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Vibriophage VcA-3 as an Epidemic Strain Marker for the U.S. Gulf Coast *Vibrio cholerae* O1 Clone

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Toxigenic and nontoxigenic *Vibrio cholerae* O1, El Tor biotype strains, which are endemic to the U.S. Gulf Coast, can be lysogenic for bacteriophage VcA-3. To evaluate the presence of VcA-3 as an indicator of toxigenicity and as an epidemic strain marker, phage production and the presence of phage and cholera toxin genes were assayed in 98 strains of *V. cholerae* O1 (35 U.S. and 63 foreign strains). By using a *Hin*dIII chromosomal digest for Southern blot analysis, 39 of the study strains hybridized with the VcA-3 probe in 10 banding patterns. The 15 toxigenic and 6 of the 20 nontoxigenic U.S. isolates gave four VcA-3-related patterns. Among the foreign isolates, 12 of 12 toxigenic classical biotype strains, 1 of 43 toxigenic El Tor biotype strains, and 3 of 8 nontoxigenic atypical strains gave six patterns that were clearly distinct from that of VcA-3. Compared with Southern blot analysis, the phage production assay had a sensitivity of 1.0 and a specificity of 0.48, while the colony hybridization assay had a sensitivity of 1.0 and a specificity of 0.77 for identification of VcA-3. Neither assay reliably identified the toxigenic Gulf Coast clone. The presence of VcA-3, as defined by Southern blot analysis, always separated toxigenic U.S. from foreign isolates and often from nontoxigenic U.S. isolates of *V. cholerae* O1.

Toxigenic *Vibrio cholerae* O1, the causative agent of cholera, has traditionally been characterized by serotype and biotype. Since the early 1960s, most isolates worldwide have belonged to the El Tor biotype and either the Inaba or Ogawa serotype. In 1984, the World Health Organization issued a report (26) outlining the need for additional methods for the subtyping of *V. cholerae* serogroup O1 isolates in order to facilitate epidemiologic studies and implement control measures such as vaccines. Phenotypic tests such as biotyping and phage typing have been used for subtyping in the past. These are often affected by culture conditions or problems of gene expression. The newer molecular techniques now available to investigators (25) may identify pathogenicity factors or at least provide more specific, stable markers for pathogenic or epidemic strains. Genotypic tests should overcome many of the limitations of phenotypic assays and have the potential to be more specific and sensitive.

Toxigenic *V. cholerae* O1 appears to have an environmental reservoir on the U.S. Gulf Coast and has caused well-documented sporadic and epidemic disease since 1973 (2–4). Kaper et al. (17) used a Southern blot analysis to demonstrate that toxigenic strains of *V. cholerae* O1 from the Gulf Coast had similar cholera toxin (CT) gene sequences and concluded that a single strain of toxigenic *V. cholerae* O1 is resident on the Gulf Coast. Epidemic investigations within the United States have been complicated by the isolation of nontoxigenic *V. cholerae* O1 from both patients and the environment; the pathogenicities of these organisms and their relationship to toxigenic strains are poorly understood and controversial (6). Furthermore, the relationship of the toxigenic Gulf Coast clone to the current (seventh) cholera pandemic remains unclear.

Goldberg and Murphy (14) reported that environmental and clinical *V. cholerae* O1 strains isolated from the U.S. Gulf Coast are lysogenic for a vibriophage which they designated VcA-3. They demonstrated by Southern blot analysis that all U.S. Gulf Coast clinical isolates carried VcA-3 phage integrated in the same chromosomal location. Later, Cook et al. (7) showed that classical biotype strains isolated during the sixth pandemic and recently in Bangladesh hybridized in Southern blots with vibriophage VcA-1 and had similar patterns, indicating similar integration sites and clonality of that biotype. In this study, we expand the work of Goldberg and Murphy (14) and also investigate the use of phenotypic and colony blot hybridization assays as epidemic strain markers for VcA-3.

MATERIALS AND METHODS

Bacteria. *V. cholerae* O1 strains were selected on the basis of epidemiologic data, case reports, environmental surveys, and geographic isolation site. Isolates were obtained from the stock collection of the Enteric Diseases Laboratory Section, Centers for Disease Control (CDC), and had been received by the CDC between 1973 and 1988. A total of 98 study strains was selected (H, human source; E, environmental source), as follows: 15 toxigenic strains (11 H; 4 E), 20 nontoxigenic strains (14 H; 6 E), 55 toxigenic foreign strains (all H), and 8 nontoxigenic foreign strains (4 H; 4 E).

Phenotypic tests. Stock cultures of *V. cholerae* O1 were examined for viability and purity and were then identified by the appropriate biochemical tests (8). Slide agglutination was used for the determination of the O1 serogroup and serotype (22). Five traditional tests were used to define the classical

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and El Tor biotypes of *V. cholerae* (8). Several CT assays were used and are described in detail in a previous report (1).

The test for production of vibriophage VcA-3 used *V. cholerae* RJ1 (16) as an indicator strain and isolate 2164-78 as a negative control. The two media used were TYC agar and TYC semisolid medium. TYC medium contained tryptone (Difco Laboratories, Detroit, Mich.), 10 g; yeast extract, 2 g; glucose, 1 g; NaCl, 8 g; and endotoxin-free water to 1,000 ml (pH 7.8). TYC agar contained 1.1% (wt/vol) agar, and TYC semisolid medium contained 0.5% (wt/vol) agar. Two tubes, each containing 3 ml of TYC semisolid medium, were warmed until the medium was in a liquid state and held at 46°C. One tube received 0.2 ml of an overnight heart infusion broth culture of indicator organism. The second tube received the same amount of negative control organism. The contents of each tube were mixed and poured over the surface of deep (5-mm) TYC agar plates. Overnight cultures of study isolates on Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) were tested for production of VcA-3 phage by stabbing a toothpick into the indicator strain and negative control overlays. A known VcA-3 producer strain was included with each batch of study isolates. The overlay plates were incubated overnight at 36°C. The appearance of a plaque in the indicator plate and the absence of clearing in the negative control plate were indicative of VcA-3 production.

Chromosomal DNA preparation. Bacterial strains were grown in brain heart infusion broth at 37°C to an A_{575} of 0.8 ± 0.02. The cells were separated from the growth medium by centrifugation and were resuspended in 1 ml of 50 mM Tris buffer (pH 8.0, 25°C). The suspension was placed in a 1.5-ml Eppendorf tube and centrifuged for 2 min at high speed in a microcentrifuge. The supernatant was discarded, and the cells were suspended in 150 μl of sucrose solution (50 mM Tris buffer [pH 8.0, 5°C] and 25% sucrose); 50 μl of lysozyme solution (5 mg of lysozyme per ml in 0.25 M Tris buffer [pH 8.0, 5°C]) was added, and the tube was placed on ice for 15 min. Then, 60 μl of 0.2 M EDTA (pH 8.0) was added and incubated for 5 min on ice. A mixture consisting of 10 μl of 1 mg of RNase per ml in 50 mM Tris buffer (pH 8.0, 25°C), 50 μl of 25 mg of pronase per ml in 50 mM Tris buffer (pH 8.0, 25°C), and 250 μl of lysis mixture (10 mM EDTA, 2% Triton X-100, 50 mM Tris buffer [pH 8.0, 25°C]) was added; and the tube was placed in a 37°C incubator for 90 min. This was followed by phenol-chloroform extraction. The purified DNA was recovered by ethanol precipitation and dissolved in a suitable volume of sterile distilled water. A 2-μg amount of DNA was digested with *Hin*dIII, according to the instructions of the manufacturer (New England Biolabs, Inc., Beverly, Mass.). After normal incubation, an additional 1 μl of *Hin*dIII was added, and the tubes were incubated for an additional 2 h.

Digests were electrophoresed through a horizontal 1% agarose (Seakem GTG, FMC Bioproducts, Rockland, Maine) gel (20) at 65 V in TBE buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid) for 18 h. Gels were stained with ethidium bromide (0.4 μg/ml) for 20 min and visualized by UV transillumination. The method of Southern (23) was used to transfer DNA fragments from the agarose gel onto a nitrocellulose membrane.

Bacterial colony DNA was prepared for hybridization by the method of Maas (18). Whatman 541 filter papers were kept at room temperature without light until hybridization.

Preparation of DNA probe for CT. The oligonucleotide probe (ctxa11) for the CT gene was produced on a model 380A polynucleotide synthesizer (Applied Biosystems, Foster City, Calif.) and was provided through the courtesy of Brian Holloway, Biotechnology Core Facility Branch, CDC. The ctxa11 probe encodes amino acids 45 through two-thirds of amino acid 51 (Ala, Arg, Gly, Thr, Gly, Thr, and the GG sequence of Gly). The exact nucleotide sequence is 5'-GCAAGAGGAACTCAGACGGG-3' (generously provided by Walter Hill, Division of Microbiology, U.S. Food and Drug Administration, Washington, D.C.). The probe was lyophilized for storage and rehydrated with sterile distilled water before end-labeling with bacteriophage T4 polynucleotide kinase (20 U/μl; Bethesda Research Laboratories, Gaithersburg, Md.). Unincorporated ^{32}P was removed by using a NACS PREPAC column (Bethesda Research Laboratories) and by eluting the labeled probe with 4.0 M ammonium acetate buffer.

Preparation of VcA-3 phage DNA probe. Phage was prepared, concentrated, and assayed by the method of Gerdes and Romig (12, 13). Phage VcA-3 DNA was purified by phenol extraction and ethanol precipitation and was stored at 4°C. The phage DNA was labeled with [α-^{32}P]dCTP by random priming following the instructions of the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany) (10, 11). Unincorporated ^{32}P was removed by binding the DNA to a NICK column (Pharmacia AB, Uppsala, Sweden) prepacked with Sephadex G-50.

Hybridization. For CT probe assays, nitrocellulose membranes were hybridized with ctxa11 overnight at 40°C in a solution containing 50 ml of hybridization mixture (15 ml of 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 ml of 50× Denhardt's solution [2 g of Ficoll, 2 g of polyvinylpyrrolidone, and 2 g of bovine serum albumin in 200 ml of distilled water, stored at −20°C in 5-ml aliquots], 0.1 ml of 0.5 M EDTA [pH 8.0], and 28.9 ml of distilled water), 1 ml of sonicated and boiled calf thymus DNA (10 mg/ml), and 10^6 cpm of labeled probe. Membranes were rinsed with 6× SSC, washed with fresh 6× SSC at 50°C for 1 h (twice), and finally rinsed with 2× SSC (19).

For VcA-3 probe assays, nitrocellulose membranes and Whatman 541 filters were hybridized with 5×10^6 cpm of labeled VcA-3 DNA as described previously (7). Membranes and filters for both probes were exposed to X-ray film overnight at −70°C.

RESULTS

In Southern blot analysis, 39 of the 98 study isolates (40%) hybridized with the VcA-3 DNA probe, yielding 10 different hybridization patterns (patterns i to x). Figure 1 shows representative VcA-3 patterns. Table 1 lists the types and numbers of study strains associated with each pattern.

All 12 classical biotype isolates hybridized with the vibriophage probe in one pattern (pattern i) that indicated the presence of VcA-1 (7). This pattern was not seen in nonclassical isolates. Previous studies have documented that VcA-1 and VcA-3 are similar morphologically and are homoimmune (14).

Each of the 15 toxigenic U.S. isolates hybridized with VcA-3 DNA, yielding three different hybridization patterns (pattern ii, 13 strains; pattern iii, 1 strain; pattern vii, 1 strain). Pattern ii is similar to that described previously (14) for VcA-3 and represents the prototype pattern in this study. Patterns iii and vii each differed from the predominant pattern ii only by the addition of a single band in the high-molecular-weight region. Pattern viii, seen in one nontoxigenic U.S. El Tor isolate, also differed from pattern ii by one high-molecular-weight band. These minor variations in

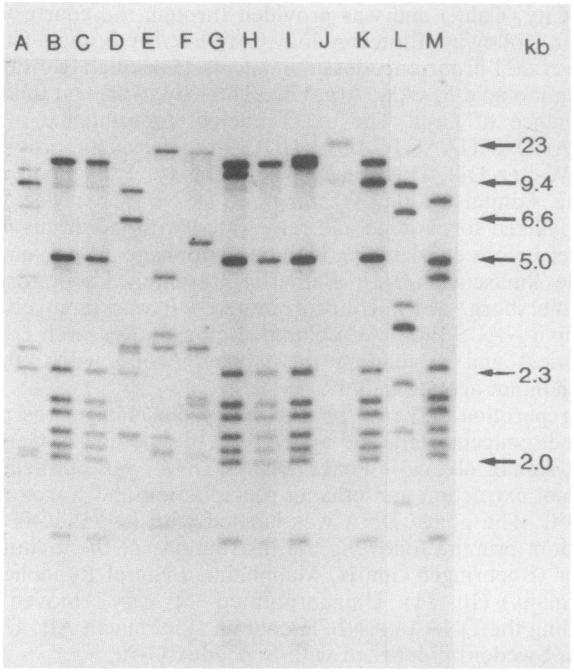

FIG. 1. Autoradiograph of *Hin*dIII-digested chromosomal DNA hybridized with the VcA-3 probe. Lane A, ATCC 14035 (9060-79) (pattern i); lane B, 2164-78 (pattern ii); lane C, 3242-73 (pattern ii); lane D, E9120 (pattern iv); lane E, 1074-78 (pattern v); lane F, 884-82 (pattern vi); lane G, 2373-81 (pattern vii); lane H, 2496-85 (pattern ii); lane I, 2496-86 (pattern viii); lane J, bacteriophage lambda DNA; lane K, 2538-86 (pattern iii), lane L, 2527-86 (pattern ix); lane M, 2584-87 (pattern x).

hybridization patterns probably reflect differences in the VcA-3 phage insertion sites. Strains with these four related hybridization patterns (patterns ii, iii, vii, viii) were also positive in colony blot assays and in the phenotypic test for VcA-3 phage production. For these reasons patterns iii, vii, and viii were all considered to be VcA-3 (pattern ii) related.

Patterns iv, v, vi, and ix (found in one strain each) and pattern x (found in two strains) were clearly different from patterns i or ii. Five of the six strains with these patterns were nontoxigenic, and three were negative in tests for VcA-3 production. These five patterns appear to be more distantly related to VcA-3 and VcA-1 and probably represent distinct and separate vibriophages.

Minor differences of one or two restriction fragments with VcA-3 hybridization patterns were predicted, since vibriophage VcA-3 is capable of integrating without classic recombination into different sites in the bacterial chromosome. This lysogeny can inactivate the particular gene into which the prophage has integrated (14). No correlation was noted between a phenotypic change, e.g., toxin production or biochemical reaction, and a variation of patterns iii, vii, and viii from VcA-3 prototype pattern ii. There were also no pattern-associated differences by year of isolation or geographic isolation site.

Table 2 shows the distribution of VcA-3 DNA probe hybridization patterns among the toxigenic El Tor isolates. The 15 U.S. isolates yielded three VcA-3 and related hybridization patterns (patterns ii, iii, and vii). The single foreign toxigenic El Tor strain (E9120) that hybridized with the VcA-3 probe in this analysis was isolated in Indonesia and had a unique hybridization pattern (pattern iv) that was clearly different from that of VcA-3 and related patterns. In summary, none of the toxigenic foreign El Tor strains yielded a VcA-3-related hybridization pattern. Therefore, the presence of VcA-3 and related hybridization patterns correlated 100% with U.S. toxigenic El Tor isolates ($P < 0.0001$ by Fisher's exact test) when U.S. and foreign toxigenic El Tor study strains were compared.

Table 3 shows that the VcA-3 production assay results disagreed with VcA-3 and related hybridization patterns in 40 of the 98 (41%) strains tested. The majority of disagree-

TABLE 1. VcA-3 DNA hybridization patterns in 39 *V. cholerae* O1 isolates

Pattern designation	No. of bands	No. of isolate and description
i	9	12 toxigenic classical strains
ii[a]	9	13 toxigenic U.S. El Tor strains, 4 nontoxigenic U.S. El Tor strains
iii[a]	10	1 toxigenic U.S. El Tor strain
iv	7	1 toxigenic foreign El Tor strain
v	8	1 nontoxigenic atypical foreign strain
vi	8	1 nontoxigenic atypical U.S. strain
vii[a]	10	1 toxigenic U.S. El Tor strain, 1 nontoxigenic U.S. El Tor strain
viii[a]	10	1 nontoxigenic U.S. El Tor strain
ix	6	1 nontoxigenic U.S. El Tor strain
x	9	2 nontoxigenic foreign El Tor strains

[a] VcA-3 and related patterns.

TABLE 2. VcA-3 DNA hybridization patterns in 58 U.S. and foreign toxigenic *V. cholerae* O1 El Tor isolates

VcA-3 probe pattern	No. (%) of:	
	U.S. strains (n = 15)	Foreign strains (n = 43)
Negative	0	42 (98)
i	0	0
ii[a]	13 (87)	0
iii[a]	1 (6.5)	0
iv	0	1 (2)
v	0	0
vi	0	0
vii[a]	1 (6.5)	0
viii[a]	0	0
ix	0	0
x	0	0

[a] VcA-3 and related patterns.

TABLE 3. Correlation of VcA-3 phage production with VcA-3 and related hybridization patterns (patterns ii, iii, vii, and viii) in 98 strains of *V. cholerae* O1

Region and toxigenicity[a] (n)	No. of isolates				No. of disagreements
	Phage production		Probe pattern		
	+	−	+	−	
U.S., tox. (15)	15	0	15	0	0
U.S., nontox. (20)	6	14	6	14	0
Foreign, tox. (55)	38	17	0	55	38
Foreign, nontox. (8)	2	6	0	8	2

[a] nontox., nontoxigenic; tox., toxigenic.

TABLE 4. Correlation of VcA-3 colony hybridization with VcA-3 and related hybridization patterns (patterns ii, iii, vii, and viii) in 98 strains of *V. cholerae* O1

Region and toxigenicity[a] (*n*)	No. of isolates				No. of disagreements
	Colony		Pattern		
	+	−	+	−	
U.S., tox. (15)	15	0	15	0	0
U.S., nontox. (20)	8	12	6	14	2
Foreign, tox. (55)	13	42	0	55	13
Foreign, nontox. (8)	3	5	0	8	3

[a] nontox., nontoxigenic; tox., toxigenic.

ments involved toxigenic foreign El Tor strains in which the VcA-3 production test result was positive yet in which no hybridization occurred with the VcA-3 DNA probe. This suggests that these plaques that were formed in the production test were caused by one or more phages that were genetically unrelated to VcA-3. The sensitivity of the VcA-3 production test compared with the hybridization pattern was 1.00; the specificity was 0.48. The accuracy of the VcA-3 production test compared with that of the "gold standard" (i.e., the VcA-3-related hybridization pattern) was 59% (58 of 98 isolates).

Table 4 shows that colony blot test results disagreed with VcA-3 and related hybridization patterns in 18 of the 98 (18%) strains tested. The sensitivity of the colony blot probe assay compared with that of the Southern blot procedure was 1.0, and the specificity was 0.77.

All 15 toxigenic U.S. El Tor strains produced plaques in the phenotypic test and were positive for VcA-3 by DNA probe. Thirty percent (6 of 20) of nontoxigenic U.S. isolates were positive for VcA-3 in the production test and for VcA-3 and related hybridization patterns. The correlation observed between toxigenicity and VcA-3 hybridization patterns as well as phage production in U.S. strains was statistically significant ($P < 0.0001$ by Fisher's exact test). Additionally, the correlation between toxigenicity in these 35 U.S. strains and colony hybridization with the VcA-3 DNA probe was statistically significant ($P = 0.0002$ by Fisher's exact test).

DISCUSSION

Farmer et al. (9) suggested that the VcA-3 production test be used as a screening test for the U.S. toxigenic *V. cholerae* O1 strain. Our data show a statistically significant correlation between toxigenicity in the U.S. study strains and VcA-3 production. When U.S. strains were tested for VcA-3 production and probed for CT genes, the results agreed for 29 of 35 (83%) strains.

Forty of 63 (63%) foreign El Tor strains from the current pandemic produced plaques in the production assay, yet only 16 were probe positive, and none had the VcA-3-related hybridization pattern. On the basis of these results, the VcA-3 production test is not a good indicator of the Gulf Coast clone. Despite the correlation between VcA-3 production and toxigenicity observed within the U.S. strains, production was not a reliable indicator of toxigenicity. The overall accuracy of the VcA-3 production test with respect to the CT probe for all 98 study strains was relatively low. Therefore, it appears that this phenotypic test may be useful only for testing U.S. isolates from persons with no foreign travel history.

Colony blot hybridization (15) was no better than the VcA-3 production test as an indicator of toxigenicity. The VcA-3 colony blot assay agreed with the CT probe results for 27 of 35 (77%) U.S. strains tested. When foreign strains were tested, the colony blot results agreed with the toxin probe results for only 18 of 63 strains. The colony blot probe assay did not prove to be a useful test for identifying the Gulf Coast clone. Although all 15 U.S. toxigenic strains were positive, 16 foreign strains (13 toxigenic, 3 nontoxigenic) tested positive as well. The colony blot probe assay did differentiate U.S. toxigenic strains from most of the foreign toxigenic strains that were positive in the production assay.

Southern blot analysis appears to be the most accurate method of defining the VcA-3 phage (gold standard). In this study, all the toxigenic U.S. isolates ($n = 15$) and 6 of the nontoxigenic U.S. isolates ($n = 20$) yielded VcA-3 and related hybridization patterns. These results are in agreement with those of Goldberg and Murphy (14) in suggesting that there is a clonal relationship among certain toxigenic and nontoxigenic strains isolated from the U.S. Gulf Coast. Morris et al. (21) also concluded that certain nontoxigenic and toxigenic *V. cholerae* O1 strains from the Gulf Coast are related. We further documented the presence of nontoxigenic *V. cholerae* O1 strains from both humans ($n = 4$) and the environment ($n = 1$) that appeared to be unrelated to the Gulf Coast clone. The hybridization pattern always separated the Gulf Coast clone from foreign strains. No foreign strain, either toxigenic or nontoxigenic, had a VcA-3 or related hybridization pattern.

Chen et al. (6) used multilocus enzyme electrophoresis to examine genetic relationships among toxigenic and nontoxigenic *V. cholerae* O1 isolates obtained from patients and the environment on the Gulf Coast. Their results support the hypothesis that the Gulf Coast strains belong to a unique "clone" (electrophoretic type) which contains toxigenic and some nontoxigenic isolates. The current epidemic of cholera in South America is caused by a strain of toxigenic *V. cholerae* O1 that can be easily distinguished from Gulf Coast isolates by phage production or colony hybridization (5). Like most of the seventh pandemic strains, it is VcA-3 negative (24). It seems that even these simple, yet less accurate, assays can be useful in specific circumstances. Certainly, Southern blot analysis of VcA-3 is the most accurate and epidemiologically useful molecular subtyping technique.

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