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Molecular Analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 Strains: Clonal Relationships between Clinical and Environmental Isolates

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A total of 26 strains of *Vibrio cholerae*, including members of the O1, O139, and non-O1, non-O139 serogroups from both clinical and environmental sources, were examined for the presence of genes encoding cholera toxin (*ctx*A), zonula occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), hemolysin (*hly*A), NAG-specific heat-stable toxin (*st*), toxin-coregulated pilus (*tcp*A), and outer membrane protein (*omp*U), for genomic organization, and for the presence of the regulatory protein genes *tcp*I and *tox*R in order to determine relationships between epidemic serotypes and sources of isolation. While 22 of the 26 strains were hemolytic on 5% sheep blood nutrient agar, all strains were PCR positive for *hly*A, the hemolysin gene. When multiplex PCR was used, all serogroup O1 and O139 strains were positive for *tcp*A, *omp*U, and *tcp*I. All O1 and O139 strains except one O1 strain and one O139 strain were positive for the *ctx*A, *zot*, and *ace* genes. Also, O1 strain VO3 was negative for the *zot* gene. All of the non-O1, non-O139 strains were negative for the *ctx*A, *zot*, *ace*, *tcpA*, and *tcp*I genes, and all of the non-O1, non-O139 strains except strain VO26 were negative for *omp*U. All of the strains except non-O1, non-O139 strain VO22 were PCR positive for the gene encoding the central regulatory protein, *tox*R. All *V. cholerae* strains were negative for the NAG-specific *st* gene. Of the nine non-*ctx*-producing strains of *V. cholerae*, only one, non-O1, non-O139 strain VO24, caused fluid accumulation in the rabbit ileal loop assay. The other eight strains, including an O1 strain, an O139 strain, and six non-O1, non-O139 strains, regardless of the source of isolation, caused fluid accumulation after two to five serial passages through the rabbit gut. Culture filtrates of all non-cholera-toxigenic strains grown in AKI media also caused fluid accumulation, suggesting that a new toxin was produced in AKI medium by these strains. Studies of clonality performed by using enterobacterial repetitive intergenic consensus sequence PCR, Box element PCR, amplified fragment length polymorphism (AFLP), and pulsed-field gel electrophoresis (PFGE) collectively indicated that the *V. cholerae* O1 and O139 strains had a clonal origin, whereas the non-O1, non-O139 strains belonged to different clones. The clinical isolates closely resembled environmental isolates in their genomic patterns. Overall, there was an excellent correlation among the results of the PCR, AFLP, and PFGE analyses, and individual strains derived from clinical and environmental sources produced similar fingerprint patterns. From the results of this study, we concluded that the non-cholera-toxin-producing strains of *V. cholerae*, whether of clinical or environmental origin, possess the ability to produce a new secretogenic toxin that is entirely different from the toxin produced by toxigenic *V. cholerae* O1 and O139 strains. We also concluded that the aquatic environment is a reservoir for *V. cholerae* O1, O139, non-O1, and non-O139 serogroup strains.

Vibrio cholerae is a natural inhabitant of the aquatic environment (9, 12, 15, 21, 24, 31, 37). This vibrio species not only survives in riverine, estuarine, and coastal waters around the world but also lives in association with crustacean copepods, such as *Acartia tonsa* and *Gerris spinolae*, and with aquatic plants, either in the viable and culturable state or in the viable but nonculturable state (13, 25, 30, 32, 33, 36, 49, 53, 66, 70). Since this human pathogen is primarily an inhabitant of the aquatic environment, water plays an important role in the transmission and epidemiology of cholera (19, 38, 72). However, the epidemiological impact of environmental *V. cholerae* strains is not clearly understood, since the majority of strains isolated from the environment do not produce cholera toxin and lack not only the virulence gene cassette (45, 64) for cholera toxin but also zonula occludens toxin and accessory cholera enterotoxin. In addition, environmental vibrios lack the toxin-coregulated pilus (TCP) that is recognized as a significant factor in the pathogenicity of toxigenic strains of *V. cholerae* serogroups O1 and O139.

Strains of *V. cholerae* belonging to serogroup O1 biotype El Tor and serogroup O139 have been described as causative

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agents of diarrhea (1, 10, 34, 42, 55). Furthermore, results of genetic and phenotypic characterizations of such strains indicate that in order to cause diarrhea, they require genes for cholera toxin, the colonization TCP, and the central regulatory protein ToxR (27, 29, 46, 50, 76). However, nontoxigenic strains isolated from the environment and lacking the virulence gene cassette have been reported to evoke the secretory response in the ligated rabbit ileal loop assay (62, 63). Nontoxigenic O1 biotype El Tor strains have also been isolated from cases of diarrhea (11, 60).

V. cholerae non-O1, non-O139 strains are recognized as causative agents of sporadic and localized outbreaks, and the diarrhea caused by these strains is sometimes characterized by blood and mucous (8, 61). However, these strains continue to be considered of negligible significance, since they have been associated with illness only in a low percentage of patients hospitalized with secretory diarrhea (52). While analyzing the nucleotide sequence of *asd* genes in *V. cholerae* strains, Karaolis et al. (43) demonstrated that the sixth- and seventh-pandemic strains and United States Gulf Coast *V. cholerae* O1 isolates may have been derived from nontoxigenic strains and postulated that horizontal gene transfer occurred in *V. cholerae*, which resulted in the emergence of a new pathogenic strain. Furthermore, *V. cholerae* non-O1 serogroups have been reported to be involved in the emergence of a newer variant of *V. cholerae*; this hypothesis is supported by the genesis of *V. cholerae* O139, a serogroup believed to have evolved by horizontal gene transfer from serogroup O1 to a non-O1 serogroup (7).

Amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE), and PCR methods have been used in epidemiological investigations and also to study relatedness among bacterial strains in order to trace the origin and geographical distribution of *V. cholerae* (40, 69, 73, 75). It is accepted that cholera is a waterborne disease, but there is no molecular evidence to prove that strains causing human infections belong to the same clones as those found in aquatic environments. To answer this vexing question, we examined a set of strains belonging to O1, O139, and non-O1, non-O139 serogroups, including both clinical and environmental isolates, to determine whether virulence and regulatory genes were present and to compare their genomic organizations.

MATERIALS AND METHODS

Bacterial strains. A total of 26 strains of *V. cholerae* were included in this study. Seven strains were serogroup O1 strains (four clinical strains and three environmental strains), eight strains were serogroup O139 strains (four strains each from clinical and environmental sources), and seven strains were non-O1, non-O139 strains (two clinical isolates and five environmental isolates) from laboratory stocks and had been identified previously by using standard bacteriological methods (77). Four additional isolates of *V. cholerae* O139, including reference strain ATCC 51394 (= MO45), provided by G. B. Nair, National Institute of Cholera and Enteric Diseases, Calcutta, India, were also included in the study. All isolates were examined for the oxidase reaction, and the identities of the *V. cholerae* O1 strains were confirmed by serogrouping by using growth from triple sugar iron agar slants with polyvalent O1 and monospecific Inaba and Ogawa antisera. *V. cholerae* strains which did not agglutinate with O1 antiserum were checked with monoclonal O139 antiserum supplied by the WHO Regional Office of South-East Asia, New Delhi, India. *V. cholerae* strains which did not agglutinate with either O1 or O139 antisera were assumed to belong to non-O1, non-O139 serogroups. The sources, years of isolation, and geographical sites of isolation of the strains are listed in Table 1. All strains were maintained in peptone agar stab cultures and did not undergo more than two subcultures prior to testing in this study.

Hemolysis. Strains of *V. cholerae* were grown in brain heart infusion broth (Difco) for 3 h and were subsequently streaked on 5% sheep blood nutrient agar plates. After overnight incubation at 37°C, colonies were examined for hemolysis on blood agar.

Preparation of culture filtrates. Culture filtrates of all nine non-cholera-toxin-producing strains, including an O1 strain and an O139 serogroup representative, were prepared by the method of Sanyal et al. (63). Briefly, 10-ml portions of AKI medium or brain heart infusion broth in 50-ml conical flasks were inoculated with approximately five colonies from an overnight nutrient agar plate culture. The flasks were incubated at 37°C in a water bath for 16 to 18 h with shaking at 80 to 120 oscillations per min. The cultures were centrifuged at 22,000 × *g* for 20 min, the supernatants were filtered through membrane filters (pore size, 0.22 μm; Millipore), and the filtrates were stored in small volumes at 4°C.

Ileal loop test and passage through rabbit ileal loops. Cultures and culture filtrates of non-cholera-toxigenic *V. cholerae* strains were tested for enterotoxin production in adult New Zealand and albino rabbits by the method of De and Chatterje (17). The strains were grown in peptone water for 3 to 4 h, diluted 10-fold in the same medium, and inoculated into rabbit ileal loops by using 1-ml portions of preparations containing 10^5 to 10^6 CFU/ml. *V. cholerae* toxigenic strain 569B grown in peptone water and uninoculated, sterile peptone water served as positive and negative controls, respectively. Culture filtrates of all strains prepared in AKI medium were tested by the same method by using 1-ml inocula. Each test was done in two rabbits; six to eight 8- to 10-cm loops were ligated in each rabbit, and the interloop length was 2 to 4 cm. The rabbits were sacrificed after 18 h.

V. cholerae strains that caused little or no fluid accumulation in the initial tests were passaged through the rabbit gut by using the method of Sanyal et al. (63). Briefly, each strain was recovered from the ileal loops on nutrient agar plates, and following overnight incubation approximately five colonies were inoculated into peptone water and incubated at 37°C for 3 to 4 h. Approximately 1 ml of diluted culture was again inoculated into rabbit ileal loops. This process was continued until unequivocal positive responses were obtained.

Ouchterlony immunodiffusion. Ouchterlony immunodiffusion tests were performed by using concentrated (10×) culture filtrates of test strains prepared in AKI medium, and the new anti-cholera toxin serum. Cholera toxin gene-negative *V. cholerae* strain X-392, which has been reported to produce the new cholera toxin (62, 63), was obtained from J. B. Kaper of the Center for Vaccine Development, University of Maryland, College Park, and was used to prepare antiserum against the new cholera toxin in purified form as described previously (67).

DNA isolation. Chromosomal DNA was extracted from each of the *V. cholerae* strains by the cetyltrimethylammonium bromide method, as described by Ausubel et al. (4). The purity of the DNA was assayed with a Beckman DU 640 spectrophotometer by using automatic calculation of the ratio of optical densities at 260 and 280 nm.

Multiplex PCR assay. A multiplex PCR assay was performed (22, 65, 74; G. R. Matte, M. H. Matte, J. Chun, A. Huq, and R. R. Colwell, submitted for publication) in order to determine the presence of toxin genes *ctx*A, *zot, ace, st, hly*A, *tcp*A, and *omp*U. In addition, all strains were examined for the presence of regulatory genes for TCP expression (*tcp*I) and the central regulatory protein (*tox*R). The primers used in this study and the expected amplicon sizes are listed in Table 2. PCR was performed with a PTC-200 thermal cycler (MJ Research, Inc., Waltham, Mass.). The instrument was programmed as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles consisting of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and a final extension step of 72°C for 10 min. For the *tcp*I gene, extension was at 72°C for 3 min and all of the other steps were the same as those described above. The amplification program for the *st* and *ace* genes began with denaturation at 94°C for 1 min, which was followed by 29 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 5 min. *V. cholerae* O139 strain MO45 (= ATCC 51394) was used as the PCR positive control for *ctx*A, *zot, ace, tcp*A, *omp*U, *tcp*I, and *tox*R. ST^+ *V. cholerae* non-O1, non-O139 strain 618, provided by A. C. P. Vicente, Department of Genetics, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, was used as a positive control for the *st* gene. Amplified products were separated on a 1% agarose gel, stained with ethidium bromide, and photographed.

Genomic fingerprinting by ERIC- and BOX-PCR. Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) was performed as described by Rivera et al. (56) by using two oligonucleotide primers (5′-ATGTAAGCTC CTGGGGATTCAC-3′ and 5′-AAGTAAGTGACTGGGGTGAGCG-3′). Box elements PCR (BOX-PCR) was performed as described by Martin et al. (48) by using a single oligonucleotide primer (5′-CTACGGCAAGGCGACGCTGACG-

TABLE 1. *V. cholerae* strains of the O1, O139, and non-O1, non-O139 serogroups included in this study

Strain	Place of isolation	Source	Year of isolation
V. cholerae O1 biotype E1 Tor strains			
VO1	Orissa, India	Diarrheal patient	1969
VO2	Varanasi, India	Diarrheal patient	1976
VO13	Varanasi, India	Diarrheal patient	1989
VO3	Varanasi, India	Diarrheal patient	1990
VO4	Varanasi, India	River water	1986
VO5	Varanasi, India	River water	1988
VO7	Varanasi, India	River water	1988
V. cholerae O139 strains			
MO45[a]	Madras, India	Diarrheal patient	1992
CO594	Calcutta, India	Diarrheal patient	1992
CO766	Calcutta, India	Diarrheal patient	1992
CO788	Calcutta, India	Diarrheal patient	1992
VO12	Varanasi, India	Diarrheal patient	1994
VO14	Varanasi, India	Diarrheal patient	1993
VO15	Varanasi, India	Diarrheal patient	1993
VO16	Varanasi, India	Diarrheal patient	1994
VO17	Varanasi, India	*Eichhornia crassipes*	1992
VO18	Varanasi, India	*Eichhornia crassipes*	1992
VO19	Varanasi, India	River water	1992
VO20	Varanasi, India	Hand pump	1992
V. cholerae non-O1, non-O139 strains			
VO22	Varanasi, India	Diarrheal patient	1979
VO23	Varanasi, India	Diarrheal patient	1979
VO24	Varanasi, India	River water	1986
VO25	Varanasi, India	River water	1988
VO26	Varanasi, India	River water	1988
VO27	Varanasi, India	River water	1986
VO28	Varanasi, India	River water	1986

[a] *V. cholerae* O139 reference strain MO45 (= ATCC 51394).

3′). The amplicons were electrophoresed in 1.8% agarose at 60 V for 6 h and stained with SYBR Green I (FMC BioProducts, Rockland, Maine). The DNA band patterns were digitized with a Bio-Imager (Molecular Dynamics) and subjected to a fingerprint analysis. A 1-kb molecular size ladder (GIBCO-BRL, Gaithersburg, Md.) was included on each gel.

AFLP fingerprinting. All AFLP procedures were performed as described by Janssen et al. (40), with the minor modifications for *V. cholerae* described by Jiang et al. (41). Briefly, 1 μg of DNA was double digested with restriction enzymes *Taq*I and *Apa*I. Following digestion, adapters were added to final concentrations of 0.4 μM for *Taq*I adapters (5′-GACGATGAGTCCTGAC-3′ and 3′-TACTCAGGACTGGC-5′) and 0.04 μM for *Apa*I adapters (5′-TCGTA GACTGCGTACAGGCC-3′ and 3′-CATCTGACGCATGT-5′). Ligation reactions were performed at 16°C overnight. Ligated template DNA was purified, stored in Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA [pH 8.0]) at −20°C, and used for PCR amplification within 48 h.

PCR was performed as described by Janssen et al. (40) by using primers A01 (5′-GACTGCGTACAGGCCCA-3′) and T01 (5′-CGATGAGTCCTGACCGA A-3′). Prior to PCR, primer T01 was end labeled with [γ-^{33}P]ATP. Two microliters of template DNA was used for amplification in a total reaction volume of 25 μl. Amplification products were separated on 5% denaturing polyacrylamide and exposed to X-ray film (Kodak Inc.). Autoradiographs were digitized for fingerprint analysis.

PFGE analysis. Intact genomic DNA was prepared by the method of Majumder et al. (47), with modifications. Briefly, vibrios were grown in Luria broth (Difco) overnight at 37°C. Cells were harvested and washed twice with 10 mM Tris-HCl (pH 7.6) buffer containing 1 M NaCl and suspended at a concentration of 10^8 CFU/ml in the same buffer. Agarose plugs were prepared by mixing equal volumes of the cell suspension and molten 1% agar (In Cert agarose; FMC BioProducts). Cells were lysed in the plugs and stored in 5 ml of buffer containing 10 mM Tris-HCl (pH 8.0) and 50 mM EDTA (pH 8.0) at 4°C until they were used.

Prior to restriction digestion, plugs containing genomic DNA were washed twice (30 min each) in 10 volumes of buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Digestion with *Sfi*I (GIBCO-BRL) was carried out at 50°C for 16 h in a solution containing 100 μg of bovine serum albumin per ml by using 40 U of enzyme per plug.

PFGE was carried out with a CHEF Mapper System (Bio-Rad, Hercules, Calif.) in 0.5× TBE buffer containing 0.1% (vol/vol) 100 mM thiourea. Electrophoresis was performed at 6 V/cm and 14°C at a field angle of 120° by using 1.2% SeaKem GTG agarose gels (FMC BioProducts). The pulse time was ramped from 2.98 to 54.17 s over 26.56 h. A lambda DNA ladder (Bio-Rad) was used as the molecular weight standard. Following electrophoresis, the gels were stained with SYBR Green I (Molecular Probes Inc., Eugene, Oreg.), the DNA band patterns were imaged with a Bio-Image analyzer (Molecular Dynamics), and the fingerprints were analyzed.

Analysis of fingerprint patterns. Digital images of ERIC-PCR, BOX-PCR, AFLP, and PFGE fingerprint patterns were analyzed by using Molecular Fingerprinting Analyst (Bio-Rad) as recommended by the manufacturer and were graphically represented as dendrograms.

RESULTS

Twenty-two *V. cholerae* strains, including both clinical and environmental strains, were hemolytic on 5% sheep blood nutrient agar (Table 3). Although two O1 strains (VO3 and VO5) and two O139 strains (CO594 and VO17) were negative for hemolysis, they were PCR positive, like all of the other strains, for the genes representing the El Tor fragment for hemolysin (*hly*A). In addition, except for two of the non-O1, non-O139 isolates (VO23 and VO28), all strains were also PCR positive for the genes encoding the classical fragment of hemolysin (Table 3).

Of the nine non-cholera-toxin-producing strains, only one environmental isolate belonging to the non-O1, non-O139 serogroup (VO24) caused fluid accumulation (range, 0.5 to 0.9

TABLE 2. Sequences of primers used for detection of selected virulence and regulatory genes

Target	Nucleotide sequence (5′-3′)	Direction[a]	Amplicon size (bp)	Reference, biotype, and/or serotype
*ctx*A	CGGGCAGATTCTAGACCTCCTG	F	564	22
	CGATGATCTTGGAGCATTCCCAC	R		
zot	TCGCTTAACGATGGCGCGTTTT	F	947	Matte et al.[b]
	AACCCCGTTTCACTTCTACCCA	R		
ace	TAAGGATGTGCTTATGATGGACACCC	F	289	65
	CGTGATGAATAAAGATACTCATAGG	R		
st	GAGAAACCTATTCATTGC	F	216	74
	GCAAGCTGGATTGCAAC	R		
*omp*U	ACGCTGACGGAATCAACCAAAG	F	869	Matte et al.[b]
	GCGGAAGTTTGGCTTGAAGTAG	R		
*tcp*A	CACGATAAGAAAACCGGTCAAGAG	F		
	TTACCAAATGCAACGCCGAATG	R	620	Matte et al.; classical[b,c]
	CGAAAGCACCTTCTTTCACACGTTG	R	453	Matte et al.; E1 Tor or O139[b,c]
*tcp*I	TAGCCTTAGTTCTCAGCAGGCA	F	862	Matte et al.[b]
	GGCAATAGTGTCGAGCTCGTTA			
*hly*A	GGCAAACAGCGAAACAAATACC	F	738	Matte et al.; classical
	GAGCCGGCATTCATCTGAAT	F	481	Matte et al.; nonclassical[b,c]
	CTCAGCGGGCTAATACGGTTTA	R		
*tox*R	CCTTCGATCCCCTAAGCAATAC	F	779	Matte et al.[b]
	AGGGTTAGCAACGATGCGTAAG	R		

[a] F, forward; R, reverse. *
[b] Matte et al., submitted.
[c] Biotype- and/or serotype-specific amplification occurs.

ml/cm) in the initial rabbit ileal loop test. However, other strains, including an O1 strain, an O139 strain, and six non-O1, non-O139 isolates from clinical sources, caused fluid accumulation after two to five serial passages through the rabbit gut, and the amount of fluid accumulated ranged from 0.5 to 1.1 ml/cm. Culture filtrates of all strains prepared in brain heart infusion broth and AKI medium also caused accumulation of fluid at volumes similar to those caused by the whole cells.

When concentrated culture filtrates of cholera toxin gene-negative *V. cholerae* O1 strain X-392 and non-cholera-toxin-producing *V. cholerae* non-O1, non-O139 strains were tested by gel diffusion, one strain (VO26) produced a precipitin band against new cholera toxin antiserum, showing a reaction of identity, whereas the other strains showed reactions of partial identity (data not shown) (Table 3).

Based on the results of the multiplex PCR, all *V. cholerae* O1 and O139 strains yielded positive results for *tcp*A, *tcp*I, and *omp*U, and one isolate each of *V. cholerae* O1 (VO1) and *V. cholerae* O139 (CO788) gave negative results for the *ctx*A, *zot*, and *ace* genes. Moreover, O1 strain VO3 was negative for the *zot* gene. However, all *V. cholerae* non-O1, non-O139 strains yielded negative results for *ctx*A, *zot, ace*, and *tcp*A, and VO26 gave a positive result for *omp*U. Further analysis showed that all non-O1, non-O139 strains were also PCR negative for the gene encoding *tcp*I, which negatively regulates expression of *tcp* (Table 3). All *V. cholerae* strains were also negative for the NAG-specific *st* gene. However, except for one non-O1, non-O139 strain (VO22), all of the isolates were positive as determined by PCR for the gene encoding the central regulatory protein, ToxR, which coordinates regulation of expression of the dynamic core genetic element, including TCP and outer membrane protein (OMP).

Genetic relatedness analyses of the clinical and environmental strains of *V. cholerae* performed by ERIC-PCR, BOX-PCR, AFLP, and PFGE methods proved to be interesting. ERIC-PCR of genomic DNA from the various *V. cholerae* strains yielded a total of 16 fingerprints with sizes ranging between 0.18 and 4.0 kb, as shown in Fig. 1. Two of the seven O1 isolates, one clinical strain (VO3) and one environmental strain (VO7), yielded similar fingerprints. Similarly, 3 of the 12 O139 serogroup strains, both clinical (VO12 and VO15) and environmental (VO20), yielded identical PCR profiles; a non-*ctx*-producing strain, CO788, yielded closely related fingerprints. The other O1 and O139 serogroup strains produced different fingerprints. A number of *V. cholerae* O1 and O139 strains had similar PCR profiles. However, they differed from each other by the presence of an analogous fragment that was approximately 0.29 kb long. When the seven *V. cholerae* non-O1, non-O139 strains were examined, clinical isolate VO22 and environmental isolate VO25 produced similar PCR profiles, clinical isolate VO23 and environmental isolate VO27 produced similar PCR profiles, and environmental isolates VO24 and VO28 strains produced similar PCR profiles.

As shown in Fig. 2, BOX-PCR of genomic DNA from various *V. cholerae* strains resulted in amplification of multiple fragments of DNA that ranged from 0.29 to 6.0 kb long. All of

TABLE 3. Selected characteristics of *V. cholerae* O1, O139, and non-O1, non-O139 strains included in this study

Strain	Hemolysis	Presence of the following genes as determined by PCR									NCT production[b]
		*ctx*A	*zot*	*ace*	*st*	*hlyA*[a]	*tcp*A	*omp*U	*tcp*I	*tox*R	
V. cholerae O1 biotype E1 Tor strains											
VO1	Beta	−	−	−	−	+	+	+	+	+	NT[c]
VO2	Beta	+	+	+	−	+	+	+	+	+	NT
VO13	Beta	+	+	+	−	+	+	+	+	+	NT
VO3	None	+	−	+	−	+	+	+	+	+	NT
VO4	Beta	+	+	+	−	+	+	+	+	+	NT
VO5	None	+	+	+	−	+	+	+	+	+	NT
VO7	Beta	+	+	+	−	+	+	+	+	+	NT
V. cholerae O139 strains											
MO45[d]	Beta	+	+	+	−	+	+	+	+	+	NT
CO594	None	+	+	+	−	+	+	+	+	+	NT
CO766	Beta	+	+	+	−	+	+	+	+	+	NT
CO788	Beta	−	−	−	−	+	+	+	+	+	NT
VO12	Beta	+	+	+	−	+	+	+	+	+	NT
VO14	Beta	+	+	+	−	+	+	+	+	+	NT
VO15	Beta	+	+	+	−	+	+	+	+	+	NT
VO16	Beta	+	+	+	−	+	+	+	+	+	NT
VO17	None	+	+	+	−	+	+	+	+	+	NT
VO18	Beta	+	+	+	−	+	+	+	+	+	NT
VO19	Beta	+	+	+	−	+	+	+	+	+	NT
VO20	Beta	+	+	+	−	+	+	+	+	+	NT
V. cholerae non-O1, non-O139 strains											
VO22	Beta	−	−	−	−	+	−	−	−	−	+
VO23	Beta	−	−	−	−	−	−	−	−	+	+
VO24	Beta	−	−	−	−	+	−	−	−	+	+
VO25	Beta	−	−	−	−	+	−	−	−	+	+
VO26	Beta	−	−	−	−	+	−	+	−	+	+
VO27	Beta	−	−	−	−	+	−	−	−	+	+
VO28	Beta	−	−	−	−	−	−	−	−	+	+

[a] Specific for *V. cholerae* classical *hly*A. All *V. cholerae* strains were positive for nonclassical specific *hly*A.
[b] NCT, new cholera toxin detected by immunodiffusion assay.
[c] NT, not tested.
[d] Reference strain MO45 (= ATCC 51394).

the O1 serogroup strains and most of the O139 serogroup strains yielded identical fingerprint patterns, regardless of source of isolation; the only exception was O139 strain CO594, which produced a closely related PCR profile. Two isolates of serogroup O139, one clinical isolate (VO16) and one environmental isolate (VO20), did not group with the large O1-O139 group. However, non-cholera-toxigenic O1 and O139 strains could not be differentiated from their toxigenic counterparts based on BOX-PCR fingerprints. Like the ERIC-PCR results, when clinical and environmental *V. cholerae* non-O1, non-O139 strains were examined, strains VO22 and VO25, strains VO23 and VO27, and strains VO24 and VO28 also produced different but closely related PCR profiles.

AFLP fragment analysis of *V. cholerae* non-O1, non-O139 strains and of O1 and O139 strains revealed that the band patterns of the non-O1, non-O139 strains tended to be more diverse, regardless of the source of isolation, while the patterns of the O1 and O139 strains were conserved (Fig. 3). However, differences in the band pattern were detected within and between the O1 and O139 serogroups.

The PFGE profiles of the *V. cholerae* strains obtained with *Sfi*I showed a variety of patterns. For example, clinical and environmental serogroup O1 isolates had similar restriction patterns. Similarly, strains of *V. cholerae* O139, whether clinical or environmental, displayed different but closely related restriction profiles (Fig. 4a). Non-cholera-toxigenic O1 and O139 strains were distinguishable from their toxigenic counterparts by the fingerprints obtained from *Sfi*I-digested genomic DNA. The PFGE profiles of non-O1, non-O139 strains differed from each other and from the patterns displayed by the strains belonging to the O1 and O139 serogroups. However, two non-O1, non-O139 isolates, a clinical isolate (VO22) and an environmental isolate (VO25), displayed closely related restriction fingerprint patterns. These data are presented in dendrogram format in Fig. 4b.

DISCUSSION

Unlike strains of the classical biotype, which are nonhemolytic, strains of the El Tor biotype produce and secrete a hemolysin into the culture medium. Historically, this feature has been used to distinguish the two biotypes. However, hemolytic activity does not always correlate with El Tor biotype, and many El Tor strains that do not produce hemolysin have been reported (6, 18, 58). The results of this study also indicate that a few strains of O1 biotype El Tor and O139, which were

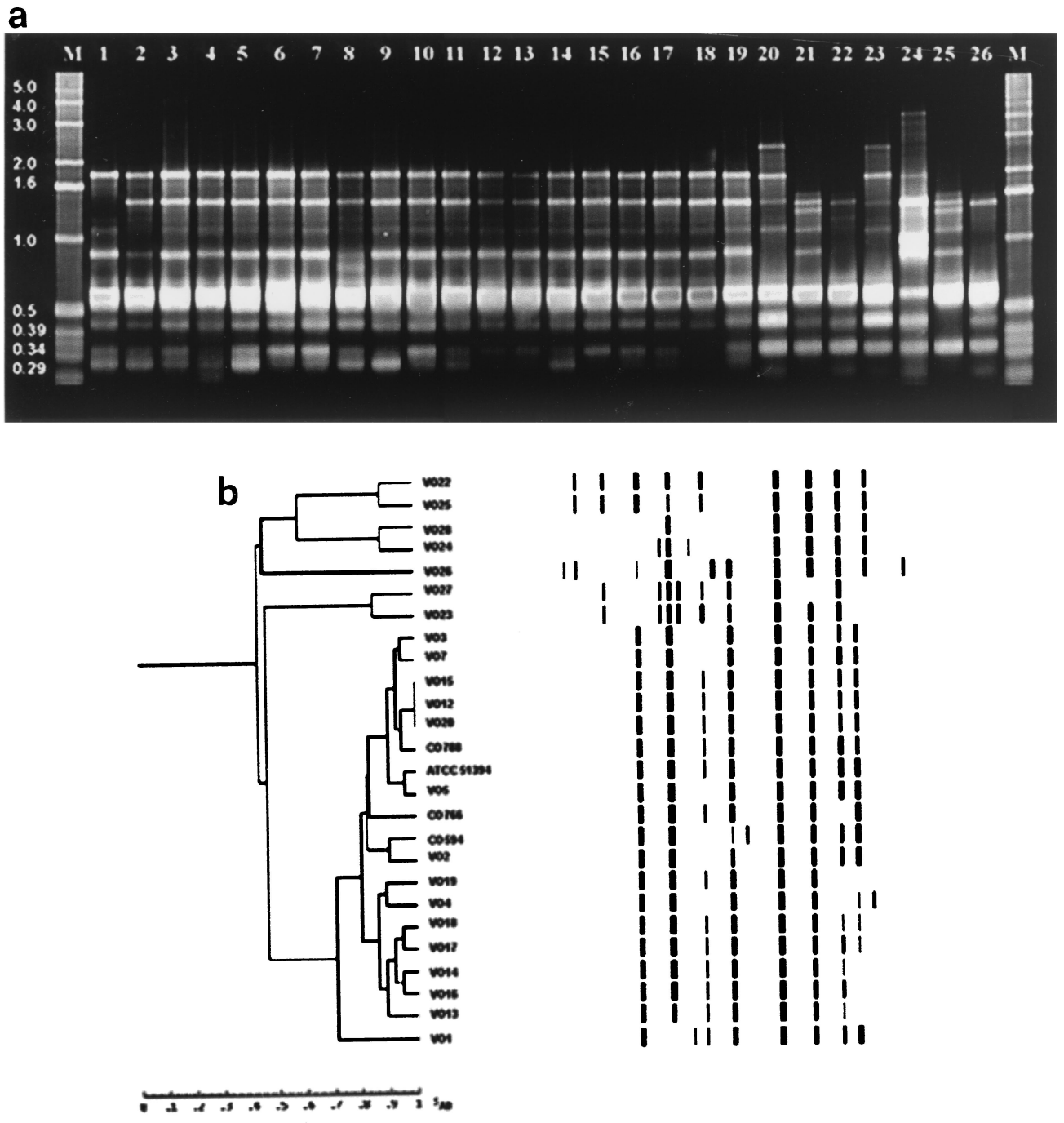

FIG. 1. (a) DNA fingerprints of clinical and environmental isolates of *V. cholerae* O1, O139, and non-O1, non-O139 strains generated by ERIC-PCR amplification. Lanes M, 1-kb molecular weight ladder; lanes 1 to 3 and 12, O1 strains VO1, VO2, VO3, and VO13, respectively; lanes 4 to 6, O1 strains VO4, VO5, and VO7, respectively; lanes 7 to 11 and 13 to 15, O139 strains MO45 (= ATCC 51394), CO594, CO766, CO788, VO12, VO14, VO15, and VO16, respectively; lanes 16 to 19, O139 strains VO17, VO18, VO19, and VO20, respectively; lanes 20 and 21, non-O1, non-O139 strains VO22, and VO23, respectively; lanes 22 to 26, non-O1, non-O139 strains VO24, VO25, VO26, VO27, and VO28, respectively. (b) Digitized ERIC-PCR profiles obtained from genomic DNA of clinical and environmental *V. cholerae* O1, O139, and non-O1, non-O139 isolates. The dendrogram was constructed by using the Molecular Fingerprinting Analyst (Bio-Rad) software with a simple-match similarity matrix, and data were clustered by the unweighted pair group method with arithmetic means.

isolated from diarrheal and environmental samples, did not show hemolysis in initial tests but were hemolytic on 5% sheep blood nutrient agar plates after serial passage through rabbit ileal loops (data not shown). All strains, whether hemolytic or nonhemolytic on 5% sheep blood nutrient agar plates, were positive for the *hly*A gene as determined by PCR. Thus, it is clear that sequences homologous to the hemolysin gene are present in both hemolytic and nonhemolytic strains of *V. chol-*

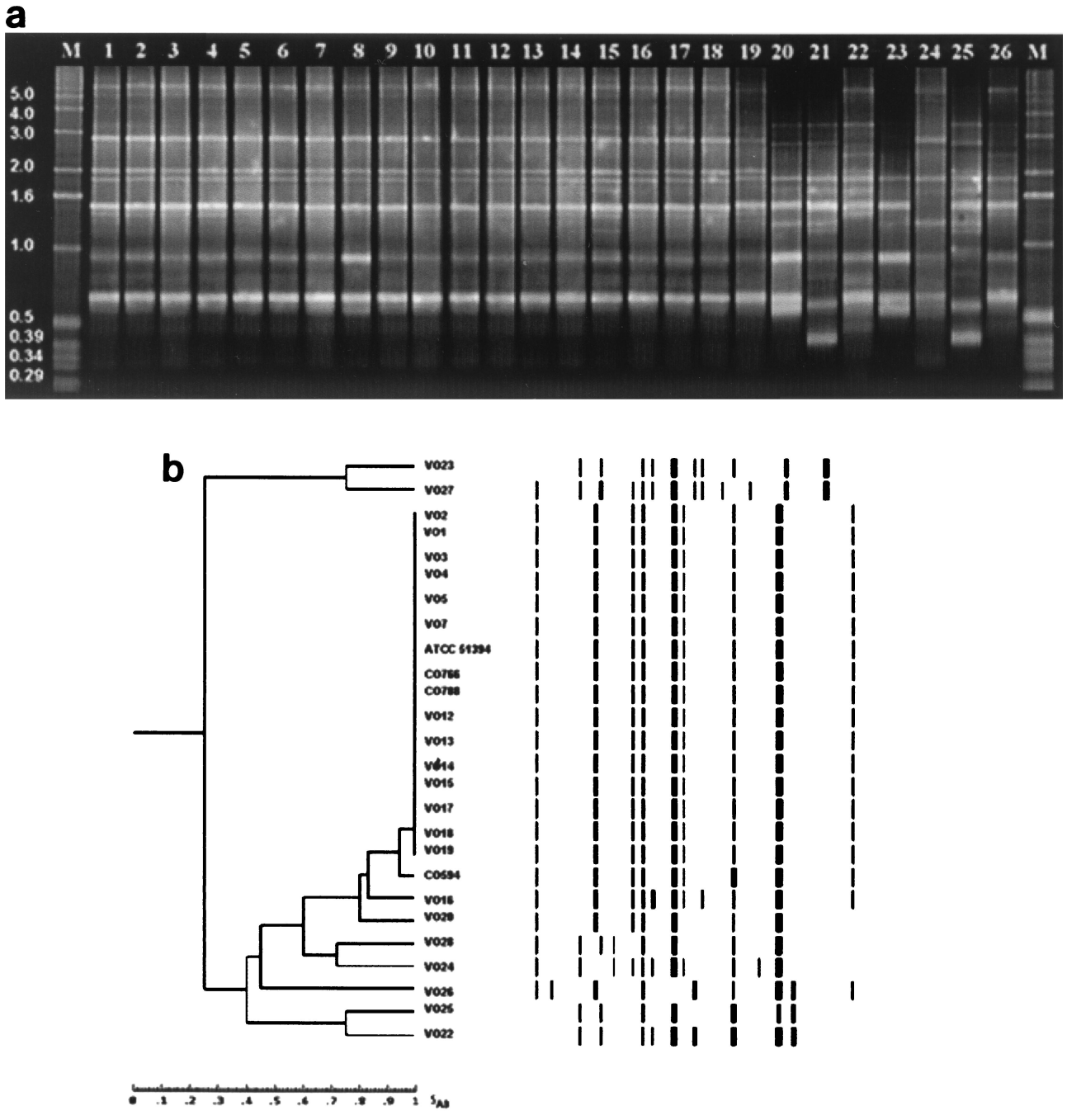

FIG. 2. (a) BOX-PCR profiles obtained by using DNA from clinical and environmental *V. cholerae* O1, O139, and non-O1, non-O139 isolates. Lanes M, 1-kb molecular weight ladder; lanes 1 to 3 and 12, O1 strains VO1, VO2, VO3, and VO13, respectively; lanes 4 to 6, O1 strains VO4, VO5, and VO7, respectively; lanes 7 to 11 and 13 to 15, O139 strains MO45 (= ATCC 51394), CO594, CO766, CO788, VO12, VO14, VO15, and VO16, respectively; lanes 16 to 19, O139 strains VO17, VO18, VO19, and VO20, respectively; lanes 20 and 21, non-O1, non-O139 strains VO22, and VO23, respectively; lanes 22 to 26, non-O1, non-O139 strains VO24, VO25, VO26, VO27, and VO28, respectively. (b) Digitized fingerprints of clinical and environmental *V. cholerae* O1, O139, and non-O1, non-O139 isolates generated by BOX-PCR amplification. The dendrogram was constructed by using the Molecular Fingerprinting Analyst (Bio-Rad) software as described in the legend to Fig. 1.

erae O1 biotype El Tor and O139 but may not be expressed in the nonhemolytic isolates.

The hemolysin produced by *V. cholerae* non-O1, non-O139 strains is identical to the El Tor hemolysin (78) and is considered to be a virulence factor that causes diarrhea (2, 35). However, we found that an El Tor-like hemolysin is not responsible for fluid production in the rabbit gut, because the nonhemolytic strains of *V. cholerae* also cause fluid accumulation. Furthermore, a number of hemolytic strains did not elicit a secretory response in the initial experiments (68, 71). All *V.*

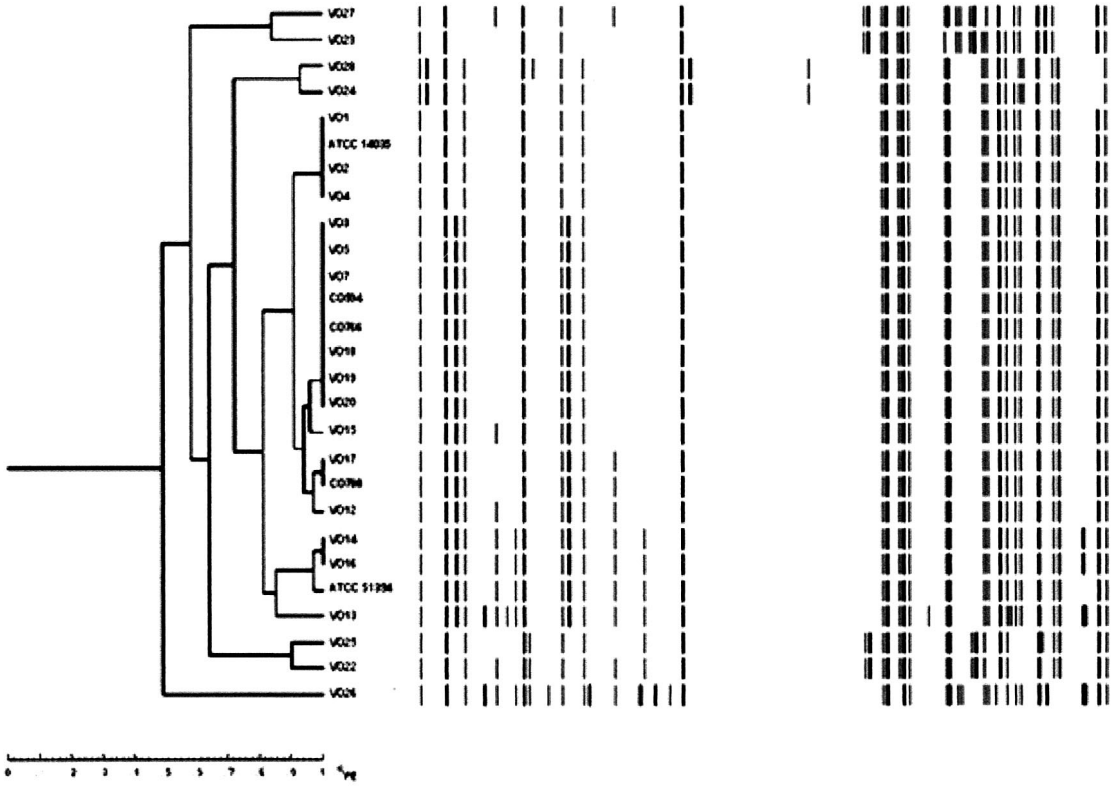

FIG. 3. Digitized images of AFLP patterns obtained from *Apa*I-*Taq*I template DNA by using the selective PCR primers A01 and T01 for clinical and environmental *V. cholerae* O1, O139, and non-O1, non-O139 isolates. The dendrogram was constructed by using the Molecular Fingerprinting Analyst (Bio-Rad) software as described in the legend to Fig. 1.

cholerae non-O1, non-O139 strains examined in this study were hemolytic on 5% sheep blood nutrient agar and had the genetic potential to produce hemolysin. However, the majority of the strains did not show a secretory response in initial tests, strongly suggesting that hemolysin does not play a role in initiating the secretory response.

The *V. cholerae* O1 biotype El Tor and O139 strains isolated from both diarrheal and environmental samples were characterized in detail to obtain an understanding of the role of the virulence traits in cholera. The environmental isolates, like the clinical isolates, were positive for *ctx*A, *zot, ace, tcp*A, and *omp*U amplicons, indicating that the genes comprising the virulence gene cassette and the genes encoding surface organelles required for intestinal adherence and colonization were intact. Thus, both the O1 biotype El Tor strains and the O139 strains retained the core of the CTX genetic element and also TCP and OMP both of which are recognized as important components in pathogenicity, even in the aquatic environment. These observations are in contrast to reports indicating that environmental isolates of *V. cholerae* O1 biotype El Tor are nontoxigenic (57) and that the hemolysin produced is most likely responsible for the enterotoxic activity (2, 35).

It is known that some *V. cholerae* non-O1, non-O139 strains have the ability to cause diarrhea-like symptoms. Recently, several localized outbreaks caused by *V. cholerae* non-O1, non-O139 have been reported (5, 16, 26, 64). Although some of these strains produce cholera or cholera-like toxin, the majority lack the virulence gene cassette but produce several other extracellular products, such as NAG-specific heat-stable toxin, a thermostable direct hemolysin, Shiga-like toxin, and hemagglutinin, which play some role in the disease process (3, 5, 23, 28, 54, 79).

We concluded from results of this study that in the absence of cholera toxin, NAG-specific heat-stable toxin, and/or TCP and OMP, *V. cholerae* O1, O139, and non-O1, non-O139 strains that have clinical or environmental origins have the ability to cause diarrhea by a mechanism entirely different from that of the toxigenic *V. cholerae* O1 and O139 strains. When culture filtrates from all of the non-cholera-toxigenic *V. cholerae* strains grown in AKI medium (39), mimicking hemolysin (59), were examined in rabbit guts, they caused fluid accumulation. The non-O1, non-O139 strains examined produced an enterotoxin that was antigenetically identifiable as a new *V. cholerae* toxin. This conclusion is supported by the fact that enterotoxic activity was completely neutralized (67) by the previously reported new cholera toxin antiserum (62, 63). Thus, we concluded that a new secretogenic toxin is the factor most likely responsible for enterotoxic activity in non-cholera-toxin-producing *V. cholerae* O1, O139, and non-O1, non-O139 strains.

Four different genetic fingerprinting methods, ERIC-PCR, BOX-PCR, AFLP analysis, and PFGE, were used to measure the relatedness of *V. cholerae* strains isolated from both clinical and environmental sources and the relatedness of O1 biotype

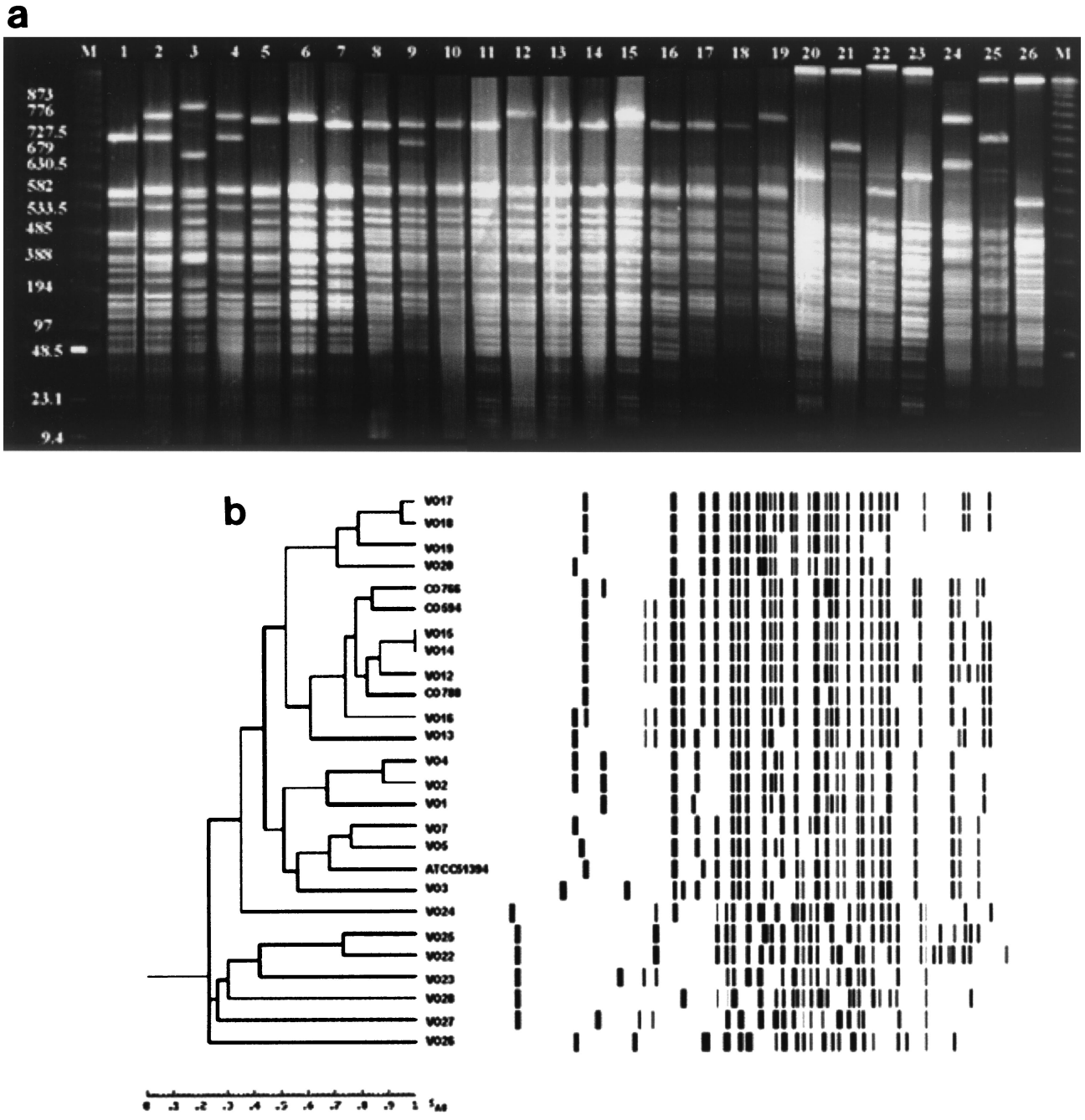

FIG. 4. (a) Fingerprint patterns obtained from PFGE of *Sfi*I-digested DNA of clinical and environmental *V. cholerae* O1, O139, and non-O1, non-O139 isolates. Lanes M, 1-kb molecular weight ladder; lanes 1 to 3 and 12, O1 strains VO1, VO2, VO3, and VO13, respectively; lanes 4 to 6, O1 strains VO4, VO5, and VO7, respectively; lanes 7 to 11 and 13 to 15, O139 strains MO45 (= ATCC 51394), CO594, CO766, CO788, VO12, VO14, VO15, and VO16, respectively; lanes 16 to 19, O139 strains VO17, VO18, VO19, and VO20, respectively; lanes 20, and 21, non-O1, non-O139 strains VO22, and VO23, respectively; lanes 22 to 26, non-O1, non-O139 strains VO24, VO25, VO26, VO27, and VO28, respectively; (b) Digitized PFGE analysis of *Sfi*I-digested profiles obtained from genomic DNA of clinical and environmental *V. cholerae* O1, O139, and non-O1, non-O139 isolates. The Dendrogram was generated by using the average percentages of matched bands summarizing the degrees of similarity of the *Sfi*I restriction patterns of genomic DNA of *V. cholerae* O1, O139, and non-O1, non-O139 strains.

El Tor, O139, and non-O1, non-O139 strains. Overall, the correlation of the results of all methods was excellent, and strains belonging to O1 biotype El Tor and O139 yielded fingerprint patterns that were closely related but distinct from those of the non-O1, non-O139 strains. The observed differences in the band patterns within and between the O1 and O139 serogroups suggested that there was divergence in genomic organization which may have arisen from genetic reassortment that took place in the environment over time. No significant difference between the clinical and environmental

isolates of *V. cholerae* was observed. The most interesting finding of the BOX-PCR, AFLP, and PFGE analyses was that environmental isolates (VO24 and/or VO28) of the non-O1, non-O139 serogroups were closely related to the toxigenic *V. cholerae* O1 and O139 strains.

Collectively, the data indicate that strains of *V. cholerae* that cause diarrhea have a clonal origin in the aquatic environment. Furthermore, clinical *V. cholerae* strains showed fingerprint patterns similar to those of strains isolated from the environment. Although minor differences in band patterns were observed among the strains, we concluded that there is a clonal relationship between clinical and environmental isolates, which supports the hypothesis of Colwell et al. (14) that *V. cholerae* strains are autochthonous to the aquatic environment and that aquatic environments serve as multiple reservoirs of toxigenic and non-cholera-toxigenic *V. cholerae* strains belonging to the O1, O139, and non-O1, non-O139 serogroups (12–14, 32, 66). The first strain of *V. cholerae* O139 isolated in our laboratory belonged to the same clone and was isolated in January 1992, when it was originally isolated from an hydrophytic plant, *Eichhornia crassipes*, in the River Ganga in Varanasi, India; this was almost 8 months before *V. cholerae* was isolated from diarrheal patients in Madras, India.

It has been reported that non-cholera-toxin-producing *V. cholerae* non-O1, non-O139 strains possess *tox*R, the central regulatory protein gene, and can acquire the *tcp* gene from toxigenic *V. cholerae* O1 by horizontal gene transfer (44) when they are exposed to the filamentous bacteriophage VPIϕ. The *ctx* element can be acquired by exposure to the toxinoferous phage CTXϕ (20, 76). Data obtained in the present study indicate that there is not a significant difference in the genomic organizations of toxigenic *V. cholerae* O1 and O139 strains, whether they are of clinical or environmental origin. Both toxigenic *V. cholerae* O1 strains and non-cholera-toxin-producing *V. cholerae* non-O1, non-O139 strains possess *tox*R, the central regulatory protein gene, and these strains are present in the aquatic environment. Thus, it can be hypothesized that under the selective pressures of the biological and physicochemical conditions of the aquatic environment, events like acquisition of *tcp* and *ctx* genetic elements by non-O1, non-O139 strains from O1 *V. cholerae* strains can occur after exposure to bacteriophages and thereby favor emergence of a novel strain. However, given the rare occurrence of non-O1, non-O139 strains in the environment that possess both the *tcp*A and *ctx* genetic elements (52), it appears that when and if such events occur, there are attendant changes in the somatic antigen or in the cell surface properties (7, 51). A similar suggestion has recently been made concerning the *asd* gene sequences of O1 and non-O1, non-O139 strains in transition for the somatic O antigen in *V. cholerae* (43).

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