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Molecular Epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast

JAMES B. KAPER,[1]* HENRY B. BRADFORD,[2] NELL C. ROBERTS,[2] AND STANLEY FALKOW[3]

Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21201[1]; Department of Health and Human Resources, Office of Health Services and Environmental Quality, Division of Laboratory Services, New Orleans, Louisiana 70112[2]; and Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305[3]

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Enterotoxigenic strains of *Vibrio cholerae* O-1, biotype El Tor, isolated from a case of cholera in Texas in 1973, an outbreak of cholera in Louisiana in 1978, and Louisiana sewage samples in 1980 and 1981 were analyzed for their genetic similarities. Chromosomal DNA was isolated from each strain, digested with restriction endonuclease, and analyzed by the Southern blot technique. A radioactive probe consisting of *Escherichia coli* heat-labile enterotoxin DNA detected cholera toxin gene sequences in these strains and demonstrated that the toxin gene sequence, if not the entire chromosomal DNA, is identical in these strains and distinctly different from other strains of *V. cholerae* isolated throughout the world. In addition, two strains of enterotoxigenic *V. cholerae* non-O-1 isolated from clinical cases, were analyzed and found to possess cholera toxin genes which differed in the DNA sequence from the *V. cholerae* O-1 strains. We concluded that a single strain of enterotoxigenic *V. cholerae* O-1 is resident in the U.S. Gulf Coast and that a second reservoir of cholera toxin genes exists in *V. cholerae* non-O-1 strains in Louisiana.

In September of 1978, an outbreak of cholera occurred in Louisiana which involved 11 people. The vehicle of transmission was shown to be inadequately cooked crabs, and strains of *Vibrio cholerae* O-1 isolated from patients bore a striking resemblance to a strain isolated from a case of cholera in Port Lavaca, Tex., in 1973. Because of the similarities in hemolytic reactions and phage type of the Louisiana and Texas strains, the possibility of an endemic focus of *V. cholerae* in the U.S. Gulf Coast was raised (2). In 1979, despite intensive surveillance, no enterotoxigenic strain of *V. cholerae* O-1 was isolated from environmental or clinical samples in Louisiana. However, in September 1980, an isolate of *V. cholerae* O-1 Inaba was recovered from a sewage sample in Lake Charles, La. This isolate proved to be enterotoxigenic, hemolytic, and of the same phage type (e5/4996/13/16/24) as the 1973 and 1978 isolates (2, 14). Recently, in the summer of 1981, further enterotoxigenic strains of *V. cholerae* O-1 Inaba were isolated from sewage samples in Louisiana. Because of the striking phenotypic similarities among these strains and their dissimilarity to other strains of *V. cholerae* isolated throughout the world, we decided to examine these isolates for their molecular genetic similarities and dissimilarities to other *V. cholerae* O-1 and non-O-1 strains. Because of the DNA sequence homology between cholera toxin (CT) and the heat-labile enterotoxin (LT) of *Escherichia coli* (5, 23), a molecular probe can be made of LT genes which hybridize with DNA extracted from *V. cholerae*, thereby detecting toxin genes in these strains (11, 19). This technique has now been utilized to yield significant epidemiological data about *V. cholerae* in the Gulf Coast.

MATERIALS AND METHODS

Bacterial strains. Several strains of *V. cholerae* O-1 were examined for the presence of genes homologous to *E. coli* LT genes (Table 1). These included the 1973 Texas isolate (E506) and stool and sewer isolates from Louisiana from the past 3 years. In addition, strains of *V. cholerae* isolated early in the current pandemic (E8439, E9120) were examined because of their similarity to the Gulf Coast strains in hemolytic reactions. Recent isolates from Australia and Japan were examined, as were two strains of *V. cholerae* non-O-1 isolated from severe cases of diarrhea in Louisiana. The well-studied toxigenic classical strain, 569B (13), was also included.

Chromosomal DNA preparation. Chromosomal DNA was extracted by the method of Brenner et al (3). One-microgram amounts of the DNA preparations were digested with *Eco*RI and *Hin*dIII restriction endonucleases (Bethesda Research Laboratories, Rockville, Md.) for approximately 2 h at 37°C in the buffers recommended by the manufacturer. The reactions were stopped by adding 4 μl of 0.07% bromophenol blue–7% sodium dodecyl sulfate in 20% Ficoll,

and restriction fragments were separated by agarose gel electrophoresis in a horizontal 0.7% agarose gel. Electrophoresis was performed in Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid), and gels were then visualized by ethidium bromide under UV light and photographed.

After electrophoresis and photography, the gels were denatured in 0.2 M NaOH–0.6 M NaCl for 45 min and then neutralized in 1 M Tris–0.6 M NaCl (pH 7.5) for 45 min, and the DNA fragments were transferred to nitrocellulose filters by the technique of Southern (22). After overnight transfer, the filters were baked at 80°C in vacuo and stored until hybridization.

Preparation and labeling of *E. coli* LT DNA. *E. coli* strain C600, containing the small, amplifiable recombinant plasmid EWD299 encoding *E. coli* LT (6), was used for preparation of labeled DNA probe. Plasmid DNA was prepared from this strain after overnight amplification and extraction as described by So et al. (21).

A molecular probe consisting of the genes encoding the *E. coli* LT A subunit was prepared as described by Kaper et al. (11). The 1,200-base-pair fragment was labeled in vitro by the nick-translation method of Maniatis et al. (17) with [α-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.).

Hybridization and autoradiography. Hybridization of the LT probe to the vibrio DNA was performed by preincubation of the filters for at least 3 h at 37°C in a solution of 25% formamide–5× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt solution (0.02% Ficoll, molecular weight 400,000; 0.02% polyvinylpyrrolidone, molecular weight 360,000; 0.02% bovine serum albumin [8])–0.1% sodium dodecyl sulfate–1 mM EDTA. After preincubation, the filters were transferred to 10 ml of fresh solution (described above) containing about 2×10^6 cpm of ^{32}P-labeled DNA and 100 μg of denatured calf thymus DNA per ml sheared by sonication in a Sonicator cell disruptor (Heat Systems Ultrasonics Inc., Plainview, N.Y.). Hybridization was carried out at 37°C for 16 h, after which the filters were rinsed in 5× SSC–0.1% sodium dodecyl sulfate at 54.5°C and then washed for 1 h in fresh 5× SSC–0.1% sodium dodecyl sulfate at 54.5°C. After a final rinse in 2× SSC at ambient temperature, the filters were air dried, placed in a cassette with Kodak X-Omat R film and Du Pont Cronex Lightning Plus intensifying screens for 24 h at −70°C, and then developed.

RESULTS AND DISCUSSION

The DNA from strains of *V. cholerae* producing CT will hybridize to an *E. coli* LT DNA probe, owing to the homology between genes encoding LT and CT (5, 11, 19, 23). Strains to which the LT probe did not hybridize did not produce CT (11). In the present study, strains Ym 331-6 and Ym 404-9 from Japan and SGN7730 from Louisiana did not exhibit homology with LT and did not produce CT in Y-1 adrenal cell and enzyme-linked immunosorbent assays (W. M. Spira and P. Ristaino, personal communication). The results of the hybridization are visualized by the radioactive probe binding to the homologous filter-bound vibrio DNA, thereby producing a darkening of the X-ray film upon autoradiography. When chromosomal DNA from the strains is digested with restriction endonucleases, separated by gel electrophoresis, and hybridized to the LT probe, a distinct autoradiographic pattern emerges, depending upon which enzyme and strain are employed. For example, with the restriction endonuclease *Hin*dIII, the classical biotype of *V. cholerae* will exhibit at least two large restriction fragments with homology to the LT (11). The strains of the El Tor biotype were found to possess only a single large *Hin*dIII fragment, with the exception of El Tor strains isolated from the 1978 Louisiana outbreak (11). In the present study, this observation was extended to a variety of other El Tor strains, including a recent isolate from Australia (Stokes 1) and strains isolated early in the current cholera pandemic (E8439, E9120). These strains were of particular interest because of the hemolytic nature of isolates from these years, in contrast to the nonhemolytic strains which are currently being isolated outside the United States (1). Most El Tor strains examined possessed only a single large *Hin*dIII fragment ranging in size from ca. 13 to 23 kilobases (kb) (Fig. 1). The exceptions to this pattern were all isolated from the U.S. Gulf Coast. Strain 4808 (Fig. 1, lane B) was isolated in 1978 from a patient in Louisiana with cholera. Figure 2 shows other isolates from the U.S. Gulf Coast, including E506 (lane a) from the 1973 Texas case; 4808 (lanes b and f); SGN7277 (lanes c and g), a 1980 sewer isolate; and two *V. cholerae* non-O-1 clinical isolates, 2002H and 2011H (lanes d, e, h, and i). The three *V. cholerae* O-1 strains plus a fourth strain, SGN 7700, a 1981 sewer isolate (data not shown), all possessed a unique *Hin*dIII restriction endonuclease site within the toxin gene which caused two small fragments (ca. 6 and 7 kb in size) to hybridize with the LT probe rather than a single large fragment. This indicates that the toxin gene base sequence has undergone evolutionary divergence and that slightly different base sequences are to be found among different strains. This genetic divergence is not surprising in light of the amino acid differences found between CT and the LT of *E. coli*. LT is very similar to CT in terms of structure, function, and antigenicity and shares approximately 79% amino acid homology with CT (5, 23).

The two non-O-1 strains (2002H and 2011H) were isolated from patients with severe diarrhea in Louisiana, and both produce CT, as evidenced by positive reactions in Y-1 adrenal cells and GM1 enzyme-linked immunosorbent assays and neutralization by anti-CT serum. Both strains hybridized with the LT probe, yielding two small *Hin*dIII fragments similar, but not

TABLE 1. Strains examined in the present study

Strain[a]	CT	Hemolysin	Source (yr)
V. cholerae O-1			
E506	+	+	Stool, Texas (1973)
4808	+	+	Stool, Louisiana (1978)
SGN7277	+	+	Sewage, Louisiana (1980)
SGN7700	+	+	Sewage, Louisiana (1981)
SGN7730	−	+	Sewage, Louisiana (1981)
30167, 62746	+	NT[b]	Stool, Bangladesh (1976)
E8439	+	+	Stool, Hong Kong (1961)
E9120	+	+	Stool, Indonesia (1961)
E9950	+	−	Stool, Philippines (1960)
Stokes 1	+	+	Stool, Australia (1977)
Ym 331-6, Ym 404-9	−	NT	Water, Japan (1978)
569B	+	−	Stool, India
V. cholerae non-O-1			
2002H, 2011H	+	+	Stool, Louisiana (1979)

[a] All strains are of the El Tor biotype except for 596B, a classical strain.
[b] NT, Not tested.

identical, to those of strains E506, 4808, and SGN7277 (Fig. 2). The toxin gene in these strains has diverged still further, since they also possess a unique *Eco*RI site (Fig. 2), unlike other O-1 and non-O-1 strains (11). These strains are also negative for sucrose fermentation and would probably be more correctly identified as *Vibrio mimicus*, a species recently described by Davis et al. (7) for sucrose-negative *V. cholerae*. The results of the hybridization studies are summarized in Table 2.

The similarity of the Gulf Coast *V. cholerae* O-1 strains is so striking that the use of a radioactive probe is not even necessary. Figure 3 shows total chromosomal DNA of various *V. cholerae* strains digested with *Hin*dIII and separated by agarose gel electrophoresis. The restriction pattern for strains SGN7700, 4808, and E506 are identical, reflecting highly similar, if not identical, DNA sequences in their chromosomes. A number of differences can be seen in other strains. None of the various enterotoxigenic strains of *V. cholerae* isolated throughout the world that we have examined possessed the exact restriction pattern seen in the enterotoxigenic U.S. strains (Fig. 3). Of particular interest is strain SGN7730, a non-enterotoxigenic O-1 Ogawa strain which did not hybridize with the LT probe. This strain was isolated at the same site as SGN7700, but 1 week later, and clearly possesses a number of genetic dissimilarities from the earlier isolate, as evidenced by the restriction pattern (Fig. 3, lane a). Similarly, strain 2002H, an enterotoxigenic non-O-1 strain of *V. cholerae*, shows a distinctly different restriction pattern from the O-1 strain (Fig. 3, lane e).

Differences among strains in restriction endonuclease patterns, particularly those fragments hybridizing with the LT probe, can be exploited as a very sensitive "molecular fingerprinting" tool for *V. cholerae*. At the present time, phage typing is the chief epidemiological typing system employed for *V. cholerae* (14), and the preliminary evidence suggests considerable common ground between these methods. The 1973, 1978, 1980, and 1981 isolates are of the same phage type (e5/4996/13/16/24), which is extremely uncommon in other strains isolated throughout the world (14). By examining different phage types of *V. cholerae* with different restriction enzymes and noting different numbers and sizes of homologous fragments, a comprehensive typing scheme could be devised. Such a study relating phage type to restriction fragment differences is currently under way (J. B. Kaper and J. Lee, manuscript in preparation). In at least one instance, however, the restriction fragment analysis has proven more reliable than phage typing. Recent isolates of *V. cholerae* O-1 from Florida were of the same phage type as the 1973, 1978, 1980, and 1981 isolates (Lee, personal communication) but did not produce CT and were negative with the LT gene probe (Kaper, unpublished observations).

In Louisiana, four different classes of *V. cholerae* have been isolated: (i) *V. cholerae* non-O-1, which do not produce CT; (ii) *V. cholerae* non-O-1 which do produce CT; (iii) *V. cholerae* O-1, which do not produce CT; and (iv) *V. cholerae* O-1, which produce CT. Strains of the first class, *V. cholerae* non-O-1 which do not produce CT, have been repeatedly isolated from both environmental and clinical samples. This reflects the widespread distribution of these strains in brackish areas (10, 25) and in diarrheal and extraintestinal disease (18, 24). Clearly, the important virulence factors for this class have

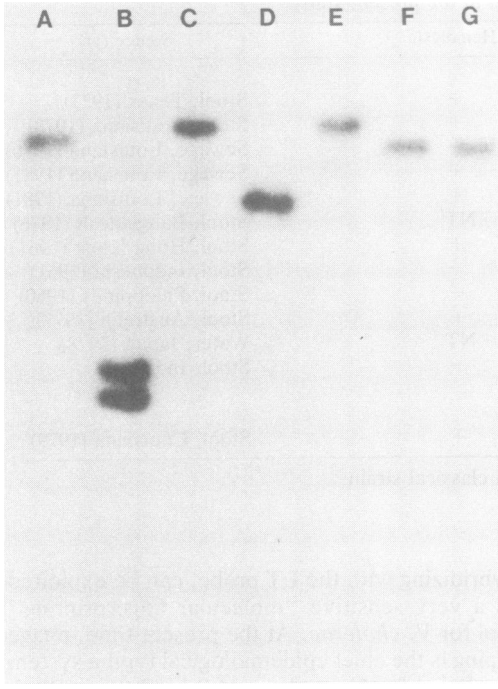

FIG. 1. Autoradiograph showing hybridization of the LT probe to *Hin*dIII restriction fragments of *V. cholerae* strains. Chromosomal DNA was extracted from each strain, digested with *Hin*dIII restriction endonuclease, separated on an agarose gel, and transferred to nitrocellulose paper. The LT probe was labeled with ^{32}P and, where it has hybridized to DNA from *V. cholerae*, is indicated by darkening of the X-ray film. Lane A, Stokes 1; lane B, 4808; lane C, E9120; lane D, E9950; lane E, E8439; lane F, 62746; lane G, 30167.

not yet been described, since they obviously cause disease without elaborating CT. This was found to be the case for the majority of non-O-1 isolates of *V. cholerae* implicated in disease (18, 24). Strains of *V. cholerae* non-O-1 which do cause diarrhea through a CT or CT-like toxin have been isolated from patients but not, to our knowledge, from uncontaminated environmental samples. A recent strain of CT-producing *V. cholerae* non-O-1 was isolated from an environmental sample in Louisiana (4) that was, however, contaminated with human feces (P. Blake, personal communication). The toxin elaborated by these strains is functionally, antigenically, and genetically similar, if not identical, to CT produced by O-1 strains (4, 11, 20) but is not apparently involved with epidemics of cholera. The failure to isolate these strains from the environment may simply be due to the failure to screen enough strains for toxin production and the inadequacy of the Y-1 adrenal cell assay for this purpose because of the masking cytotoxins produced by many of these strains (16). A solution to this problem would be to use the LT probe to screen colonies grown on filter paper (11). This technique will detect the toxin genes directly, without interference from the cytotoxins, and would allow hundreds of isolates to be tested in a day. Such a project is ongoing in our laboratories to detect a reservoir among the thousands of environmental isolates of *V. cholerae* non-O-1 we have accumulated. This approach is even more powerful and specific than before, owing to the recent report of cloned CT genes used as hybridization probes (9).

In recent years, a great deal of attention has been focused on strains of *V. cholerae* O-1 which do not produce CT. These strains have been isolated chiefly from water and seafood samples from locations such as England, Guam, Brazil, Maryland, and Louisiana, usually in the absence of cholera in the nearby community. The role of these isolates in the epidemiology of cholera as a possible reservoir was uncertain until molecular genetic and human volunteer studies demonstrated that these strains did not possess any genetic material capable of CT production (11) and that, aside from the non-

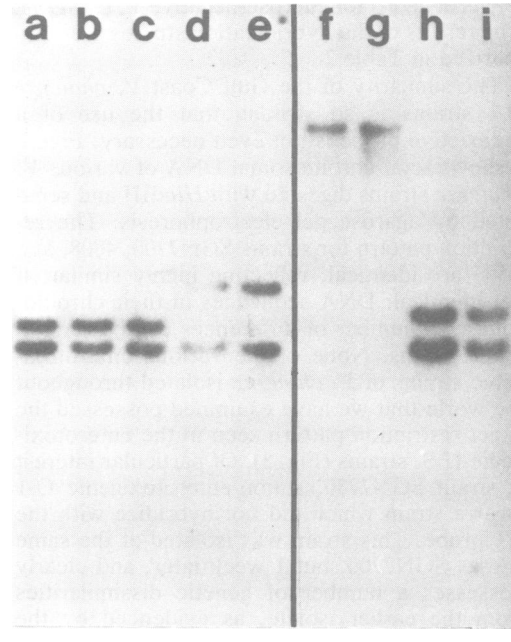

FIG. 2. Autoradiograph showing hybridization of the LT probe to *Hin*dIII and *Eco*RI restriction endonuclease fragments. Lanes a through e, *Hin*dIII digestion; lanes f through i, *Eco*RI digestion. Lane a, E506; lane b, 4808; lane c, SGN7277; lane d, 2002H; lane e, 2011H; lane f, 4808; lane g, SGN7277; lane h, 2002H; lane i, 2011H.

TABLE 2. Summary of DNA hybridization results with LT genes and enterotoxigenic *V. cholerae* El Tor strains

Strain	Source (yr)	Restriction pattern[a]
V. cholerae O-1		
E506	Stool, Texas (1973)	*Hin*dIII, 6 and 7 kb; *Eco*RI, 26 kb
4808	Stool, Texas (1978)	*Hin*dIII, 6 and 7 kb; *Eco*RI, 26 kb
SGN7277	Sewage, Louisiana (1980)	*Hin*dIII, 6 and 7 kb; *Eco*RI, 26 kb
SGN7700	Sewage, Louisiana (1981)	*Hin*dIII, 6 and 7 kb; *Eco*RI, 26 kb
30167, 62646	Stool, Bangladesh (1976)	*Hin*dIII, 19 kb
E8439	Stool, Hong Kong (1961)	*Hin*dIII, 24 kb
E9120	Stool, Indonesia (1961)	*Hin*dIII, 24 kb
E9950	Stool, Phillipines (1960)	*Hin*dIII, 13 kb
Stokes 1	Stool, Australia (1977)	*Hin*dIII, 20 kb
V. cholerae non-O-1		
2002H, 2011H	Stool, Louisiana (1979)	*Hin*dIII, 6 and 8 kb; *Eco*RI, 6 and 7.5 kb

[a] Restriction pattern refers to the size (in kb) of the *V. cholerae* DNA fragment homologous to the *E. coli* LT DNA probe. Chromosomal DNA from every *V. cholerae* strain was digested with the restriction endonuclease *Hin*dIII and with *Eco*RI in some cases, separated by agarose gel electrophoresis, and hybridized as described in the text.

toxigenicity, these strains did not colonize the human gut, thus lacking other essential virulence factors (15). In addition, several isolates of non-enterotoxigenic *V. cholerae* O-1 have been recovered from extraintestinal infections and patients with diarrhea (Blake and Lee, personal communication), so some additional unknown virulence factor may be involved with sporadic infections. In contrast to non-enterotoxigenic *V. cholerae* O-1, few strains of *V. cholerae* O-1 producing CT have been isolated from Louisiana, and no isolates have been obtained from areas free of fecal contamination. The 1978 environmental strains were isolated subsequent to the cholera outbreak from areas high in coliform counts (2). The other isolates were from sewage, presumably from an unreported case of cholera in the nearby community. Clearly, environmental sources, and seafood in particular, are important in the epidemiology and reservoir of enterotoxigenic *V. cholerae* in the U.S. Gulf Coast, but the failure despite intensive efforts to isolate these strains from uncontaminated environmental samples is puzzling if the environment is the sole reservoir. A simple mutation from *V. cholerae* O-1 to non-O-1 and vice versa is an unlikely explanation in light of the significant genetic differences found in the present study. Thus, a single enterotoxigenic strain of *V. cholerae* is definitely resident in the U.S Gulf Coast and apparently unique to this area. This strain continues to cause disease in the Gulf Coast area, as recently reported by Kelly et al. (12). Although both fecal dissemination and an environmental reservoir appear to be involved, the relative contributions of these two factors in the epidemiology of cholera in the United States remain to be fully elucidated.

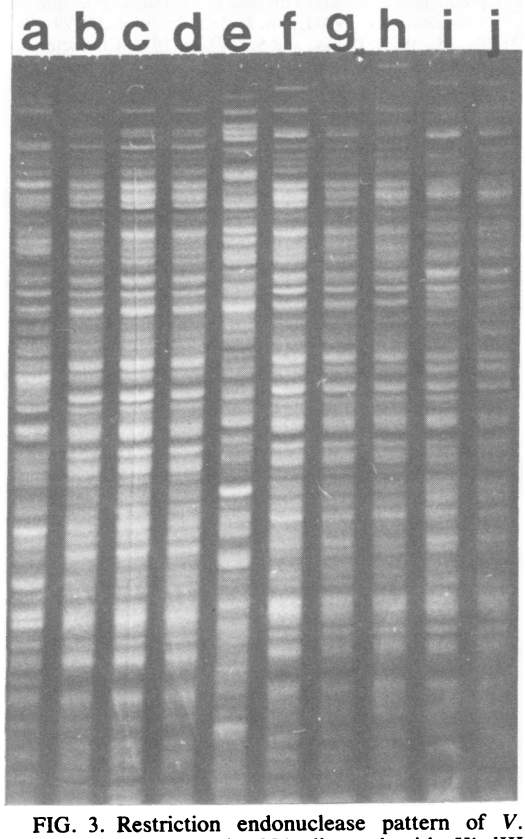

FIG. 3. Restriction endonuclease pattern of *V. cholerae* chromosomal DNA digested with *Hin*dIII and separated by agarose gel electrophoresis. Lane a, SGN7730; lane b, SGN7700; lane c, 4808; lane d, E506; lane e, 2002H; lane f, 62746; lane g, Stokes 1; lane h, E9120; lane i, E9950; lane j, 569B.

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