MEE or ribotyping schemes described previously (3). However, *V. cholerae* O1 isolates from the Latin American epidemic were indistinguishable by MEE, ribotyping, or PFGE (3). Arbitrary primer PCR and priming efficiency in various PCR experiments carried out by other investigators were also found to be low in sensitivity and did not allow distinction of strains within a species (2, 42). In contrast, the approaches applied here and by Versalovic et al. (42) are a simple and useful alternative to the other methods used to date, because ERIC primers are highly specific and the procedure rapidly distinguishes toxigenic strains from nontoxigenic strains of *V. cholerae*. Multiple colonies isolated from the same culture, as well as repeated isolation of the same strain over time, revealed a consistent pattern, demonstrating that the fingerprint is stable and specific to a given bacterial strain (28). ERIC-PCR is proving to be a powerful tool for molecular analysis of toxigenic and nontoxigenic *V. cholerae*. Our results to date demonstrate that the fingerprints generated significantly facilitate cholera surveillance in environmental studies as well as molecular epidemiological investigations.

The ERIC-PCR methods applied here for *V. cholerae* are applicable to other microbial species and to strains in the viable but nonculturable state, as has been shown for *V. cholerae*, which can exist in the environment in the viable but nonculturable state under conditions adverse for growth (12). Detection of such cells and of the emergence of newly virulent strains has been very difficult. The ERIC-PCR technique is currently being applied in our laboratory to analyze environmental specimens. We speculate that the application of ERIC-PCR techniques to environmental samples may aid in understanding the molecular ecology of the cholera agent and related enteric pathogens in the environment.

In summary, we report the first application of ERIC-PCR-generated fingerprints to differentiate toxigenic and nontoxigenic strains of *V. cholerae* serogroup isolates from a variety of sources, including environmental. With this tool, health officials should be able to monitor environments and thereby predict the emergence of virulent strains, which in turn will allow the institution of preventive measures in cholera-endemic regions.

ACKNOWLEDGMENTS

We are grateful to G. B. Nair (National Institute of Cholera and Enteric Diseases, Calcutta, India), P. Sanchez, M. I. Z. Sato (CETESB, S. P.-Brazil), and R. Glavur (F. Saude Publica-USP, S. P.-Brazil) for providing strains of *V. cholerae*. We thank Bing Xu for technical assistance.

I. G. Rivera was supported by a fellowship from UNESCO during the research period and by CNPQ (National Council for Research Support, Brazil) for her graduate study. The research was supported, in part, by the Thrasher Research Fund, grant UM SAI 930720-8011.

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