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Development and Testing of a Nonradioactive DNA Oligonucleotide Probe That Is Specific for *Vibrio cholerae* Cholera Toxin

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An alkaline phosphatase-labeled oligonucleotide DNA probe (CTAP) that was specific for the cholera toxin gene (*ctxA*) was identified. All cholera toxin-producing strains of *Vibrio cholerae*, regardless of serotype, hybridized with the CTAP probe, while nontoxigenic strains from either environmental sources or from deletion or substitution mutations did not hybridize. Unlike the whole-gene probes for either *ctxA* or for the heat-labile toxin or *Escherichia coli* (*eltA*), this 23-base sequence did not hybridize with *E. coli* or with vibrios other than *V. cholerae* that produce related toxins. By using CTAP to identify colonies grown on nonselective medium, *V. cholerae* was enumerated at concentrations of 10^3 to 10^7/g from stool samples of volunteers who had ingested *V. cholerae* O1 strain 569B. CTAP provides a specific and sensitive tool for diagnosis and environmental monitoring of cholera toxin-producing *V. cholerae*.

Recent epidemics of cholera throughout South America have underscored the need for rapid, accurate means of identification of pathogenic *Vibrio cholerae* for diagnostic purposes, food and water quality assurance, and environmental monitoring. Cholera is caused by strains of *V. cholerae* O1 that produce cholera toxin (CT) (15). Identification of *V. cholerae* is usually based on a combination of biochemical and immunological assays, with pathogenic *V. cholerae* strains traditionally defined by their agglutination with O1 antisera (19, 21). However, there can be problems with this approach, particularly when working with environmental strains or in areas with a low level of cholera endemicity. Several studies (4, 14, 25) have shown that the majority of environmental O1 strains outside an endemic area do not carry the genes for CT; several of these strains have been tested in volunteers and did not produce disease (7). At the same time, there are *V. cholerae* strains in groups other than O1 (non-O1 *V. cholerae*) which produce CT and probably do cause human illness (4–6, 22).

These observations suggest that identification of pathogenic *V. cholerae* would ideally be based on the ability of a strain to produce CT. However, assays for CT are not routinely conducted because methods of detection are complex (requiring the use of animal models, tissue culture, enzyme-linked immunosorbent assay [ELISA], or radioactive DNA probes) and not suitable for many laboratories. DNA probes and ELISA have also shown cross-reactivity with the heat-labile enterotoxin (LT) of *Escherichia coli* (10, 11, 20, 28) and the CT-like toxin of *V. mimicus* (3, 5, 23).

To address these problems, we identified a 23-base portion of the CT gene sequence (*ctxA*) that could be used as a synthetic oligonucleotide which would retain sensitivity for *V. cholerae* strains but show increased specificity for the species. We incorporated this probe into a nonradioactive detection method that had been developed previously for *V. vulnificus* by using an alkaline phosphatase label and inexpensive Whatman filter paper (26). We also used the alkaline phosphatase-labeled oligonucleotide probe derived from the *ctxA* gene (CTAP) to identify bacteria from stool samples of volunteers who had ingested *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains and media. Strains used in this study were from the Center for Vaccine Development culture collection and represented 11 *Vibrio* species and both toxigenic and nontoxigenic strains of *E. coli* (Table 1). The *V. cholerae* O1 strains included both classical and El Tor biotypes and Inaba and Ogawa serovars collected from the United States, Africa, and Asia, as well as recent isolates from the 1991 epidemic in South America. Strains were grown on L agar or thiosulfate citrate bile salts sucrose agar (TCBS) (Difco, Detroit, Mich.) at 30°C and stored at −70°C in L broth with 50% glycerol.

CTAP oligonucleotide probe. The CT-specific alkaline phosphatase-labeled 23-base oligonucleotide probe (Fig. 1, CTAP) was derived from a region of the *ctxA* sequence that differed from the corresponding region of both the porcine (LTp) and human (LTh) *E. coli* LT genes (28). It was synthesized with a 5′ amino linker by the Biopolymer Laboratory, University of Maryland at Baltimore, and alkaline phosphatase label was attached by using E-link (Cambridge Research Biochemicals, Wilmington, Del.).

The method for colony blot hybridization and detection of alkaline phosphatase probe has been previously described (1a, 17, 18, 26). Briefly, colonies grown overnight on L agar were overlaid with Whatman #541 filters for 30 min to transfer the colonies to the filters. The #541 filters (colony side up) were microwaved for 1 to 4 min on #3 Whatman filters prewetted with a 0.5 M NaOH–1.5 M NaCl solution (alkaline conditions). The blots were then neutralized by placing them (colony side up) on #3 filters prewetted with 2 M ammonium acetate. After three rinses in 0.15 M NaCl–

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TABLE 1. Specificity of CTAP

Species	No. of strains with response to probe[a]							
	CTAP		CT		LT		ST	
	+	−	+	−	+	−	+	−
V. cholerae								
O1	66	3	66	3	ND[b]	ND	0	36
Non-O1	3	202	3	202	ND	ND	13	189
E. coli	0	52	22	30	22	30	24	28
V. mimicus	0	7	3	4	ND	ND	2	3
V. vulnificus	0	18	0	18	ND	ND	0	18
V. hollisae	0	3	0	3	ND	ND	0	3
V. fluvialis	0	6	0	6	ND	ND	5	1
V. alginolyticus	0	1	0	1	ND	ND	0	1
V. damsela	0	2	0	2	ND	ND	2	0
V. harveyii	0	1	0	1	ND	ND	0	1
V. metschnikovii	0	1	0	1	ND	ND	0	1
V. parahaemolyticus	0	6	0	6	ND	ND	0	8
V. furnissii	0	1	0	1	ND	ND	0	1

[a] Number of strain within each species that gave a positive (+) or negative (−) response for either the CTAP oligonucleotide probe or the whole-gene probes for CT or LT. *E. coli* strains were probed with an oligonucleotide for *E. coli* ST and *Vibrio* species were probed with an oligonucleotide for NAG-ST (heat-stable enterotoxin of non-O1 *V. cholerae*) as described in the text.

[b] ND, not done.

0.015 M sodium citrate, pH 7.0 (SSC), colony blots were treated with proteinase K (40 μg/ml) at 42°C for 30 min, followed by three rinses in SSC.

Prior to hybridization, filters were incubated in hybridization buffer (bovine serum albumin [0.5%], sodium dodecyl sulfate [SDS] [1%], polyvinylpyrrolidone [0.5%] in SSC) for 30 min at 56°C. CTAP (10 μg) was added to filters for hybridization in fresh buffer (prewarmed to 56°C), and the filters were incubated at 56°C for 1 h. Filters were rinsed two times (10 minutes each) in SSC–1% SDS at 56°C, followed by three rinses at room temperature in SSC. Phosphatase activity was assayed in prewarmed (37°C) diethanolamine buffer (100 mM, pH 9.5) (5 mM $MgCl_2$, 0.2% NaN_3) with nitroblue tetrazolium (75 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml). Blots were developed in the dark for 1 h.

Other enterotoxin probes. *E. coli* strains were probed with an alkaline phosphatase-labeled oligonucleotide probe derived from the gene sequence for the *E. coli* heat-stable toxin (ST) (Molecular Biosystems, San Diego, Calif.) (17, 18). *Vibrio* species were probed with an oligonucleotide probe derived from the nucleotide sequence of the non-O1 *V. cholerae* heat-stable enterotoxin (NAG-ST) (2, 16). Strains were also probed with either CT or LT whole-gene probes as previously described (3) using #541 Whatman filters and random priming (GIBCO BRL, Gaithersburg, Md.) to label probes with ^{32}P.

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CTAP    C T C C G G A G C A T A G A G C T T G G A G G
CT*     C T C C G G A G C A T A G A a C T T G G A G G
LTh     C a C C G G A t C A c c a A G C T T G G A G a
LTp     C a C C G G A t C A c c a A G C T T G G A G a
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FIG. 1. Comparison of nucleotide sequence from the region of *V. cholerae ctxA* used for the alkaline phosphatase-labeled DNA probe (CTAP) to the sequence of the same region of DNA from *V. cholerae* Texas Star (CT*) and to the corresponding region of the LT genes from human-colonizing enterotoxigenic *E. coli* (LTh) and swine-colonizing enterotoxigenic *E. coli* (LTp) (21). Nucleotides that differ from the *ctxA* sequence are in lowercase type.

CTAP probe specificity and sensitivity. Strains were inoculated to L agar, incubated overnight at 30°C, transferred to filter paper, and hybridized with CTAP, LT, CT, or ST probes as described above. *V. cholerae* O1 strain 569B was used as a positive control, and non-O1 *V. cholerae* NRT36S and *V. vulnificus* MO6-24 were used as negative controls. Serial 10-fold dilutions of a 10^{10} broth culture were spotted (10 μl) to L agar, and colony blots were prepared from plates incubated 3, 5, or 16 h at 30°C.

Volunteer study. *V. cholerae* 569B, serogroup O1 Inaba, classical biotype, was administered orally to 11 volunteers; these volunteers served as unvaccinated controls for ongoing vaccine trials at the Center for Vaccine Development, University of Maryland School of Medicine (8, 9). Stool samples were collected daily and diluted 1:10 in phosphate-buffered saline. Serial 10-fold dilutions were spread on TCBS or L agar for enumeration of viable bacteria, and 10 μl of a 1:10 (either unfiltered or filtered through a 1-μm-pore-size filter) or a 1:1,000 (unfiltered) dilution of stool sample was spotted on L agar for colony blots. Sucrose-positive colonies on TCBS were confirmed as *V. cholerae* by agglutination with Inaba-specific antiserum.

RESULTS

Species specificity and sensitivity of CTAP probe. The CTAP probe was 100% specific for wild-type CT-producing *V. cholerae* (Table 1). The CTAP probe identified strains of both O1 (all strains) and non-O1 (strains S-21, 1236, and CA89A455) *V. cholerae* that were toxigenic as determined by hybridization to the whole-gene CT probe; however, the oligonucleotide did not react with O1 strains (CVD 101, 103, 105) which have deletions of the *ctxA* gene (9) or with CT-negative strains of O1 or non-O1 *V. cholerae*. It also did not detect O1 *V. cholerae* Texas Star, a nontoxigenic strain that was isolated following nitrosoguanidine mutagenesis (1). This strain has been shown to have a single-base-pair mismatch that was coincidentally in the middle of the CTAP sequence (Fig. 1). All other *Vibrio* species examined were negative with the CTAP probe.

From serial dilutions of *V. cholerae* spotted on L agar, 10^7 bacteria in 10 μl were detectable by CTAP after only 3 h of bacterial growth on agar. An initial inoculation of 3 bacteria in 10 μl was detectable after 16 h of incubation.

Cross-reactivity with CT, LT, and ST genes. As shown in Table 1, three *V. mimicus* strains (61956, 2002H, and 2011H) which hybridized with the whole-gene CT probe and 22 *E. coli* strains which were LT positive (as well as 30 strains which were LT negative) did not hybridize with the CTAP probe. Strains of *E. coli* (n = 24) or *V. cholerae* (n = 13) that were ST positive did not hybridize with the CTAP probe.

Detection of *V. cholerae* from human volunteers by using CTAP. For stool samples collected from day 1 to day 4 after bacterial challenge, the concentration of *V. cholerae* as detected by CTAP ranged between 10^3 and 10^7/g of stool. Table 2 shows a comparison of the number of *V. cholerae* colonies detected in stools from human volunteers at day 3 of infection. On average, the viable count of *V. cholerae* on L agar, as detected by CTAP, was about 100-fold higher than that recovered on TCBS by using standard methods. In

TABLE 2. Detection of *V. cholerae* in stool

Patient	Total viable count[a]	CTAP count[b]	TCBS[c]
1	6×10^4	5×10^4	6×10^2
2	2×10^6	2×10^5	1×10^4
3	6×10^6	7×10^4	2×10^3
4	6×10^8	1×10^5	8×10^2
5	4×10^6	2×10^4	1×10^3
6	3×10^7	2×10^7	5×10^5
7	2×10^6	3×10^5	1×10^3
8	9×10^7	7×10^7	4×10^5
9	2×10^6	NR[d]	NR
10	4×10^6	NR	NR
11	3×10^6	NR	NR

[a] Total aerobic plate count per gram of stool, as estimated by serial dilutions plated on L agar.
[b] Number of CTAP probe-positive colonies per gram of stool from serial dilutions plated on L agar.
[c] Number of sucrose-positive, Inaba-positive colonies per gram of stool as estimated by dilutions spread on TCBS.
[d] NR, none recovered.

general, it was not possible to enumerate *V. cholerae* by CTAP on L agar when concentrations were $<10^3$/g, because of overgrowth of other bacteria. *V. cholerae* was isolated from a total of 22 stool samples from the 11 volunteers; 20 (91%) of these samples were also positive on direct screening with CTAP. Stool samples in which *V. cholerae* was not detected by CTAP had concentrations of *V. cholerae* on TCBS of $<3 \times 10^2$/g of stool. One sample which was positive by CTAP did not yield detectable *V. cholerae* by direct plating on TCBS; however, *V. cholerae* O1 Inaba was identified after enrichment. Spotting 10 μl of a 1:10 dilution of stool on L agar produced some samples that were uninterpretable because of pigments of stool that were retained on the filter paper and produced colony blots that looked positive before the addition of substrate. However, spotting either 10 μl of a filtered 1:10 sample or 10 μl of an unfiltered 1:1,000 dilution gave results that easily discriminated CTAP-positive versus negative samples (Fig. 2).

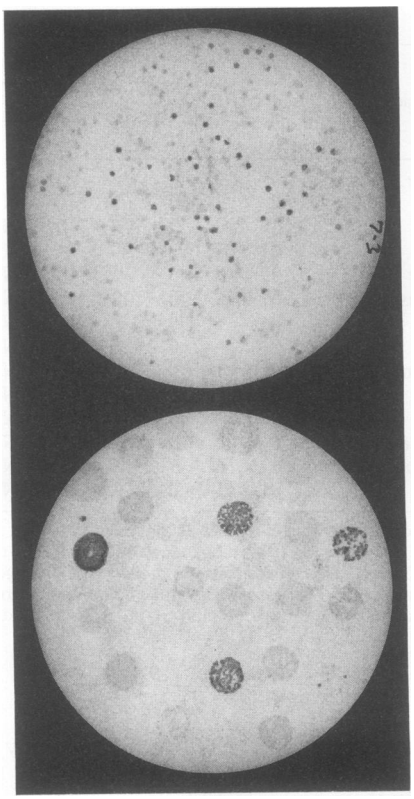

FIG. 2. Colony blots of 100 μl of 10^{-3} dilution of stool sample spread on L agar (top) and 10-μl samples of 10^{-3} dilutions of stool spotted on L agar (bottom). Blots were probed with CTAP as described in the text, and darker colonies are positive for CTAP probe.

DISCUSSION

The CTAP oligonucleotide probe correctly identified all CT-positive strains of O1 *V. cholerae* from both El Tor and classical biotypes and Inaba and Ogawa serotypes. These strains were collected from both clinical and environmental sources in the United States, Africa, Asia, and South America, including isolates from the 1991 South American epidemic. Constructions of *V. cholerae* with deletions of *ctxA* did not hybridize. Interestingly, the nontoxigenic O1 strain *V. cholerae* Texas Star, which was isolated following nitrosoguanidine mutagenesis, was negative for CTAP and positive for the whole gene probe. The observation that this strain, which contains a single-base substitution within the region encompassing the CTAP sequence (1), did not hybridize with the oligonucleotide raises the possibility of false negatives. However, our data and previous reports of identical sequences of *ctxA* from classical strain 569B (13) and El Tor strains 62746 (11, 12), 2125 (13), and 3083 (1) indicate that variation in wild-type *ctxA* is not common.

Further, we found that all CT-positive non-O1 strains were also identified by the CTAP oligonucleotide probe. Non-O1 *V. cholerae* strains show more strain variation in restriction enzyme patterns when probed with the CT gene than O1 strains (5), and physicochemical differences in toxin preparations have been reported (27). Although the non-O1 *V. cholerae* CT or CT-like genes have not been sequenced, these data also suggest the *ctxA* is well conserved within the species.

CTAP did not hybridize with strains of *V. mimicus* and *E. coli* that produce highly related enterotoxins; however, as expected, strains of *E. coli* or *V. mimicus* that produce LT or CT did hybridize with the whole-gene CT probe. The homology between *ctxA* sequence and the analogous sequence (*eltA*) from *E. coli* is 78% (8). We selected the CTAP probe sequence on the basis of the dissimilarity between *ctxA* and *eltA* in that region; therefore, the lack of hybridization of CTAP with enterotoxigenic *E. coli* producing LT was not surprising. The CT or CT-like genes for *V. mimicus* have not been sequenced, but previous reports have shown *V. mimicus* enterotoxin to have biochemical and immunologic identity with CT (23); however, some diversity in restriction fragment patterns between *V. cholerae* and *V. mimicus* has been reported (5). Our results confirm that differences do exist between these species, at least in the location of the CTAP sequence, but that non-O1 and O1 *V. cholerae* are probably identical in this area. All other species of vibrio assayed were negative for CTAP.

Previously, it has been shown that some strains of *E. coli* which produce LT also produce ST and that genes for both toxins are carried on a plasmid. Strains of non-O1 and O1 *V. cholerae* that produce a heat-stable toxin (NAG-ST) that is closely related to *E. coli* ST have been identified (16); however, unlike *E. coli*, this gene is chromosomal (24). We

examined our strain collection for homology to a NAG-ST oligonucleotide probe (2, 16) to determine whether any of the *Vibrio* strains coproduced both toxins. *E. coli* strains that produce ST alone were negative both for the CT whole gene probe and for CTAP. We found several *Vibrio* strains that hybridized with the NAG-ST probe; however, none of these were positive for CTAP.

By using CTAP, it was possible to directly screen stool samples from human volunteers with *V. cholerae* infection for the presence of viable *V. cholerae* without the use of enrichment or selective medium. Although *V. cholerae* is not fastidious and is relatively easy to identify, there are inherent problems with most identification schemes. Detection methods have typically relied on enrichment in an alkaline peptone broth followed by growth on selective media such as TCBS. Enrichment can result in overgrowth of other organisms, and the recovery rate for *V. cholerae* on TCBS can be <70% (21). Although different methods of enumeration were used, the number of *V. cholerae* strains recovered on L agar appeared to be considerably greater than that on TCBS. However, samples with $<10^3$ *V. cholerae* strains could not be enumerated on L agar because of overgrowth of background bacteria on the nonselective medium. It may be possible to increase the sensitivity of this method for diagnostic purposes or for enumeration of *V. cholerae* from stool samples by using the CTAP probe in combination with a selective medium, perhaps one which is not as harsh as TCBS.

Probably one of the more pertinent applications of CTAP will be environmental monitoring of water and seafood samples. As most O1 *V. cholerae* strains from environmental sources are CT negative (14, 25), this method would allow direct identification of potentially pathogenic (i.e., CT-positive) strains. It is feasible with DNA probes to identify and enumerate isolates without enrichment or additional assays. Recovery of CT-positive isolates, by using probe results to guide selection of colonies from the plate from which the blot was originally prepared, is also possible. Previously, we had shown that a similar probe for the *V. vulnificus* cytolysin gene was a sensitive and accurate tool for the identification of this bacterium in the environment, and we were able to detect *V. vulnificus* at concentrations ranging from 20/ml in the water column to 10^4/ml in oysters without enrichment or selective media (26). While these studies need to be repeated with the CTAP probe, we would anticipate that CTAP would have a sensitivity comparable to that of the *V. vulnificus* probe.

The development of a more economical method that replaces the hazardous and technically difficult radioactive labeling with a stable enzymatic method of detection would be of particular value for field research in areas that are endemic for *V. cholerae*. CTAP offers advantages over traditional methods in the ability to evaluate large numbers of samples in less than 24 h, as well as in the increased accuracy inherent to oligonucleotide probes.

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