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Use of Automated Sequencing of Polymerase Chain Reaction-Generated Amplicons To Identify Three Types of Cholera Toxin Subunit B in *Vibrio cholerae* O1 Strains

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Cholera toxin is the principal factor causing the profuse intestinal fluid secretion that is characteristic of cholera. The DNA sequences of the cholera toxin subunit B structural genes from 45 *Vibrio cholerae* O1 strains isolated in 29 countries over a period of 70 years were determined by automated DNA sequencing of polymerase chain reaction-generated amplicons. Three types of cholera toxin B subunit gene (*ctxB*) were identified. Genotype 1 was found in strains of classical biotype worldwide and El Tor biotype strains associated with the U.S. Gulf Coast, genotype 2 was found in El Tor biotype strains from Australia, and genotype 3 was found in El Tor biotype strains from the seventh pandemic and the recent Latin American epidemic. All base changes correspond to an amino acid substitution in the B subunit of the cholera toxin. Heterogenicity in the B subunit could have implications for vaccine development and diagnostic tests for cholera toxin and antitoxin. We conclude that this technology provides timely and potentially useful epidemiological information.

Cholera, a highly epidemic diarrheal disease, is caused by toxin-producing strains of *Vibrio cholerae* O1. These strains are further classified into two biotypes, classical and El Tor, and into two serotypes, Inaba and Ogawa. Cholera toxin is a 85-kDa protein composed of two functional units, an enzymatic A subunit of 27 kDa and an intestinal receptor-binding B subunit consisting of five identical 11.6-kDa peptides (18). Infected individuals mount an immune response to the toxin; antitoxin antibodies, primarily recognizing the B subunit, have been used to detect past exposure to the organism (22). Because of its possible role in immunity to natural infections, the B subunit has been incorporated in a new vaccine formulation recently tested in Bangladesh (3). Previous reports have noted antigenic heterogeneity in the B subunit of toxins from various *V. cholerae* strains (5, 9, 10, 17). We employed automated DNA sequencing of polymerase chain reaction (PCR)-generated amplicons to determine the DNA sequence of the gene encoding the B subunit (*ctxB*) from a diverse collection of strains to demonstrate the extent of heterogeneity and any association with the temporal or geographic origins of the strains.

MATERIALS AND METHODS

Strains. A collection of 45 toxigenic *V. cholerae* O1 strains isolated from infected individuals worldwide over a period of 70 years and representing both serotypes and biotypes was investigated (Table 1).

PCR. The strains were verified to produce the cholera toxin by an enzyme-linked immunosorbent assay, and the presence of *ctxB* was demonstrated by a PCR assay. Bacterial colonies from an overnight cultivation on blood agar plates (tryptic soy agar II with 5% sheep blood) were suspended in 0.5 ml of sterile water to a concentration of 10^5 to 10^6 organisms per ml, boiled for 30 min, and used as a template for the PCR. The primers CTX7 (GGT TGC TTC TCA TCA TCG AAC CAC) and CTX9B (biotin-GAT ACA CAT AAT AGA ATT AAG GAT G) (15) were used for amplification of a 460-bp segment of *ctxB* (Fig. 1). The cycling conditions were 25 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Reagents and other conditions were as described by Fields et al. (6).

DNA sequencing. The amplicon from the *ctxB* PCR was used as a template in a robot-assisted DNA sequencing reaction and analyzed on an automated DNA sequencer. The biotin-labelled amplicons from the *ctxB* genes were first purified with a Gene Clean spin column (Bio 101 Inc., La Jolla, Calif.). Three hundred nanograms of the amplicon was mixed with 250 μg of streptavidin-coated magnetic beads (Dynabead M280 Streptavidin; Dynal, Oslo, Norway) in 50 μl of binding buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl [pH 7.5]), and kept at room temperature for 5 min. The amplicon was then made single stranded by denaturing with 0.125 M NaOH–0.1 M NaCl for 5 min, and both the bead-bound and dissolved strands were recovered for sequencing (7). The supernatant containing one strand was collected after the beads were attached to the side of the tube by a magnet (MPC-E; Dynal) and neutralized by adding 1 μl of 1 M HCl. The magnetic beads with the other strand of DNA attached were washed once with 50 μl of 0.1 M NaOH, once with 50 μl of binding buffer, and once with 50 μl of TE buffer before being resuspended in 13 μl of distilled water. Both the supernatant and the bead-associated DNA strands were transferred to microtiter plates and used as templates in the robot (Biomek-1000 and HCB-1000; Beckman Instruments, Fullerton, Calif.) facilitated sequencing reaction. Reagents from an Auto Read T7 sequencing kit (Pharmacia, Uppsala, Sweden) were used as described by Hultman et al. (7). The sequencing primers (CTX7 for solid-phase-bound strand and CTX9 for supernatant strand) were labelled with isothiocyanate (Pharmacia). The products of the sequencing reactions were separated on a 6% polyacrylamide gel in an

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TABLE 1. *V. cholerae* O1 strains used in this study and their cholera toxin subunit B genotypes

Strain	Origin and year of isolation	Serotype	*ctxB* genotype[a]
75	Japan, 1921	Inaba[b]	1
569B	India, 1940	Inaba[b]	1
9060-79	India, 1949	Ogawa[b]	1
H23337	Bangladesh, 1970	Inaba[b]	1
3242-73	Texas, 1973	Inaba	1
2164-78	Louisiana, 1978	Inaba	1
1428-81	Texas, 1981	Inaba	1
776-83	Mexico, 1983	Inaba	1
2538-86	Georgia, 1976	Inaba	1
2270-77	Australia, 1977	Inaba	2
2463-88	Australia, 1988	Inaba	2
E9112	Malaya, 1963	Ogawa	3
1623-80	Hong Kong	Ogawa	3
E9956	Philippines, 1963	Ogawa	3
X25514	Bangladesh, 1982	Ogawa	3
9912-84	Mali, 1984	Ogawa	3
2472-86	Philippines, 1986	Ogawa	3
2484-86	Thailand, 1986	Inaba	3
2459-90	India, 1990	Ogawa	3
E9121	Iran, 1965	Ogawa	3
E8249	Lebanon, 1970	Ogawa	3
E8257	Kuwait, 1977	Ogawa	3
2673-81	Saudi Arabia, 1981	Inaba	3
E8252	Spain, 1971	Ogawa	3
E8248	Libya, 1970	Ogawa	3
E8264	Ivory Coast, 1971	Inaba	3
E8262	Algeria, 1972	Inaba	3
E8253	Tunisia, 1973	Ogawa	3
2213-80	Cameroon, 1980	Inaba	3
2432-88	Rwanda, 1988	Ogawa	3
C6610	Malawi, 1990	Inaba	3
E8260	Gilbert Islands, 1966	Inaba	3
1800-82	Truk, 1982	Inaba	3
C5631	Truk, 1990	Inaba	3
2560-90	Guam, 1990	Ogawa	3
2097-80	New Zealand, 1980	Inaba	3
C6706	Peru, 1991	Inaba	3
C6707	Peru, 1991	Inaba	3
C7267	Peru, 1991	Ogawa	3
C7268	Peru, 1991	Ogawa	3
C7174	Chile, 1991	Inaba	3
C7331	Mexico, 1991	Inaba	3
C7403	Alabama, 1991	Inaba	3
C7150	Colombia, 1991	Inaba	3
C7128[c]	New York, 1991	Inaba	3

[a] See Table 2.
[b] Classical biotype; all other strains were of the El Tor biotype.
[c] Epidemiologically linked to Latin America.

ALF automated laser fluorescence apparatus (Pharmacia). The sequences were aligned by using the computer program DNASIS (Hitachi Software Engineering America Ltd., Brisbane, Calif.). An outline of the technology used is presented in Fig. 1.

RESULTS

The DNA sequence data obtained spanned a 300-base segment from positions 60 to 360 of the *ctxB* gene. The sequences obtained from the solid-phase-linked strands were complementary to the soluble strand for each strain. Sequences spanning from positions 108 to 207 are shown in Figure 1. Specific base substitutions at positions 115 (C or T), 138 (T or G), and 203 (C or T) among the 45 strains were identified. Each base change resulted in a change in the deduced amino acid sequence (Table 2). These sequence differences divided the strains into three genotypes of *ctxB* (Table 2). All classical strains and El Tor strains from the U.S. Gulf Coast had *ctxB* of genotype 1; El Tor strains from Australia had *ctxB* of genotype 2; and El Tor strains from the seventh pandemic and from the recent Latin American epidemic had *ctxB* of genotype 3.

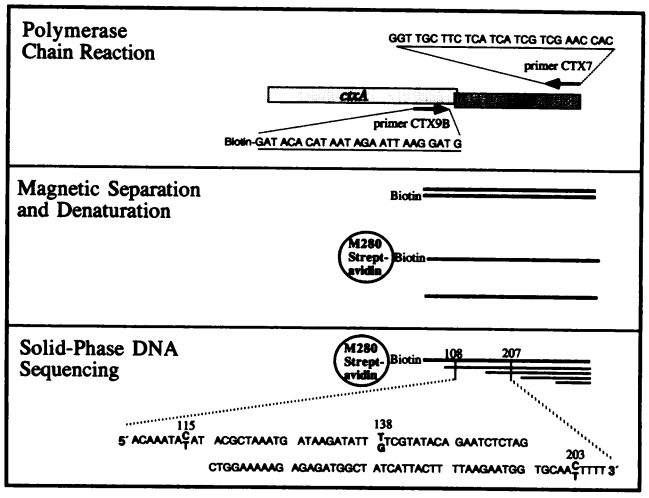

FIG. 1. Automated sequencing of a PCR-generated *ctxB* amplicon. Details and reagents are described in the text. A 100-base segment of the 300 bases sequenced is shown. The locations of the three substitutions are shown in boldface type. The DNA sequence obtained is identical to that reported by Lockman and Kaper (11) and differs from that reported by Mekalanos et al. (15) at two bases that most likely represent misprints or sequencing errors.

DISCUSSION

DNA sequence analysis of *ctxB* from 45 strains has revealed three base changes representing three different alleles at this locus. The strains used in this work were both geographically and temporally diverse. The classical strains were isolated over a 60-year period, the seventh-pandemic strains were isolated over a 30-year period, and the two Australian strains were isolated 11 years apart. This suggests that random mutations in the *ctxB* gene are not common. However, a more extensive study of the molecular epidemiology of cholera in Latin America suggests that although toxin genotype seems to be conserved, other portions of the *V. cholerae* genome are more complex and variable (19).

The sequences obtained in our study were very reproducible, documented by both the corresponding sequence from each strand sequenced and the same sequence in the high number of strains from each group. Our sequence is identical to that presented by Lockman and Kaper (11) but differs from that presented by Mekalanos and coworkers (15) in only two positions. As we sequenced the same strains, we consider this to be a combination of misprints and incorrect sequencing.

The sequencing approach used can identify sequence differences when the two genes are present in equal amounts (21). We were unable to detect any sequence differences among the nine strains tested that possessed two copies of the *ctx* operon, suggesting that the copies are identical. All

TABLE 2. Characteristics of *V. cholerae* O1 strains and toxins based on the DNA sequence of toxin subunit B genes

Strain description	No. of strains	Nucleotide[a] at position:			Amino acid[b] at position:			*ctxB* genotype
		115	138	203	18	25	47	
Classical, worldwide	4	C	T	C	His	Phe	Thr	1
El Tor, U.S. Gulf Coast[c]	5	C	T	C	His	Phe	Thr	1
El Tor, Australia	2	C	G	C	His	Leu	Thr	2
El Tor, seventh pandemic	25	T	T	T	Tyr	Phe	Ile	3
El Tor, Latin American epidemic	9	T	T	T	Tyr	Phe	Ile	3

[a] The numbers refer to the nucleotide positions in the *ctxB* sequence (one overlap with the *ctxA* sequence) (15).
[b] Deduced from the nucleic acid sequence; numbers refer to amino acid positions in the B-subunit polypeptide sequence (63 bases downstream from the start of *ctxB*) (15).
[c] Includes one strain from Mexico.

classical strains and about 30% of the El Tor strains possess two copies of the *ctx* operon (13–15). Mekalanos (13, 14) came to a similar conclusion after cloning and partially sequencing each of them.

An interesting and unexpected observation was the identical *ctxB* sequence for the classical strains and the U.S. Gulf Coast El Tor strains. Although strains from these two groups have different phenotypic characteristics, they both have two copies of the cholera toxin operon (13, 20). The origin of the toxigenic El Tor strains associated with the U.S. Gulf Coast is uncertain, but it is possible that a nontoxigenic marine strain acquired toxin genes from classical strains during cholera outbreaks in the late 1800s. Nontoxigenic El Tor strains of the same multilocus-enzyme type as the toxigenic strains have been recovered from the U.S. Gulf Coast (2), supporting this hypothesis.

Recently, toxigenic *V. cholerae* O1 strains were isolated from oysters in Mobile Bay, off the coast of Alabama (4). These strains were indistinguishable from the Latin American outbreak strains with respect to phenotypic characterization, *ctxB* DNA sequence (type 3), and number of copies of the cholera toxin operon (4, 20). The appearance of the Latin American strain in the Gulf of Mexico might be explained by the isolation of such strains from ballast water of ships coming to Mobile Bay from cholera-affected Latin American ports (12).

Heterogeneity in the B subunit could have implications for vaccine development and diagnostic tests for cholera toxin and antitoxin (8, 14, 22). Although laboratory experiments indicate a high degree of antigenic similarity between the toxins from El Tor and classical strains (5), vaccines partly based on the B subunit from classical strains have been shown to protect less efficiently against El Tor strains than against classical strains in field trials (3). This finding might be explained by the B-subunit differences we have identified. However, the vaccine formula is complex, and other antigenic determinants may contribute to differences in protection.

New technology using automated sequencing of PCR amplicons has made it possible to obtain DNA sequences from many strains within a few days (1, 7). An advanced microbiology laboratory equipped with PCR thermocycler, robot station, and automatic DNA sequencer could sequence 300 to 400 bases from up to 20 strains within 12 h with the methodology described herein. DNA sequences from many strains should be invaluable in addressing the distribution and evolution of genes and bacterial strains. Data from our study and from other studies (1, 16) also indicate that DNA sequencing is becoming a useful epidemiological for the identification and characterization of clinically important isolates.

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