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Polymerase Chain Reaction for Detection of the Cholera Enterotoxin Operon of *Vibrio cholerae*

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We report a set of oligonucleotide primers and amplification conditions for the polymerase chain reaction to detect the *ctx* operon of *Vibrio cholerae*. The results of this assay on strains of *V. cholerae* and related organisms were identical with those obtained by the DNA colony hybridization test with the polynucleotide probe. The detection limit of this system was 1 pg of chromosomal DNA or broth culture containing three viable cells. The polymerase chain reaction method could directly detect the *ctx* operon sequences in rice-water stool samples from patients with cholera.

Cholera enterotoxin (CT) is a major virulence determinant of *Vibrio cholerae* O1 (4). However, most *V. cholerae* O1 strains isolated from the environment do not produce CT, nor do they possess the genetic potential to produce CT, while some *V. cholerae* non-O1 strains produce CT (3, 7, 15, 20). These findings necessitate regular examination of *V. cholerae* isolates for their ability to produce CT in order to assess their clinical significance. The DNA probe method to detect the operon (*ctx*) encoding CT is a specific and sensitive method which allows examination of isolates for their potential ability to produce CT (6). The polymerase chain reaction (PCR) method would be more rapid than the DNA probe method in detecting the *ctx* operon because PCR amplifies the target sequence in vitro to a detectable level in a short time (16). Oligonucleotide probes have some advantages over polynucleotide probes, but oligonucleotide probes can be so specific when employed under high-stringency conditions that they may miss varied sequences in the target genes. On the other hand, the oligonucleotide probes may hybridize to nucleotide sequences unrelated to the target genes under reduced-stringency conditions. To circumvent this problem, it is considered better to target relatively specific and conserved sequences (13). The same principle can be applied to the PCR method. In this article, we describe a set of oligonucleotide primers and amplification conditions that yielded results identical to those obtained with the hybridization test using a polynucleotide probe in detecting the *ctx* operon. Also, a successful application of the PCR method directly to cholera stool samples is reported.

(A preliminary account of this work was presented elsewhere [17].)

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* O1 strain 61H-151 was originally isolated in a clinical case of cholera at the Osaka Prefectural Institute of Public Health and was found to possess the *ctx* operon by the DNA colony hybridization test described below. Other test strains were obtained from James B. Kaper or were laboratory stock strains at the Department of Microbiology, Faculty of Medicine, Kyoto University, or at the National Institute of Cholera and Enteric Diseases of India.

Collection of stool samples and examination for enteropathogens. Stool samples from patients with acute diarrhea admitted to the Infectious Diseases Hospital, Calcutta, India, were collected in sterile McCartney bottles by inserting sterile catheters into patients' rectums. The stool samples were examined immediately at the National Institute of Cholera and Enteric Diseases, Calcutta, India, for enteropathogens, such as *V. cholerae* O1 and non-O1, enterotoxigenic *Escherichia coli*, and *Shigella*, *Salmonella*, and *Campylobacter* spp., by standard techniques (19). For identification of enterotoxigenic *E. coli*, *E. coli* isolates were examined by the DNA colony hybridization test with polynucleotide probes specific to the genes encoding heat-labile enterotoxin (LT) and heat-stable enterotoxins (STh and STp) (described below). For isolation of *V. cholerae* O1, apart from direct culture, all stool samples were enriched in alkaline peptone water (1% peptone, 1% NaCl [pH 8.5]) and plated on thiosulfate-citrate-bile-sucrose agar (Eiken, Tokyo, Japan).

Extraction of chromosomal DNA. Chromosomal DNA of *V. cholerae* O1 strain 61H-151 was extracted by the mini-preparation method described by Ausubel et al. (1).

Synthetic oligonucleotides. Oligodeoxyribonucleotides used as primers for PCR and a hybridization probe were synthesized on a Cyclone Plus DNA Synthesizer (MilliGen/Biosearch) and purified by chromatography on Oligonucleotide Purification Cartridges (Applied Biosystems) according to the manufacturer's specifications. The sequences of the synthesized oligonucleotides used as PCR primers are listed in Table 1.

DNA colony hybridization test. DNA colony blots were prepared, polynucleotide probes were labeled, and hybridization was carried out as described previously (12). The polynucleotide probes used were specific to the *ctx* operon (6) and the genes encoding LT or STh and STp of enterotoxigenic *E. coli* (11).

PCR with purified chromosomal DNA. PCR was carried out as described by Saiki et al. (16) with minor modifications. To 49.5 μl of template DNA solution (purified chromosomal DNA dissolved in 10 mM Tris-HCl [pH 8.0]–1 mM EDTA), 10 μl of buffer solution (100 mM Tris-HCl [pH 8.5], 500 mM

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TABLE 1. Synthetic oligonucleotides used as primers for PCR

Primer	Sequence	Corresponding positions in *ctx* operon[a]	No. of mismatches with LT operon[b]
1	5′-CTCAGACGGGATTTGTTAGGCACG-3′	712–735	8
2	3′-GCATTATCCCCGATGTCTCTATCT-5′	990–1013	7
3	5′-TGACCGAGGTACTCAAATGAATAT-3′	662–685	4
4	3′-AAGACGAAATCCACCCTAAGGTAT-5′	908–931	3
5	5′-TGTTAGGCACGATGATGGATATGT-3′	725–748	5
6	3′-CCGTATGTCAGGAGTAGGTCTACT-5′	875–898	5

[a] *ctx* operon nucleotide sequence reported in reference 9.
[b] LT operon nucleotide sequence reported in reference 21.

KCl, 15 mM $MgCl_2$, 2 mg of gelatin per ml), 10 μl of each primer (10 mM), and 20 μl of deoxynucleoside triphosphate mix (containing 1 mM dATP, 1 mM dCTP, 1 mM dTTP, and 1 mM dGTP) were added. This reaction mix was heated at 94°C for 3 min, and 0.5 μl (2.5 U) of *Taq* DNA polymerase (Perkin-Elmer Cetus) was added to make a final volume of 100 μl. The mixture was overlaid with 50 μl of mineral oil, and the amplification was carried out in a TempCycler (Coy Laboratory Products Inc.). The samples were subjected to rounds of amplification steps consisting of a 1-min denaturation step at 94°C, a 1.5-min annealing step at 60°C, and a 1.5-min extension step at 72°C, and the steps were repeated for 29 or 39 more cycles after the first cycle.

PCR with broth cultures. *V. cholerae* O1 61H-151 was grown in LB medium (10) with shaking (250 rpm) for 8 h at 37°C and was serially diluted in LB medium. For determination of the number of viable cells, portions of each dilution were plated onto LB agar and incubated overnight at 37°C. Other test organisms were grown in LB medium overnight at 37°C. Ten microliters of undiluted (all of the test organisms including strain 61H-151) or diluted (strain 61H-151) culture was mixed with 39.5 μl of distilled water and heated at 94°C for 5 min. To this suspension, 10 μl of the buffer solution, 10 μl of each primer (10 mM), 20 μl of dNTP mix, and 0.5 μl (2.5 U) of *Taq* DNA polymerase were added to make a final volume of 100 μl, and the reaction mix was subjected to amplification as described above.

PCR with stool samples. The stool samples were transported to the Department of Microbiology, Faculty of Medicine, Kyoto University, Kyoto, Japan, for the PCR assay. The transit time from collection of the stools to processing by PCR was about 72 to 96 h. The samples were maintained at ambient temperature during transportation. The stool samples were then heated at 94°C for 5 min and stored at −20°C until use. For the PCR assay, the samples were diluted with 10 mM Tris-HCl (pH 8.5)–1 mM EDTA, and 10 μl of the diluted sample was subjected to the PCR assay with primers 1 and 2 (Table 1) as described for the broth culture samples above.

Detection and confirmation of PCR products. For detection of the PCR products by gel electrophoresis, 1/10 (10 μl) of the amplification mixture and molecular weight markers (*Hae*III digest of ϕX174 DNA) were subjected to electrophoresis through a 5% polyacrylamide gel. Amplified DNA fragments of specific sizes were visualized by UV fluorescence after being stained with ethidium bromide.

For confirmation of the amplified DNA by Southern blot hybridization, 1/10 (10 μl) of the PCR products was resolved on a NuSieve GTG agarose gel (FMC BioProducts), transferred from the gel onto a nitrocellulose membrane by the method of Southern (18), and subjected to the hybridization test with an oligonucleotide probe specific to the amplified sequence. A synthetic oligonucleotide (5′-ACTATATTGTC TGGTCATTCTACT-3′, corresponding to positions 792 to 815 in the reported nucleotide sequence of the *ctx* operon [9]) was labeled at the 5′ end with [γ-^{32}P]ATP with T4 polynucleotide kinase (Takara, Shuzo Co., Kyoto, Japan). The blot was hybridized with the ^{32}P-labeled oligonucleotide probe (10^6 cpm) in 0.3% sodium dodecyl sulfate (SDS)–1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin)–5× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate [pH 7.0]) in the presence of denatured salmon sperm DNA (100 μg/ml) at 37°C overnight. After hybridization, the blot was washed two times in 5× SSC–0.1% SDS at 60°C for 30 min, air dried, and subjected to autoradiography at −80°C overnight with a single intensifying screen.

RESULTS

Optimization of PCR with purified chromosomal DNA. The various parameters of the PCR method were evaluated with the chromosomal DNA extracted from *V. cholerae* O1 strain 61H-151. Experiments with five combinations of six different primers (primers 1 and 2, 3 and 4, 3 and 6, 5 and 4, and 5 and 6 [Table 1]) showed that primers 1 and 2 and the reaction conditions described in Materials and Methods yielded the best result in terms of specificity (amplification of a single specific sequence) and sensitivity (amount of amplified sequences).

The results of the PCR obtained with amounts of purified DNA ranging from 0.1 pg to 1 ng as the template are shown in Fig. 1A. An amplified fragment of 302 bp (expected size for primers 1 and 2) was detected from 10 and 1 pg of chromosomal DNA after 30 and 40 cycles, respectively (Fig. 1A, lanes 4 and 8). To confirm the specificity of the amplification, the amplified products were subjected to Southern blot hybridization. The results, shown in Fig. 1B, revealed that the 302-bp fragment was a *ctx*-specific sequence and that no other fragment homologous to the *ctx* operon was amplified.

PCR with broth cultures. First, broth culture of *V. cholerae* O1 61H-151 was used as the template for the PCR, and it was found that broth cultures containing as few as 30 or 3 viable cells yielded positive results after 30 or 40 cycles of amplification, respectively (Fig. 2, lanes 4 and 10). In this experiment, a bacterial suspension was heated at 94°C for 5 min prior to the amplification. However, freezing at −20°C followed by thawing at room temperature yielded similar results (data not shown). The results indicated that broth culture, even if diluted considerably, can serve as the PCR template in place of purified chromosomal DNA.

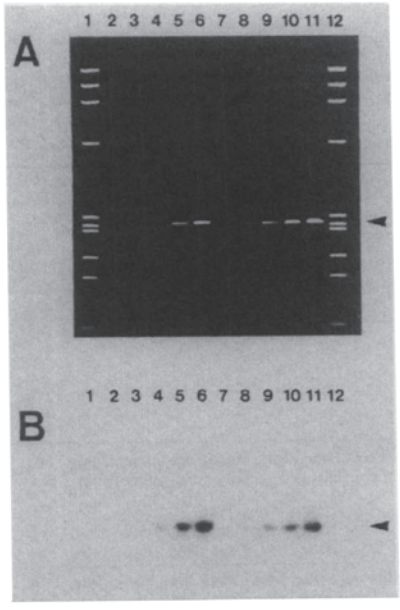

FIG. 1. Analysis of the PCR products of chromosomal DNA of *V. cholerae* O1 strain 61H-151. The amounts of chromosomal DNA used for the PCR were 0.1 pg (lanes 2 and 7), 1 pg (lanes 3 and 8), 10 pg (lanes 4 and 9), 100 pg (lanes 5 and 10), and 1 ng (lanes 6 and 11). Amplification was performed for 30 cycles (lanes 2 to 6) or 40 cycles (lanes 7 to 11). One-tenth of the PCR products (see text) was analyzed. The size of the amplified fragment (302 bp, indicated by the arrowheads) was confirmed by the positions of the bands relative to those of the molecular size markers (*Hae*III digest of ϕX174 DNA [lanes 1 and 12]). (A) The PCR products were separated by 5% polyacrylamide gel electrophoresis and were visualized by staining with ethidium bromide and illumination with UV light. (B) The PCR products were separated by 4% NuSieve agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled oligonucleotide probe specific to the *ctx* operon.

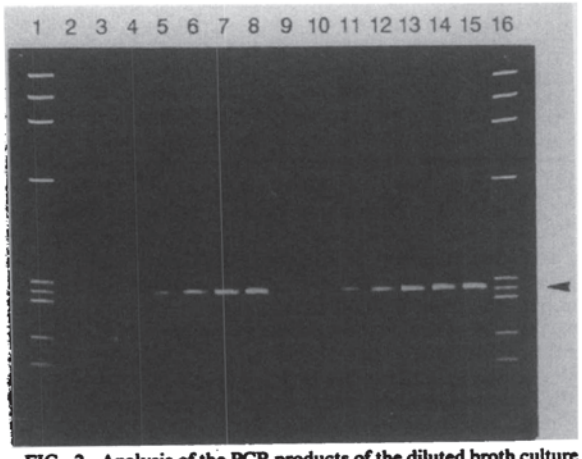

FIG. 2. Analysis of the PCR products of the diluted broth culture of *V. cholerae* 61H-151. The estimated numbers of viable cells present in each of initial PCR mixtures were 0 (lanes 2 and 9), 3 (lanes 3 and 10), 30 (lanes 4 and 11), 3×10^2 (lanes 5 and 12), 3×10^3 (lanes 6 and 13), 3×10^4 (lanes 7 and 14), and 3×10^5 (lanes 8 and 15). The number of cycles of amplification was 30 (lanes 2 to 8) or 40 (lanes 9 to 15). One-tenth of the PCR products was analyzed by 5% polyacrylamide gel electrophoresis (see legend to Fig. 1). The size of the amplified fragment (302 bp, indicated by the arrowhead) was confirmed by the positions of the bands relative to those of the molecular size markers (*Hae*III digest of ϕX174 DNA [lanes 1 and 16]).

Correlation of this PCR method (broth culture amplified with primers 1 and 2) with the DNA colony hybridization test using the *ctx*-specific polynucleotide probe was investigated next. Clinical and environmental strains of *V. cholerae* O1 and related organisms isolated from various geographical areas including India, Indonesia, the United States, and Japan were examined for the *ctx* operon by the two tests. As shown in Table 2, results of detection of the *ctx* operon in the test strains by the PCR method and by the DNA colony hybridization test were in complete concordance; only probe-positive *V. cholerae* O1 strains were positive in the PCR test, and the other organisms, which belonged to five species of the genus *Vibrio* and LT-producing *E. coli*, were negative in both tests.

LT-producing *E. coli* strains yielded negative results in this PCR test with primers 1 and 2. However, the PCR test with primers 3 and 4 and the same annealing temperature yielded a positive result with LT-producing *E. coli* (data not shown), indicating that this PCR system allowed three or four mismatches in a 24-bp sequence (Table 1).

Application of PCR to stool samples. To investigate whether the PCR can be used directly for stool samples, 25 stool samples were collected from patients with secretory diarrhea or dysentery. By the culture methods, of the 25 samples examined, 7 were positive for *V. cholerae* O1 (Ogawa serogroup, eltor biotype) (both by direct and by enrichment culture), 4 were positive for *Shigella dysenteriae* type 1, 2 were positive for *Shigella flexneri*, 1 was positive for LT- and STh-producing *E. coli*, and 1 was positive for STh- and STp-producing *E. coli*. Immediately before the PCR assay (72 to 96 h after stool collection), survival of *V. cholerae* O1 in the stool samples was examined by the direct culture method, but *V. cholerae* O1 was not recovered from any of the samples.

The results of the PCR tests for the stool samples are summarized in Table 3. Five of the seven *V. cholerae* O1 culture-positive samples yielded positive results after 30

TABLE 2. Results of PCR and the DNA colony hybridization tests to detect the *ctx* operon

Organism	No. of strains	Result of:	
		PCR[a]	DNA colony hybridization test[b]
V. cholerae O1	32	+[c]	+
	15	−[d]	−
V. cholerae non-O1	1	−	−
V. mimicus	4	−	−
V. parahaemolyticus	5	−	−
V. fluvialis	3	−	−
V. furnissii	4	−	−
E. coli (LT$^+$)[e]	5	−	−

[a] Specific amplification of the 302-bp fragment was examined as described in the text.
[b] Performed as described previously (12) with the polynucleotide probe specific to the *ctx* A subunit gene (6).
[c] +, the *ctx* operon was present.
[d] −, the *ctx* operon was absent.
[e] Strains producing LT (5).

TABLE 3. Results of PCR assay with stool samples

Stool sample no.	Isolation of *V. cholerae* O1[a]	Result[b] of PCR assay after:						
		30 cycles of amplification of sample diluted at:						40 cycles of amplification of undiluted sample
		1/1 (undiluted)	1/2	1/10	$1/10^2$	$1/10^3$	$1/10^4$	
1, 2	+	+++	+++	+++	+++	++	+	NT
3	+	+++	+++	+++	++	+	−	NT
4	+	+++	++	++	+	−	−	NT
5	+	++	++	++	+	−	−	NT
6, 7	+	−	−	−	−	−	−	+
8	−[c]	+	−	−	−	−	−	+
9–11	−[c]	−	−	−	−	−	−	+
12–15	−[d]	−	−	−	−	−	−	−
16, 17	−[e]	−	−	−	−	−	−	−
18	−[f]	−	−	−	−	−	−	−
19	−[g]	−	−	−	−	−	−	−
20–25	−[c]	−	−	−	−	−	−	−

[a] *V. cholerae* O1 was isolated (+) or not isolated (−) from the stool sample immediately after collection of the stool from the patient.
[b] A very intense (+++), intense (++), or weak (+) band or no band (−) of amplified 302-bp fragment was detected by the gel electrophoresis assay. NT, not tested.
[c] No enteropathogen was isolated.
[d] *S. dysenteriae* 1 was isolated.
[e] *S. flexneri* was isolated.
[f] LT- and STh-positive *E. coli* was isolated.
[g] STh- and STp-positive *E. coli* was isolated.

cycles of amplification (samples 1 to 5 in Table 3). These samples contained enough template DNA, and therefore these samples, when diluted to considerable degrees (up to 1:100 to 1:10,000), still yielded positive results. Two *V. cholerae* O1 culture-positive samples, which were negative after 30 cycles of amplification, yielded positive results after 40 cycles of amplification of the undiluted samples (samples 6 and 7 in Table 3). In addition, 4 of the 18 *V. cholerae* O1 culture-negative samples yielded positive results after 30 or 40 cycles of amplification of the undiluted samples (samples 8 to 11 in Table 3). No enteropathogens (see Materials and Methods) had been isolated from these four samples. All of the amplimers from the stool samples, including samples 8 to 11, were confirmed to be true positives by the Southern blot hybridization analysis. Representative electrophoretic and hybridization patterns of amplified DNA fragments are shown in Fig. 3.

DISCUSSION

We have used the PCR to detect the *ctx* operon of *V. cholerae* O1. This method was successfully used with broth cultures of *V. cholerae* O1. While our work was in progress, Kobayashi et al. (8) reported a PCR method with a set of primers which could detect strains producing cholera toxin in culture medium. Comparison of our results obtained by the PCR method using primers 1 and 2 and the DNA colony hybridization test (Table 2) indicated that our PCR method can be an alternative to the DNA colony hybridization method, which uses a polynucleotide probe to detect the *ctx* operon in the test organisms.

Our PCR method was also successfully applied to stool samples from patients with cholera. Stool samples were thought to contain a substance(s) which interferes with hybridization and PCR assays, and thus nucleic acids extracted from stool samples were used in assays by other investigators (2, 14). However, rice-water stools from patients with cholera do not appear to contain such a substance(s) at levels inhibitory to the PCR assay, because, in

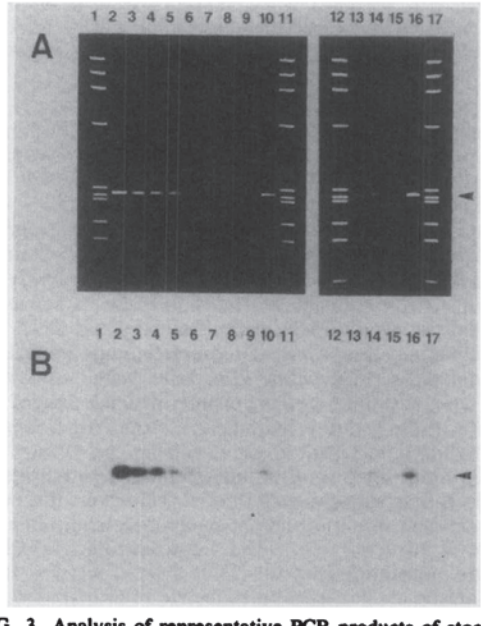

FIG. 3. Analysis of representative PCR products of stool samples obtained from patients with cholera. One-tenth of the PCR products was analyzed by 5% polyacrylamide gel electrophoresis (panel A) and Southern blot hybridization (panel B) as described in the legend to Fig. 1. Undiluted stool samples were subjected to 30 (lanes 2 to 9; samples 1 to 6, 9, and 20, respectively) or 40 (lanes 13 to 15; samples 6, 9, and 20, respectively) cycles of amplification. See Table 3 for sample designations. Chromosomal DNA (1 ng) of *V. cholerae* O1 61H-151 was subjected to 30 (lane 10) or 40 (lane 16) cycles of amplification. Lanes 1, 11, 12, and 17, molecular size markers (*Hae*III digest of ϕX174 DNA).

this study, undiluted stool samples could be successfully used for PCR amplification (Table 3).

V. cholerae O1 could be cultured from stool samples 1 to 7 (Table 3) immediately after stool collection but not after 72 to 96 h. *V. cholerae* O1 was not isolated from samples 8 to 11 (Table 3), even immediately after stool collection. However, the PCR assay performed 72 to 96 h after the collection of the samples detected the *ctx* operon sequences in samples 1 to 11. These results indicate that the PCR method could detect the *ctx* operon sequences even when *V. cholerae* O1 was dead and the chromosomal DNA may have been released by autolysis. Two of the *V. cholerae* O1 culture-positive samples (samples 6 and 7 in Table 3) contained very low levels of template DNA, suggesting that chromosomal DNA released by autolysis had probably been degraded to a significant degree by the time of the PCR assay. Nevertheless, the extremely high sensitivity of the PCR method could obviate the need for stool examination immediately after stool collection. Thus, the PCR method has some advantages over the culture method.

The PCR method can be more sensitive, quicker, and easier to perform than the DNA colony hybridization test. Thus, the PCR method holds promise as a diagnostic tool, particularly because it can be used directly on stool samples. We are in the process of expanding our study with a larger number of stool samples in various conditions to optimize the PCR conditions (maintenance and processing of samples and number of amplification cycles, etc.) for stool samples.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Coll, P., K. Phillips, and F. C. Tenover.** 1989. Evaluation of a rapid method of extracting DNA from stool samples for use in hybridization assays. J. Clin. Microbiol. **27**:2245–2248.
3. **Craig, J. P., K. Yamamoto, Y. Takeda, and T. Miwatani.** 1981. Production of cholera-like enterotoxin by a *Vibrio cholerae* non-O1 strain isolated from the environment. Infect. Immun. **34**:90–97.
4. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. **2**:553–623.
5. **Honda, T., M. Arita, Y. Takeda, and T. Miwatani.** 1982. Further evaluation of the Biken test (modified Elek test) for detection of enterotoxigenic *Escherichia coli* producing heat-labile enterotoxin and application of the test to sampling of heat-stable enterotoxin. J. Clin. Microbiol. **16**:60–62.
6. **Kaper, J. B., J. G. Morris, Jr., and M. Nishibuchi.** 1988. DNA probes for pathogenic *Vibrio* species, p. 65–77. *In* F. C. Tenover (ed.), DNA probes for infectious disease. CRC Press, Inc., Boca Raton, Fla.
7. **Kaper, J. B., S. L. Moseley, and S. Falkow.** 1981. Molecular characterization of environmental and nontoxigenic strains of *Vibrio cholerae*. Infect. Immun. **32**:661–667.
8. **Kobayashi, K., K. Seto, S. Akasaka, and M. Makino.** 1990. Detection of toxigenic *Vibrio cholerae* O1 using polymerase chain reaction for amplifying the cholera enterotoxin gene. J. Jpn. Assoc. Infect. Dis. **64**:1323–1329. (In Japanese with English summary.)
9. **Mekalanos, J. J., D. J. Swartz, and G. D. N. Pearson.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
10. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Moseley, S. L., P. Echeverria, J. Seriwatana, C. Tirapat, W. Chaicumpa, T. Sakuldaipeara, and S. Falkow.** 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. **145**:863–869.
12. **Nishibuchi, M., M. Ishibashi, Y. Takeda, and J. B. Kaper.** 1985. Detection of the thermostable direct hemolysin gene and related DNA sequences in *Vibrio parahaemolyticus* and other *Vibrio* species by the DNA colony hybridization test. Infect. Immun. **49**:481–486.
13. **Nishibuchi, M., A. Murakami, M. Arita, H. Jikuya, J. Takano, T. Honda, and T. Miwatani.** 1989. Detection with synthetic oligonucleotide probes of nucleotide sequence variations in the genes encoding enterotoxins of *Escherichia coli*. J. Clin. Microbiol. **27**:2272–2276.
14. **Olive, D. M.** 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. J. Clin. Microbiol. **27**:261–265.
15. **Roberts, N. C., R. J. Siebeling, J. B. Kaper, and H. B. Bradford, Jr.** 1982. Vibrios in the Louisiana gulf coast environment. Microb. Ecol. **8**:299–312.
16. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
17. **Shirai, H., Y. Takeda, and M. Nishibuchi.** 1990. Detection of cholera enterotoxin gene by DNA amplification (PCR) method. Jpn. J. Bacteriol. **45**:434. (Abstract for the 63rd Congress of Japanese Society for Bacteriology; in Japanese.)
18. **Southern, E. M.** 1980. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
19. **World Health Organization.** 1983. Manual for laboratory investigations of acute enteric infections. World Health Organization, Geneva.
20. **Yamamoto, K., Y. Takeda, T. Miwatani, and J. P. Craig.** 1983. Purification and some properties of a non-O1 *Vibrio cholerae* enterotoxin that is identical to cholera enterotoxin. Infect. Immun. **39**:1128–1135.
21. **Yamamoto, T., T. Tamura, and T. Yokota.** 1984. Primary structure of heat-labile enterotoxin produced by *Escherichia coli* pathogenic for humans. J. Biol. Chem. **259**:5037–5044.