TaqMan PCR for Detection of *Vibrio cholerae* O1, O139, Non-O1, and Non-O139 in Pure Cultures, Raw Oysters, and Synthetic Seawater†

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Received 7 May 2001/Accepted 25 June 2001

Vibrio cholerae is recognized as a leading human waterborne pathogen. Traditional diagnostic testing for *Vibrio* is not always reliable, because this bacterium can enter a viable but nonculturable state. Therefore, nucleic acid-based tests have emerged as a useful alternative to traditional enrichment testing. In this article, a TaqMan PCR assay is presented for quantitative detection of *V. cholerae* in pure cultures, oysters, and synthetic seawater. Primers and probe were designed from the nonclassical hemolysin (*hlyA*) sequence of *V. cholerae* strains. This probe was applied to DNA from 60 bacterial strains comprising 21 genera. The TaqMan PCR assay was positive for all of the strains of *V. cholerae* tested and negative for all other species of *Vibrio* tested. In addition, none of the other genera tested was amplified with the TaqMan primers and probe used in this study. The results of the TaqMan PCR with raw oysters and spiked with *V. cholerae* serotypes O1 and O139 were comparable to those of pure cultures. The sensitivity of the assay was in the range of 6 to 8 CFU g⁻¹ and 10 CFU ml⁻¹ in spiked raw oyster and synthetic seawater samples, respectively. The total assay could be completed in 3 h. Quantification of the *Vibrio* cells was linear over at least 6 log units. The TaqMan probe and primer set developed in this study can be used as a rapid screening tool for the presence of *V. cholerae* in oysters and seawater without prior isolation and characterization of the bacteria by traditional microbiological methods.

Vibrio cholerae is a waterborne pathogen that causes gastrointestinal disorders with a wide range of clinical manifestations, including vomiting and rice-like diarrhea (24). The association of human illness with consumption of *V. cholerae*-contaminated oysters, seawater, and other shellfish is well documented (29, 37). Consumption of raw oysters correlates strongly with gastrointestinal infections, and several *Vibrio* species, including strains of *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *V. cholerae*, have been implicated as the causative agents.

Coastal areas with brackish waters and estuarine regions are niches for many *Vibrio* species, including strains of toxigenic O1 *V. cholerae*. Epidemic cholera strains are endemic in several regions, including the U.S. gulf coast and Australia, and are occasionally involved in illnesses in these regions (24). Because *Vibrio* species attach to material suspended in water, shellfish and mollusks that are in these environments can be expected to consume *Vibrio* during feeding (24).

Traditional identification methods currently used are time-consuming and laborious, requiring prolonged incubation and selective enrichment to reduce the growth of background flora. *Vibrio* cells may also enter a viable but not culturable (VBNC) state, caused by nutrient starvation and physical stress. This may explain the failure of traditional culture techniques to isolate this organism from contaminated water and food samples implicated in food-borne outbreaks (10, 27, 47). Several investigators have de-

veloped PCR and DNA probe techniques for the detection of pathogenic *Vibrio* species (7, 12, 14, 19, 23, 25, 40, 41, 44, 46; S. C. Arya, Letter, J. Clin. Microbiol. 35:3364, 1997). DNA-based methods such as PCR have been increasingly used for rapid, sensitive analysis, but are nonquantitative in their detection of *V. cholerae* cells (7, 12, 14, 19, 23, 25, 34, 40, 41, 44, 46; Arya, Letter). The presence of PCR products must also be verified by subsequent procedures such as gel electrophoresis and Southern hybridization. All of these additional steps are time-consuming and laborious, and the additional procedures add to the overall cost of the test. The TaqMan assay utilizes the 5'-exonuclease activity of *Thermus aquaticus* DNA polymerase (17, 21, 35, 43; K. J. Livak, L. Marmaro, and S. J. A. Flood, Perkin-Elmer Research News, p. 1–12, Perkin-Elmer Corp., Norwalk, Conn., 1995) to hydrolyze an internal TaqMan probe labeled with a fluorescent reporter dye (FAM-6-carboxyfluorescein) and a quencher dye (TAMRA-6-carboxy-*N,N,N',N'*-tetramethylrhodamine) (User bulletin 2, ABI Prism 7700 Sequence Detection System, PE Applied Biosystems, Foster City, Calif., 1997). The probe is designed to hybridize to the DNA sequence between the PCR primers (User bulletin 2, PE Applied Biosystems). During PCR amplification, cleavage of the TaqMan probe separates the reporter dye and quencher dye, which results in increased fluorescence. In contrast, when the probe is intact, the proximity of the reporter dye to the quencher dye results in blockage of the reporter fluorescence (User bulletin 2, PE Applied Biosystems). TaqMan PCR eliminates the need for subsequent PCR product verification that is required by other PCR amplifications, thereby reducing the amount of time needed for sample analysis.

Several investigators have discussed the major obstacles encountered with the current DNA-based tests. These obstacles include the separation of culturable, VBNC, and dead micro-

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† This is journal paper no. 120 of the Louisiana Agricultural Experiment Station, Baton Rouge, La. (project: S-263).

organisms (30, 33). In addition to culturable versus VBNC versus dead cells, PCR inhibition is a major problem, especially in samples from the environment or food (30, 33). The nucleic acids in living cells are protected because the cell walls and membranes are intact. In dead cells, the cell membranes are compromised, and the nucleic acids are thus exposed to compounds added to the sample (30, 33). The exposure of DNA in dead cells may be utilized to destabilize or inactivate the nucleic acids, while the nucleic acids within living cells are protected from the treatment by the cell membrane and wall. Nogva et al. (30) also addressed the fact that bacterial DNases may cleave exposed DNA and determined that addition of DNase to the samples prior to DNA extraction did not have any effect on intact live cells, but did result in a 1-log reduction in the cell counts (i.e., dead cells) compared to that of control samples not treated with DNase.

Another major concern with VBNC cells is the potential for reversion to a metabolic state that can cause disease in humans after consumption of VBNC cells. Colwell et al. (10, 11) first reported that *V. cholerae* cells rendered nonculturable by incubation in estuarine water retained virulence when tested in rabbit ligated ileal loop assays and could cause disease when fed to human volunteers. Therefore, it is important that VBNC cells are enumerated and taken into consideration when evaluating foods that could be potentially contaminated with VBNC *Vibrio* cells.

Several investigators have developed TaqMan assays for detection of *Salmonella* species, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, and Shiga-like toxin genes in various foods (5, 9, 30, 31, 45). Among the various quantitative PCR strategies available, those based on real-time monitoring of the amplification reactions are the most accurate (20, 21, 35; Livak et al., Perkin-Elmer Research News). However, a real-time assay is still not available for *V. cholerae*. TaqMan PCR assays are also desirable for rapid analysis of foods that are consumed raw without further processing. Rapid methods that can quickly address the presence of *Vibrio* in fresh raw products such as oysters would be beneficial in eliminating release of contaminated products into the marketplace, which could result in potential food-borne outbreaks.

An elegant paper written by Singh et al. (42) evaluated the virulence of *V. cholerae* O1, O139, non-O1, and non-O139 strains isolated from various environmental sources. They found that environmental *V. cholerae* strains do not possess the virulence gene cassette (26) that contains the genes that encode the cholera toxin (*ctx*), zonula occludens toxin (*zot*), and accessory cholera toxin (*ace*). Furthermore, environmental vibrios lack the gene coding for toxin-coregulated pilus (*tcp*), which is recognized as a virulence factor in the pathogenicity of toxigenic *V. cholerae* serotypes O1 and O139 (26, 42). The genes coding for cholera toxin, the colonization toxin-coregulated pilus, and central regulatory protein (*toxR*) are required by virulent strains of *V. cholerae* O1 and O139 for production of the diarrheal syndrome (16, 18, 26, 27). In contrast, *V. cholerae* non-O1 and non-O139 strains lack the virulence genes (i.e., *ctx*, *zot*, and *ace*); however, these strains do contain genes that encode other products, such as NAG-specific heat-stable toxin (*st*), thermostable direct hemolysin, shigella-like toxin, and the classical hemolysin (4, 15, 32, 42, 48). The presence of these toxins has been correlated with the disease symptoms

produced by *V. cholerae* non-O1 and non-O139 strains (22, 24). Singh et al. (42) determined that both environmental and clinical *V. cholerae* O1, O139, non-O1, and non-O139 strains contain the nonclassical fragment of the hemolysin (*hlyA*) gene. We further evaluated the *hlyA* gene for the feasibility of using this gene in the development of a TaqMan PCR for *V. cholerae* strains.

This paper describes the development and evaluation of a primer and probe system that is rapid, sensitive, and quantitative for *V. cholerae* cells in pure cultures, seawater, and raw oysters. The primers and probe are directed towards the nonclassical specific *hlyA* gene of *V. cholerae* O1, O139, non-O1, and non-O139 strains. This primer-probe set can be used in the quantification of *V. cholerae* in the presence of other contaminating *Vibrio* species. A TaqMan primer and probe system that is a rapid screening tool for the presence of *V. cholerae* in oysters and seawater would eliminate the need for isolation and characterization by classical microbiological methods.

MATERIALS AND METHODS

Bacterial strains, media, and cultures. Sixty-seven strains of *Vibrio* were used to test the specificity of the primers and the probe (Table 1). The isolates were collected from patients and food products within the United States, India, Peru, and England. Sixty bacterial strains not belonging to the genus *Vibrio* were used to evaluate the specificity of the TaqMan primers and probe developed in this study. A complete list of bacterial strains that were evaluated is given in Tables 1 and 2.

The following media were used and prepared according to the manufacturer's instructions: alkaline peptone water (APW) (3), *Arcobacter* broth (Oxoid Ltd., Ogdensburg, N.Y.), bile-oxalate-sorbose broth (BOS) (13), buffered peptone water (BPW; Difco, Detroit, Mich.), brain heart infusion (BHI; Difco) broth, heart infusion (HI; Difco) broth, Preston broth (Difco), thiocitrate bile sucrose cholera agar (TBSC; Oxoid, Ltd.), synthetic seawater (SSW) (3), tryptic soy agar (TSA; Difco), MRS broth (Difco), and Trypticase peptone glucose yeast extract (TPGY) (3) medium. *Vibrio* species were grown aerobically in BHI broth supplemented with 3% (wt/vol) NaCl at 35°C, serially diluted into APW (3), and plated onto TBSC and BHI supplemented with 3% (wt/vol) NaCl. The numbers of CFU per milliliter were obtained and used to determine the number of cells used in the DNA isolation procedure described below.

DNA isolation. Three 1.0-ml samples (1.0 ml, 10^7 CFU ml⁻¹) of 18-h culture were centrifuged at $6,000 \times g$ for 5 min, and the supernatants were discarded. Cell pellets were resuspended with 200 μ l of Dynabeads DNA Direct I solution (DynaL AS, Oslo, Norway). The bacterium-bead suspensions were incubated at 65°C for 20 min, followed by incubation at room temperature for another 2 min. DNA bound to magnetic beads was then drawn to the wall of the microcentrifuge tube by a magnet (MPC-E; DynaL AS) for 2 min. The supernatant containing salts, detergent, and cell debris was carefully removed without disrupting the Dynabead-DNA complex. The beads were washed twice with a wash buffer provided in the kit. The DNA was removed from the beads by resuspension of the bead-DNA complex in 20 μ l of 10 mM Tris HCl (pH 8.0). The bead complexes were incubated at 65°C for 5 min to release the DNA from the beads. The beads were collected with the magnet, the DNA-containing supernatant was transferred to a fresh tube, and 2.5 μ l of the DNA suspension was used directly in PCRs. The DNA extraction efficiency was determined by the method described by Heid et al. (17). DNA was extracted from three different 1.0-ml aliquots of each sample, and the DNA concentrations were determined with a Hoefer DyNa Quant 200 fluorometer as described by the manufacturer (Amersham Pharmacia Biotech, Piscataway, N.J.).

TaqMan probe and primer design. The probe region used in this study was localized within a sequence region coding for the nonclassical region of *hlyA* (42) shown in Table 3. BLAST N, BLAST P, and BLAST X database searches (2) were done, and the primers and probes were designed within a region that had no homology with other known proteins in the database. The Primer Express (version 1.5) ABI Prism system (PE Applied Biosystems, Foster City, Calif.) was used for the primer-probe design, together with guidelines from PE Applied Biosystems (User bulletin 2, PE Applied Biosystems; Livak et al., Perkin-Elmer Research News).

TaqMan assay probe for detection of *V. cholerae*. Amplification reaction mixtures (50 μ l) contained a DNA sample (2.5 μ l, 100 ± 0.08 ng/ μ l); 1 $\times$ TaqMan buffer A; 5 mM MgCl₂; 200 μ M (each) dATP, dCTP, and dGTP; 400 μ M dUTP;

TABLE 1. *Vibrio* strains evaluated and Δ RQ values generated in PCR with TaqMan primers designed to detection *V. cholerae* strains

Bacteria evaluated	No. of isolates tested	Collection or isolation source ^a	Δ RQ value ^b	Interpretation ^c	Bacteria evaluated	No. of isolates tested	Collection or isolation source ^a	Δ RQ value ^b	Interpretation ^c
<i>Vibrio alginolyticus</i>	5	WL104, L, O	1.0	Negative	<i>Vibrio cholerae</i> sero-type non-O1	9	ATCC 25872, C	35.0	Positive
		85-2-4, L, CF	1.2	Negative			ATCC 25873, C	34.0	Positive
		WL118, CF, L	0.0	Negative			N2030	35.4	Positive
		WL121	0.8	Negative			WL102, L, EN	36.0	Positive
		WL210, L, O	0.9	Negative			WL105, L, O	35.2	Positive
<i>Vibrio anguillarum</i>	5	WL83, L, S	0.1	Negative			WL111	32.2	Positive
		WL87, O	0.2	Negative			WL115	35.0	Positive
		WL85, L, O	0.0	Negative			WL116	34.4	Positive
		WL89, L, CF	0.4	Negative			WL119	35.2	Positive
		99-053B	0.0	Negative	<i>Vibrio cholerae</i> sero-type non-O139	3	WL42, L, O	32.2	Positive
<i>Vibrio cholerae</i> serotype O1	13	ATCC 14035, I	30.1	Positive			NCTC 8457, C, I	33.0	Positive
		ATCC 14735, C, I	33.2	Positive			WL29, C, I	34.0	Positive
		0395N1	34.0	Positive	<i>Vibrio damsela</i>	3	ATCC 33539, CF, CA	0.0	Negative
		WL11, C, P	35.4	Positive			CDC 23-81, S, F	0.0	Negative
		WL13, C	34.0	Positive			CDC 183-79, C, H	0.0	Negative
		WL26, S, P	35.2	Positive	<i>Vibrio fluvialis</i>	2	ATCC 33809, C, I	0.0	Negative
		WL31, C, I	32.2	Positive			CDC 1280-78, C, I	0.0	Negative
		WL34	33.0	Positive	<i>Vibrio mimicus</i>	2	ATCC 33653, C, NC	2.0	Negative
		WL36, C, M	35.8	Positive			CDC 269-80, S, LA	1.8	Negative
		WL41	32.4	Positive	<i>Vibrio parahaemolyticus</i>	6	ATCC 17802, C, J	0.0	Negative
		WL65	34.4	Positive			ATCC 27519, S, LA	0.0	Negative
		WL69	33.8	Positive			WL78, L, C	0.1	Negative
		WL99	31.4	Positive			90-299	0.2	Negative
<i>Vibrio cholerae</i> serotype O139	13	ATCC 51394	32.1	Positive			WL90, L, S	0.0	Negative
		ATCC 25874, C	30.1	Positive			ATCC 49398, C, M	0.3	Negative
		WL29, C, I	33.1	Positive	<i>Vibrio vulnificus</i>	6	ATCC 27562, C	0.0	Negative
		WL32	33.2	Positive			CDC A1402, C	0.1	Negative
		WL33	32.0	Positive			89-142	0.1	Negative
		WL34	35.4	Positive			WL43, L, O	0.0	Negative
		WL42, L, O	34.4	Positive			CDC A8694, C, F	0.0	Negative
		WL50	35.2	Positive			WL76, L, S	0.2	Negative
		WL37	32.2	Positive					
		WL38	33.0	Positive					
		WL39	35.5	Positive					
		WL40	36.8	Positive					
		NCTC 8457, I, C	35.8	Positive					

^a ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; NCTC, National Collection of Type Cultures, London, England. Bacterial strains without letter designations are from John Hawke at Louisiana State University Veterinary Microbiology Department. WL, Louisiana State University Rapid Microbial Detection Facility's culture collection. WL strains without a history attached were obtained from the Department of Food Science Collection at Louisiana State University. Geographic origin abbreviations: EN, England; I, India; P, Peru, CA, California; F, Florida; L, Louisiana; M, Maryland; NC, North Carolina. Isolation source abbreviations: O, oysters; C, clinical; CF, saltwater catfish; E, eel; S, seawater.

^b Positive values were assigned when the Δ RQ was greater than the Δ RQ threshold value (2.80), based on the average value of the no-template controls ($n = 4$). Δ RQ values are the arithmetic mean of the triplicate DNA samples in each PCR analysis.

^c Negative, no PCR amplification; positive, PCR amplification occurred.

0.02 μ M *V. cholerae*-specific fluorogenic probe; 0.3 μ M (each) *V. cholerae*-specific primers; 1 U of AmpErase uracil *N*-glycosylase; and 2.5 U of AmpliTaq Gold DNA polymerase. All PCR samples and controls were prepared in triplicate by using 0.2 ml of MicroAmp Optical reaction tubes and MicroAmp Optical tube caps (PE Applied Biosystems).

The PCR mixture was held at 50°C for 5 min and denatured at 95°C for 10 min. Forty amplification cycles were carried out at 95°C for 20 s followed by 60°C for 1 min. All PCRs were performed with the ABI Prism 7700 sequence detection system (PE Applied Biosystems). Data were analyzed on a power Macintosh G4 (Apple Computer, Santa Clara, Calif.) linked directly to the ABI Prism 7700 sequence detection system by using the SOS (version 1.7) application software (PE Applied Biosystems) as described by the manufacturer. PCR products were detected directly by monitoring the increase in fluorescence from the dye-labeled *V. cholerae*-specific DNA probe. The TaqMan *V. cholerae*-specific probe is an oligonucleotide with a 5' reporter dye (FAM-6-carboxyfluorescein) and a 3' quencher dye (TAMRA-6-carboxy-*N,N,N',N'*-tetramethylrhodamine). Fluorescence was detected with the ABI Prism 7700 sequence detection system (PE Applied Biosystems) by using the equation Δ RQ = $RQ^+ - RQ^-$ (User bulletin 2, PE Applied Biosystems; Livak et al., Perkin-Elmer Research News). A positive interpretation for *V. cholerae* was based on a threshold of four times the average Δ RQ value (39, 45) of no-template controls from each 96-well optical reaction plate (i.e., three no-template controls per plate; PE Applied Biosystems). The

amplification was plotted as Δ RQ, which was the normalized reporter signal. The data were used to develop a standard curve against the log of cell numbers per PCR (17; User bulletin 2, PE Applied Biosystems; Livak et al., Perkin-Elmer Research News). All PCR products were verified with ethidium bromide-stained 4% agarose E-Gels (Invitrogen, Carlsbad, Calif.). Agarose gel electrophoresis was performed essentially as described by Sambrook et al. (38).

Specificity studies with pure cultures. Genomic DNA was isolated from 67 *Vibrio* strains (Table 1) and 60 other bacteria (Table 2). PCR was run with the DNA from each strain to determine the specificity of the TaqMan probe for *V. cholerae* species. Detection sensitivity studies were done to identify the lower detection limit of the TaqMan PCR analyses. *V. cholerae* O1 and O139 (ATCC 14035 and ATCC 51394, respectively) were incubated at 37°C in BHI broth containing 3% (wt/vol) NaCl until the mid-logarithmic growth phase (10^7 CFU ml⁻¹). Three 1.0-ml samples were taken from each of the cultures, serially diluted (10-fold to 10^{-7}) in SSW, and enumerated on BHI broth supplemented with 3% NaCl. Three 1.0-ml samples were taken from each serial dilution; the cells were pelleted by centrifugation ($6,000 \times g$ for 5 min), and the DNA was extracted from each cell pellet.

All other bacterial species were grown for 6 h (10^7 CFU ml⁻¹) in the media indicated and incubated at the temperatures indicated in Table 2. The cultures were serially diluted (10-fold to 10^{-7}) in BPW and enumerated on the nonselective medium shown in Table 2. Three 1.0-ml portions from each serial dilution were

TABLE 2. Bacterial strains evaluated with TaqMan primers and a probe specifically designed for detection of *V. cholerae* DNA and Δ RQ values generated during PCR amplification

Bacteria evaluated	No. of isolates	Medium and growth temp ^a	Collection or isolation source ^b	Δ RQ value ^c	Interpretation ^d
<i>Aeromonas hydrophila</i>	2	BHI broth, 35°C	ATCC 49140	4.1	Negative
			WL220	4.2	Negative
<i>Arcobacter butzleri</i>	1	<i>Arcobacter</i> broth, 35°C	ATCC 49615	4.1	Negative
<i>Bacillus cereus</i>	2	BHI broth, 35°C	WL322	4.1	Negative
			WL333	4.2	Negative
<i>Bacillus subtilis</i>	1	BHI broth, 35°C	ATCC 6633	4.3	Negative
<i>Campylobacter jejuni</i>	2	BHI broth, 35°C	ATCC 29428	4.7	Negative
			ATCC 49349	3.9	Negative
<i>Citrobacter freundii</i>	2	TSB, 35°C	ATCC 8090	3.7	Negative
			WL242	3.5	Negative
<i>Escherichia coli</i>	6	TSB, 35°C	ATCC 11775	4.2	Negative
			ATCC 23562	3.8	Negative
			ATCC 12792	4.4	Negative
			ATCC 23980	3.2	Negative
			ATCC 35150	3.3	Negative
			ATCC 43889	4.2	Negative
<i>Escherichia coli</i> O157:H7	3	TSB, 35°C	NADC 3073	4.7	Negative
			NADC 2939	4.5	Negative
			NADC 2909	4.4	Negative
<i>Clostridium botulinum</i> type E	2	TPGY broth, 30°C	ATCC 17786	4.1	Negative
			ATCC 17854	4.5	Negative
<i>Enterobacter aerogenes</i>	1	BHI broth, 35°C	WL243	3.6	Negative
<i>Enterobacter agglomerans</i>	1	BHI broth, 35°C	WL245	3.5	Negative
<i>Enterobacter avium</i>	1	BHI broth, 35°C	ATCC 14025	3.8	Negative
<i>Enterococcus faecalis</i>	2	TSB, 35°C	ATCC 19433	4.0	Negative
			WL348	4.4	Negative
<i>Enterococcus faecium</i>	1	BHI broth, 37°C	ATCC 19434	3.8	Negative
			ATCC 882	3.9	Negative
<i>Enterococcus iniae</i>	2	BHI, 37°C	345–78	3.7	Negative
			356–89	3.5	Negative
<i>Enterococcus pneumoniae</i>	1	BHI broth, 37°C	ATCC 33400	3.8	Negative
<i>Klebsiella pneumoniae</i>	3	TSB, 35°C	ATCC	4.1	Negative
			WL349	4.6	Negative
			WL352	4.0	Negative
<i>Klebsiella oxytoca</i>	1	TSB, 35°C	ATCC 13182	3.4	Negative
<i>Lactobacillus acidophilus</i>	2	MRS broth, 30°C	ATCC 4356	3.4	Negative
			WL567	3.8	Negative
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	2	MRS broth, 30°C	ATCC 19435	4.0	Negative
			WL678	4.5	Negative
<i>Listeria ivanovii</i>	1	BHI broth, 37°C	ATCC 19119	4.2	Negative
<i>Listeria monocytogenes</i>	3	BHI broth, 37°C	WL178 (Scott A)	3.8	Negative
			NADC 2283	3.5	Negative
			NADC 2276	3.7	Negative
<i>Pasteurella multocida</i>	1	BHI broth, 35°C	WL780	4.0	Negative
<i>Pediococcus acidilactici</i>	2	MRS broth, 35°C	ATCC 33314	4.2	Negative
			WL890	4.0	Negative
<i>Plesiomonas shigelloides</i>	2	TSB with 3% NaCl, 35°C	ATCC 14029	3.8	Negative
			WL689	4.2	Negative
<i>Pseudomonas aeruginosa</i>	1	BHI broth, 32°C	ATCC 15442	4.0	Negative

Continued on following page

TABLE 2—Continued

Bacteria evaluated	No. of isolates	Medium and growth temp ^a	Collection or isolation source ^b	ΔRQ value ^c	Interpretation ^d
<i>Salmonella enterica</i> serovar Derby	1	TSB, 37°C	ATCC 6960	3.8	Negative
<i>Salmonella enterica</i> serovar Enteritidis	1	TSB, 37°C	ATCC 4931	3.6	Negative
<i>Salmonella enterica</i> serovar Heidelberg	1	TSB, 37°C	ATCC 8326	3.9	Negative
<i>Salmonella enterica</i> serovar Typhimurium	1	TSB, 37°C	ATCC 13311	4.1	Negative
<i>Shigella sonnei</i>	1	TSB, 37°C	ATCC 11060	3.8	Negative
<i>Staphylococcus aureus</i>	2	BHI broth, 37°C	ATCC 25923	3.8	Negative
<i>Staphylococcus epidermidis</i>	1	BHI broth, 37°C	WL699	4.2	Negative
<i>Yersinia enterocolitica</i>	1	BOS broth, 35°C	ATCC 9612	3.5	Negative
			ATCC 23715	3.8	Negative

^a All cultures were grown aerobically, with the exception of *A. butzleri*, *C. jejuni*, and *C. botulinum* strains, which were grown under microaerobic and anaerobic conditions, respectively. Cultures were grown in a Gas-Pak jar (BBL) with *Campylobacter* and anaerobic gas-generating envelopes (BBL).

^b ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; NADC, USDA National Animal Disease Center, Ames, Iowa; NCTC, National Collection of Type Cultures, London, England. Bacterial strains without letter designations are from John Hawke at Louisiana State University Veterinary Microbiology Department. WL, Louisiana State University Rapid Microbial Detection Facility's culture collection.

^c Positive values were assigned when the ΔRQ was greater than the ΔRQ threshold value (2.84), based on the average value of the no-template controls ($n = 4$). ΔRQ values are the arithmetic mean of the triplicate DNA samples in each PCR analysis.

^d Negative, no PCR amplification; positive, PCR amplification occurred.

centrifuged ($6,000 \times g$ for 5 min), and the DNA was extracted from each cell pellet. DNA was also extracted from three 1.0-ml samples of the undiluted culture.

A 2.5-μl sample of extracted DNA (i.e., DNA extracted from 1.0 ml of undiluted and serially diluted samples) was analyzed with the TaqMan probe and primers. All DNA samples were analyzed in triplicate along with appropriate PCR controls as described by the manufacturer (PE Applied Biosystems).

DNA extraction from VBNC *V. cholerae* cells. *Vibrio* species are known to exist in a VBNC state when subjected to stress (i.e., storage at cold temperature in the presence of NaCl). The following experiments were done to evaluate whether the resuscitation of VBNC cells was necessary prior to DNA extraction and to determine if the DNA extraction procedure was effective in extracting DNA from VBNC cells. DNase was also added to samples to eliminate the contribution of DNA from dead cells during cell quantification.

V. cholerae O1 and O139 (ATCC 14035 and ATCC 51394, respectively) were incubated at 37°C in HI broth until the early logarithmic growth phase (10^7 CFU ml⁻¹). Three 1.0-ml samples were taken from each of the cultures; the cells were pelleted by centrifugation ($6,000 \times g$ for 5 min). One cell pellet was used to extract DNA. This DNA sample was considered to be the control sample that was not treated with DNase. Another cell pellet was resuspended in 1.0 ml of 1× DNase buffer (Promega, Madison, Wis.) and 10 U of DNase (Promega). The suspension was mixed and incubated at 25°C for 5 min, and the DNA was extracted from the DNase-treated cells. DNA concentrations were determined with a Hoefer DyNa Quant 200 fluorometer as described by the manufacturer (Amersham Pharmacia Biotech). The initial number of CFU per milliliter was obtained by plating the 10-fold SSW dilutions on HI plates in duplicate followed by incubation at 37°C for 18 h.

The final cell pellet was washed twice with SSW to remove trace nutrients, resuspended in 1.0 ml of SSW, and transferred into 99 ml of SSW to form a 100-ml nutrient-free microcosm as described by Whitehead and Oliver (47). The microcosm was incubated at 5°C for 7 days (i.e., cold stress to induce a VBNC state), and 10.0-ml samples were taken at day 7 and filtered through 0.2-μm-pore-size polyether sulfone (PES) filters (Millipore Corporation, Bedford, Mass.). Each of the filters was placed onto HI plates and incubated at 25°C for 25 h. One-milliliter aliquots were also removed for DNase treatment and DNA extraction as described earlier.

DNA detection sensitivity studies were done for all samples to determine the lower detection limit (i.e., number of *Vibrio* cells) that could be detected by the TaqMan PCR. All DNA samples were run in triplicate. The entire experiment was replicated twice.

Sensitivity studies with pure cultures spiked into raw oysters and SSW. Freshly harvested shellstock oysters obtained from three different docks in Louisiana were bagged in polyethylene bags, transported on ice to the Louisiana Department of Agriculture & Forestry, Louisiana State University Rapid Microbial Detection Laboratory, stored at refrigeration temperature (5°C), and subjected to microbiological analysis within 4 to 8 h. Fresh shellstock oysters were scrubbed with a brush under running tap water to remove mud and debris from the shells prior to shucking. *V. cholerae* serotype O1 (ATCC 14035) or *V. cholerae* serotype O139 (ATCC 51394) cultures were grown overnight in BHI, serially diluted (10-fold) in SSW, and enumerated on BHI supplemented with

3% (wt/vol) NaCl. Twenty-five grams of oysters was spiked with either *V. cholerae* serotype O1 (6.2×10^7 CFU) or *V. cholerae* serotype O139 (6.7×10^7 CFU), placed into a sterile stomacher bags (Seward, Inc.; model 400 filter bags), and homogenized with 225 ml of APW for 2 min in a stomacher (Tekmar, Inc.). One-milliliter samples from of the oyster homogenates were serially diluted 10-fold in APW. One-milliliter samples were taken from each of the APW dilutions, the cells were pelleted by centrifugation ($6,000 \times g$ for 5 min), and the DNA was extracted from the cells.

The SSW experiments were set up at the same time, except SSW was used as the diluents. Unspiked oyster samples were used as control samples for determination of the amount of DNA that was extracted from the oyster cells. Twenty-five grams of unspiked oysters was placed into sterile stomacher bags (Seward, Inc.), homogenized with 225 ml of either APW or SSW for 2 min, and serially diluted 10-fold in APW or SSW. One-milliliter samples were taken from each of the serial dilutions and centrifuged ($6,000 \times g$ for 5 min), and the DNA was extracted from the pellet. Each DNA sample was run in triplicate. The entire experiment was replicated twice.

RESULTS

TaqMan PCR specificity. Specific PCR primers and a probe were designed for detection of *V. cholerae*. The probe region was chosen to optimize specificity and amplification efficiency. The putative primers and probe were constructed with the primer express program, and then these DNA sequences were subjected to BLAST N, BLAST X, and BLAST P database searches (2) to find any sequence similarities. A 70-bp chromosomal DNA fragment of the nonclassical *hlyA* gene was found to be unique for all *V. cholerae* O1, O139, non-O1, and non-O139 strains tested. There were no known DNA sequences or

TABLE 3. TaqMan probe and primers for the nonclassical hemolysin (*hlyA*) gene region are specific for detection of *V. cholerae* O1, O139, non-O1, and non-O139 species

Probe or primer	Sequence (5'→3')	Denaturation temp (°C) ^a
<i>hlyA</i> primer		
Forward	TGC GTT AAA CAC GAA GCG AT	58
Reverse	AAG TCT TAC ATT GTG CTT GGG TCA	59
TaqMan probe	TCA ACC GAT GCG ATT GCC CAA GA	69

^a Calculated by the nearest-neighbor algorithm with the Primer Express (PE Applied Biosystems) program.

TABLE 4. Effectiveness of DNase in elimination of nonviable *Vibrio* cells prior to DNA extraction

Strain	Treat- ment	No. of CFU g ⁻¹		DNA concn (ng μ l ⁻¹) ^a	Δ RQ value ^b
		Initial	Final		
<i>V. cholerae</i> O1 (ATCC 14035)	None	7.2×10^7	6.5×10^7	108	35.2
				111	35.4
				105	35.8
	DNase	7.2×10^7	6.2×10^6	90	37.5
				92	37.8
				89	37.0
<i>V. cholerae</i> O139 (ATCC 51394)	None	6.4×10^7	5.8×10^7	100	35.2
				104	35.5
				96	35.4
	DNase	6.4×10^7	5.3×10^6	89	37.0
				91	37.2
				87	37.4

^a DNA was amplified with the *hlyA* TaqMan primers in the presence of the *hlyA* fluorogenic probe. The values shown are from three separate experiments. The DNA samples were analyzed in triplicate for each PCR.

^b Positive values were assigned when the Δ RQ was greater than the Δ RQ threshold value, based on the average value of the no-template controls ($n = 4$). Δ RQ values are the arithmetic mean of the triplicate DNA samples in each PCR analysis.

protein sequences in the BLAST N, BLASTX, and BLAST P databases with homology to this primer-probe region for *Vibrio cholerae* O1, O139, non-O1, and non-O139 species.

After the probe region was identified, the specificity of the selected primers and probe was evaluated. Thirty-eight *V. cholerae* strains were tested, and DNA from each strain was amplified with the TaqMan primers and probe. The specificity of the primers and probe was tested against 29 *Vibrio* strains (Table 1) and a set of 60 strains of bacteria belonging to other genera, some of which are common food-borne organisms and pathogens (Table 2). The DNA extracted from these tested organisms was not amplified in the TaqMan PCR. Three-microliter samples from each TaqMan PCR run were analyzed in 4% agarose E-Gels (Invitrogen), and nonspecific PCR products were not detected (data not shown). The agarose gels showed a fragment of the expected length of 70 bp when compared to PCR molecular weight markers (Sigma, St. Louis, Mo.) run in parallel (data not shown).

Effect of externally added DNase to samples during DNA extraction. The effect of externally added DNase was compared to that in control samples to which no DNase was added to the cell pellets prior to extraction of the DNA. Sixteen-hour cultures of *V. cholerae* serotype O1 (ATCC 14035; 7.2×10^7 CFU ml⁻¹) and *V. cholerae* O139 (ATCC 51394; 6.4×10^7 CFU ml⁻¹) were serially diluted 10-fold in SSW, and DNA was extracted from 1.0 ml of cells for each *Vibrio* culture. DNA (2.5 μ l) extracted from these cells was amplified by using the TaqMan PCR assay as described earlier. There was a 1-log reduction in CFU per milliliter in DNase-treated samples when compared to the non-DNase-treated control samples (Table 4). DNA extracted from *V. cholerae* O1 cells (ATCC 14035) that were treated with DNase had an average DNA concentration of 90 ± 5 ng/ μ l, and the DNA samples from cells not treated with DNase had a DNA concentration of 108 ± 3 ng/ μ l (Table 4). The Δ RQ values for samples that received DNase treatment were 37.5, 37.8, and 37.0, and samples that did not

receive DNase treatment had Δ RQ values of 35.2, 35.4, and 35.8 (Table 4). Similar results were obtained with *V. cholerae* O139 (ATCC 51394) cells. The DNA concentration of DNase-treated cells was 89 ± 2 ng/ μ l, and the DNA concentrations of cells not treated with DNase was 100 ± 4 ng/ μ l (Table 4). The Δ RQ values for samples that received DNase treatment were 35.2, 35.5, and 35.4, and samples that did not receive DNase treatment had Δ RQ values of 37.0, 37.2, and 37.4 (Table 4). TaqMan PCR sensitivity was able to quantify a 1-log reduction in cells; this was indicated by an increase in the RQ values for both *V. cholerae* O1 and O139 serotypes (Table 4).

Sensitivity studies with pure cultures of *V. cholerae*. Sensitivity studies were performed with pure cultures of *V. cholerae* serotypes O1 (ATCC 14035; 6.2×10^7 CFU ml⁻¹) and O139 (ATCC 51394; 6.7×10^7 CFU ml⁻¹) to test the lower detection limit of the TaqMan PCR. All *V. cholerae* strains tested gave a positive reaction with the Δ RQ values above the threshold of 2.80 (four times the average Δ RQ of the no-template controls; Table 1) (39, 45). Other bacterial and non-*V. cholerae* species tested gave a negative interpretation, with Δ RQ values below the threshold of 2.80 (Table 1). These results are similar to the data described by Ibrahim et al. and Vishnubhatla et al. (20, 45). The sensitivity curves based on the dilutions of *V. cholerae* O1 (ATCC 14035) cells and *V. cholerae* O139 (ATCC 51394) cells are shown in Fig. 1. Division of the lowest dilution of the culture that gave a positive reaction by the approximate final volume of the DNA extraction (CFU per microliter) used in the reaction gave a lower detection limit of >7.0 CFU ml of reaction mixture⁻¹. In our assay, the lowest detection limits were 7.3 and 8.2 CFU ml of reaction mixture⁻¹ for *V. cholerae* serotype O1 (ATCC 14035) and *V. cholerae* O139 (ATCC 51394), respectively.

Sensitivity of the TaqMan assay for detection of DNA extracted from VBNC *V. cholerae* cells. *V. cholerae* cells (O1, ATCC 14035; O139, ATCC 51394) were inoculated in SSW and stored at 5°C.

FIG. 1. Sensitivity of the fluorogenic TaqMan PCR assay for detection of pathogenic 10-fold dilutions of *V. cholerae* serotype O1 ($\square$; ATCC 14035) and O139 ($\circ$; ATCC 51394) in SSW. Experiments were done in triplicate. DNA was amplified with the *hlyA* TaqMan primers in the presence of the *hlyA* fluorogenic probe. The average Δ RQ values determined from DNA recovered from both serotypes were plotted against the CFU per milliliter. The average Δ RQ threshold values at four times the average of no-template controls were calculated to be 2.80 and 2.83 for *V. cholerae* O1 and O139, respectively (line across the graph). Error bars indicate the standard deviations of the means.

TABLE 5. Efficacy of isolation of DNA from VBNC *V. cholerae* O1 and O139 cells and amplification with the TaqMan primers and probe

Strain used	Day of incubation (5°C)	No. of CFU ml ⁻¹		DNA concn (ng µl ⁻¹) ^b	ΔRQ value ^c
		Initial	Final ^a		
<i>V. cholerae</i> O1 (ATCC 14035)	0	7.2×10^7	<0.1	100	34.2
				95	35.4
				105	35.8
	7	7.2×10^7	<0.1	96	33.5
				95	33.2
				101	34.0
<i>V. cholerae</i> O139 (ATCC 51394)	0	6.4×10^7	<0.1	100	33.2
				104	33.8
				96	34.4
	7	6.4×10^7	<0.1	105	33.2
				100	33.5
				103	33.8

^a This level of detection was determined by filtering 10.0-ml samples of cells.

^b DNA was amplified with the *hlyA* TaqMan primers in the presence of the *hlyA* fluorogenic probe. The values shown are from three separate experiments. The DNA samples were analyzed in triplicate for each PCR.

^c Positive values were assigned when the ΔRQ was greater than the ΔRQ threshold value, based on the average value of the no-template controls ($n = 4$). ΔRQ values are the arithmetic mean of the triplicate DNA samples in each PCR analysis.

Refrigerated *V. cholerae* cultures were sampled at days 0 and 7. For *V. cholerae* O1 cultures, there were 3.8×10^7 and <0.1 CFU ml⁻¹ at days 0 and 7, respectively. For *V. cholerae* O139 (ATCC 51394) cultures, there were 2.8×10^7 CFU ml⁻¹ and <0.1 CFU g⁻¹ at days 0 and 7, respectively. DNA was extracted from three 1.0-ml samples of each culture at days 0 and 7. TaqMan PCR analysis was run on all of the DNA samples, and the data are shown in Table 5. Each of the DNA preparations (2.5 µl; 100 ± 5 ng µl⁻¹) was analyzed in triplicate, and the experiment was repeated three times (Table 5). These data indicate that the *Vibrio* cells induced by exposure to NaCl and storage at 5°C were in a VBNC state and that the amounts of DNA isolated were similar to that of day 0. VBNC *Vibrio* cells were not detected by traditional culturing methods when plated immediately after cold storage (Table 5). However, the VBNC cells were resuscitated on filters placed on HI plates after an incubation of 25 h at 25°C. As expected, these results are similar to the results found by other investigators (27, 47), indicating that when *Vibrio* strains are placed under physiochemical stresses, they are induced into a VBNC state and can be resuscitated by raising the temperature to 25°C. VBNC *V. cholerae* cells were detected by using the TaqMan PCR assay (Table 5). The ΔRQ values were similar to that of *Vibrio* cells that had not been induced into a VBNC state (Table 5).

Sensitivity studies with spiked raw oysters. Raw oysters obtained from three different docks in Louisiana were confirmed to be culture negative for *V. cholerae*. Twenty-five-gram samples were spiked with 6.2×10^7 CFU of *V. cholerae* serotype O1 (ATCC 14035). The presence of oyster homogenate did not affect the ability to isolate comparable amounts of *Vibrio* DNA. The average amounts of DNA isolated from three 1.0-ml samples of pure cultures of *V. cholerae* O1 and O139 were 100 ± 5 and 102 ± 3 ng µl⁻¹, respectively. The average amounts of DNA isolated from three 1.0-ml samples of spiked oyster ho-

mogenate were 105 ± 2 and 103 ± 4 ng µl⁻¹ for *V. cholerae* O1 and O139, respectively (data not shown). The contribution of the DNA from the oyster cells was determined by serially diluting the nonspiked oyster homogenate 10-fold in SSW. The DNA concentrations were 0.09 ± 0.1 and 0.001 ± 0.003 ng µl⁻¹ in the 1:10 and 1:100 dilutions, respectively (data not shown). The oyster cell DNA was considered to be an additional control and was run in triplicate with each PCR run. The ΔRQ values for oyster homogenates were 0.30, 0.28, and 0.32. The average ΔRQ value for oyster homogenates was 0.32; this value was subtracted from amplification ΔRQ values obtained

FIG. 2. Sensitivity of the fluorogenic TaqMan PCR assay for detection of pathogenic *V. cholerae* O1 and O139 in spiked oyster samples (A) and spiked SSW (B). Tenfold dilutions of *V. cholerae* serotype O1 (□; ATCC 14035) and O139 (○; ATCC 51394) in SSW were done in triplicate. DNA was amplified with the *hlyA* TaqMan primers in the presence of the *hlyA* fluorogenic probe. The average ΔRQ values determined from DNA recovered from both serotypes were plotted against either the CFU per gram or CFU per milliliter. Delta RQ average threshold values are defined as four times the average of no-template controls and are shown for spiked oyster samples (A) and SSW samples (B) as 3.3 and 2.5 (line across the graphs), respectively. Error bars indicate the standard deviations of the means.

for each *Vibrio* DNA dilution prior to plotting, which was the ΔRQ (Fig. 2A).

The sensitivity of the TaqMan PCR in identifying *V. cholerae* in spiked raw oyster samples was found to be 6 to 8 CFU g⁻¹ (Fig. 2A). The sensitivity in identifying *V. cholerae* serotypes O139 (ATCC 51394; 6.8×10^7 CFU) in the spiked raw oyster sample was similar to the sensitivity of *V. cholerae* (ATCC 14035) in raw oysters (Fig. 2A). *V. cholerae* O139 was detected at the level of 6 to 8 CFU g⁻¹, with an average ΔRQ value of 3.25 (Fig. 2A).

Sensitivity of the TaqMan PCR for isolation of *V. cholerae* cells from SSW. Sensitivity studies were performed with pure cultures of *V. cholerae* serotype O1 to test the lower detection limit of the TaqMan PCR. Division of the lowest dilution of the culture that gave a positive reaction by the approximate final volume of the DNA extraction (CFU per microliter) used in the reaction gave a lower detection limit of approximately 10 CFU ml of reaction mixture⁻¹ with a threshold ΔRQ of 2.50 for *V. cholerae* serotype O1 cultures (ATCC 14035; 6.2×10^7 CFU) spiked into SSW (Fig. 2B). Similar results were obtained with *V. cholerae* O139 (ATCC 51394; 6.8×10^7 CFU), which had a detection limit of 10 CFU ml⁻¹ in spiked SSW. The reproducibility was good among three experiments, with ΔRQ threshold values of 2.50, 2.46, and 2.53 and 2.50, 2.46, and 2.56 for *V. cholerae* O1 and *V. cholerae* O139, respectively. Theron et al. (44) obtained a comparable detection level of 1 CFU ml⁻¹ for *V. cholerae* in environmental water samples.

DISCUSSION

There is a requirement for rapid, quantitative, and accurate measurements of target organisms responsible for food poisoning. In the present study, a TaqMan PCR system was constructed and applied to specifically detect and quantify *V. cholerae* strains. The preferred targets for pathogen detection are pathogen virulence genes. However, the need for a specific probe that targets all *V. cholerae* species would be valuable because of the variation between the different *V. cholerae* serotypes. For example, the *ctx* genes are expressed in some of the serotypes of *V. cholerae* O1/O139 and some strains of non-O1/O139, but not at all in other non-O1/O139 strains (10, 27, 47). Therefore, it appears that the virulence genes (*ctx*, *zot*, and *ace*) would be unsuitable for the design of primers and probes for detection of *V. cholerae* strains. The sequence region of the nonclassical *hlyA* region (70 bp) in *V. cholerae* is specific for this organism and not other *Vibrio* species or other bacterial genera. The 70-bp target region within the nonclassical *hlyA* region was compared with the most recently published sequences in the National Center for Biotechnology Information database (BLAST N, BLAST P, and BLAST X), and no homologous protein sequences were found within this region, with the exception of the structural subunit of the longus pili of *E. coli*, which had less than 38% homology. This region of the *hlyA* gene was not included in the design of the primers and probe. The specificity of the constructed primers and probe was tested both by homology searches of protein and nucleotide databases and by screening a number of *V. cholerae* strains isolated from patients, oysters, shellfish, and water from several parts of the world. No false positives were recorded among the 61 bacterial species belonging to other genera, and

no false positives were recorded among the other *Vibrio* strains tested, demonstrating the high specificity of the designed primer-probe set for *V. cholerae* strains (Tables 1 and 2).

Because PCR operates with constant efficiency, it is well suited for quantitative measurements. The detection limits of the PCR assay were estimated to be approximately 10 CFU ml⁻¹ in synthetic seawater and 6 to 8 CFU ml⁻¹ in oysters. These reported limits of detection are similar to those in other reports obtained with a TaqMan PCR assay for endpoint detection (5, 9, 30, 31, 45). For *Listeria monocytogenes*, Bassler et al. (5) and Nogva et al. (30) obtained detection levels of approximately 50 CFU ml⁻¹ and 6 CFU ml⁻¹, respectively. Chen et al. (9) and Vishnubhatla et al. (45) showed a detection limit as low as 2 CFU ml⁻¹ from a pure culture of *Salmonella enterica* serovar Typhimurium and 9.4 CFU ml⁻¹ for *Yersinia enterocolitica* in spiked ground pork, respectively.

Our data indicate a good correlation between CFU counts and the TaqMan assay for *V. cholerae* incubated in synthetic seawater at 5°C (Fig. 2B and Table 5). These results are in agreement with earlier experiments in which *Vibrio* cells (i.e., VBNC cells) were recovered from refrigerated seafoods by incubation in SSW at 5°C (10, 27, 47). After 7 days, there was a 7-log reduction in the direct viable counts and plate counts of *V. cholerae* O1 and O139 (Table 5). These data indicate that traditional plating can result in underestimation of the number of potentially infectious *V. cholerae* cells present in samples stored under refrigeration. In contrast, VBNC cells are detectable through the use of the TaqMan PCR (Table 5).

It is also important to know the history of the food product prior to analysis. Products such as raw oysters stored on ice may contain a large numbers of cold stress-induced VBNC *Vibrio* cells. The history of the food product is especially important if the product was heat treated or underwent a process that could cause cell death of the target organism. Nogva et al. (30) found that use of DNase in the sample prior to isolation of the DNA appears to reduce the amount of nucleic acid isolated from dead bacteria. In this study, it appears that the aspect of living versus dead cells was important, because *Vibrio* cells isolated from spiked oysters appear to contain both viable and dead cells. Treatment with DNase prior to DNA isolation resulted in a 1-log reduction in the cell counts and subsequently resulted in elimination of DNA from dead *Vibrio* cells (Table 4).

The use of the TaqMan PCR is a sensitive and quantitative method that is useful for estimating the number of cells of a specific pathogen in a food product (5, 9, 30, 31, 45). The main advantage of the TaqMan PCR is that it is very rapid and is a valuable method for screening a large number of samples. Traditional culturing methods require time-consuming enrichments and labor-intensive procedures that require several days. However, the TaqMan PCR developed in this study requires only 3 h. Other molecular systems for identifying *V. cholerae* in food and water, such as PCR fingerprinting and ribotyping (1, 41) and detection of virulence genes by PCR (6, 8, 28, 36, 44, 46; Arya, Letter), require additional verification steps that increase analysis time. The real value of the TaqMan PCR is the potential for rapid analysis of numerous pathogen-free samples, thereby allowing laboratories the ability to quickly screen products before they are released for human consumption. Putative positive food samples can also be quickly identified and pulled for further analysis.

Future developments. The use of TaqMan technology for quantification of *V. cholerae* in other foods and environmental water samples should be feasible. Work is under way to develop TaqMan assays for *V. vulnificus* and *V. parahaemolyticus*. Once these nuclease assays are developed, it should be a simple procedure to screen for all three species by using one enrichment technique and one DNA sample extracted from bacterial cells in the food sample.

REFERENCES

- Aidara-Kane, A., C. S. Boye, S. Koblavé, F. Grimont, and P. Grimont. 1997. Isolation of a new variant of *Vibrio cholerae* O1: *V. cholerae* O1 ribotype B27 toxinogenotype TB31 during the last cholera epidemic in Senegal. *Jpn. J. Med. Sci. Biol.* **50**:227–232.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Atlas, R. M. 1995. Handbook of microbiological media for examination of food. CRC Press, Inc., Boca Raton, Fla.
- Bagchi, K., P. Echeverría, J. D. Arthur, O. Sethabutr, O. Serichantalergs, and C. W. Hoge. 1993. Epidemic diarrhea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. *J. Clin. Microbiol.* **31**:1315–1317.
- Bassler, H. A., S. J. A. Flood, K. J. Livak, J. Marmaro, R. Knorr, and C. A. Batt. 1995. Use of a fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **61**:3724–3728.
- Basu, A., A. K. Mukhopadhyay, P. Garg, S. Chakraborty, T. Ramamurthy, S. Yamasaki, Y. Takeda, and G. B. Nair. 2000. Diversity in the arrangement of the CTX prophages in classical strains of *Vibrio cholerae* O1. *FEMS Microbiol. Lett.* **182**:35–40.
- Chakraborty, S., J. Khanam, Y. Takeda, and G. B. Nair. 1999. Application of PCR for detection of toxigenic *Vibrio cholerae* O1 in water samples during an outbreak of cholera. *Trans. R. Soc. Med. Hyg.* **93**:527–538.
- Chakraborty, S., A. K. Mukhopadhyay, R. K. Bhadra, A. N. Ghosh, R. Mitra, T. Shimada, S. Yamasaki, S. M. Faruque, Y. Takeda, R. R. Colwell, and G. B. Nair. 2000. Virulence genes in environmental strains of *Vibrio cholerae*. *Appl. Environ. Microbiol.* **66**:4022–4028.
- Chen, S., A. Yee, M. Griffiths, C. Larkin, C. T. Yamashiro, R. Behari, C. Paszko-Kolva, K. Rahn, and S. A. De Grandis. 1997. The evaluation of fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. *Int. Food Microbiol.* **35**:239–250.
- Colwell, R. R., P. R. Brayton, and D. Herrington. 1996. Viable but nonculturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World J. Microbiol. Biotechnol.* **12**:28–31.
- Colwell, R. R., and A. Huq. 1994. *Vibrios* in the environment: viable but nonculturable *Vibrio cholerae*, p. 117–133. In I. K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington, D.C.
- DePaola, A., and G. C. Hwang. 1995. Effect of dilution, incubation time, and temperature of enrichment on cultural and PCR detection of *Vibrio cholerae* obtained from the oyster *Crassostrea virginica*. *Mol. Cell. Probes* **9**:75–81.
- Elliot, E. L., C. A. Kaysner, L. Jackson, and M. L. Tamplin. 1995. Bacteriological analytical manual, 8th ed. AOAC International, Gaithersburg, Md.
- Fields, P. I., T. Popovic, K. Wachsmuth, and Ø. Olsvik. 1992. Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae* O1 strains from the Latin American cholera epidemic. *J. Clin. Microbiol.* **30**:2118–2121.
- Finkelstein, R. A., and S. Mukerjee. 1963. Haemagglutination: a rapid method for differentiating *Vibrio cholerae* and *El Tor* vibrios. *Proc. Soc. Exp. Biol. Med.* **112**:355–359.
- Hall, R. H., F. M. Khambaty, M. H. Kothary, S. P. Keasler, and B. D. Tall. 1994. *Vibrio cholerae* non-O1 serogroup associated with cholera gravis genetically and physiologically resembles O1 *El Tor* cholera strains. *Infect. Immun.* **62**:3859–3863.
- Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. *Genome Res.* **6**:986–994.
- Herrington, D., R. H. Hall, G. Losonsky, J. J. Mekalanos, and R. K. Taylor. 1988. Toxin, toxin co-regulated pili, and toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J. Exp. Med.* **168**:1487–1492.
- Hoshino, K., S. Yamasaki, A. K. Mukhopadhyay, S. Chakraborty, A. Basu, S. K. Bhattacharya, G. B. Nair, T. Shimada, and Y. Takeda. 1998. Development and evaluation of multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunol. Med. Microbiol.* **20**:201–207.
- Ibrahim, M. S., R. S. Lofts, P. B. Jahrling, E. A. Henchal, V. W. Weedn, M. A. Northrup, and P. Belgrader. 1998. Real-time microchip PCR for detecting single-base differences in viral and human DNA. *Anal. Chem.* **70**:2013–2017.
- Kalinina, O., I. Lebedeva, J. Brown, and J. Silver. 1997. Nanoliter scale PCR with TaqMan detection. *Nucleic Acids Res.* **25**:1999–2004.
- Kaper, J. B., A. Fasano, and M. Trucksis. 1994. Toxins in *Vibrio cholerae*, p. 145–176. In I. K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington, D.C.
- Karunasagar, I., G. Sugumar, I. Karunasagar, and A. Reilley. 1995. Rapid detection of *Vibrio cholerae* contamination of seafood by polymerase chain reaction. *Mol. Mar. Biol. Biotechnol.* **4**:365–368.
- Kayner, C. A. 2000. *Vibrio* species. Apsen Publications, Gaithersburg, Md.
- Koch, W. H., W. L. Payne, B. A. Wentz, and T. A. Cebula. 1993. Rapid polymerase chain reaction method for detection of *Vibrio cholerae* in foods. *Appl. Environ. Microbiol.* **59**:556–560.
- Kurazono, H., A. Pal, P. K. Bag, G. B. Nair, T. Karasawa, T. Mihara, and Y. Takeda. 1995. Distribution of genes encoding cholera toxin, zonula occludens toxin, accessory cholera toxin, and *El Tor* hemolysin in *Vibrio cholerae* of diverse origins. *Microb. Pathog.* **18**:231–235.
- Miller, C. J., B. S. Drasar, and R. C. Feachem. 1984. Response of toxigenic *Vibrio cholerae* to physio-chemical stresses in aquatic environments. *J. Hyg.* **93**:475–495.
- Miyagi, K., K. Sano, C. Morita, S. Imura, S. Morimatsu, T. Goto, Y. Nakano, K. Omura, Y. Matsumoto, K. Maeda, S. Hashimoto, and T. Honda. 1999. An improved method for detecting faecal *Vibrio cholerae* by PCR of the toxin A gene. *J. Med. Microbiol.* **48**:883–889.
- Morris, J. G. 1994. Non-O1 group *Vibrio cholerae* strains not associated with epidemic disease, p. 103–115. In I. K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington, D.C.
- Nogva, H. K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug. 2000. Application of 5'-nuclease PCR assay for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl. Environ. Microbiol.* **66**:4266–4271.
- Norton, D.-M., and C. A. Batt. 1999. Detection of viable *Listeria monocytogenes* with a 5' nuclease PCR assay. *Appl. Environ. Microbiol.* **65**:2122–2127.
- O'Brien, A. D., M. E. Chen, R. K. Holmes, J. Kaper, and M. M. Levine. 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce *Shigella dysenteriae* type 1 (Shiga)-like toxin. *Lancet* **11**:958.
- O'Brien, M. C., and W. E. Bolton. 1995. Comparison of cell viability probes compatible with fixation and permeabilization for combined surfaces and intracellular staining in flow cytometry. *Cytometry* **19**:243–255.
- Olsen, J. E., S. Aabo, W. Hill, S. Notermans, K. Wernars, P. E. Granum, T. Popovic, H. N. Rasmussen, and Ø. Olsvik. 1995. Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. *Int. J. Food Microbiol.* **28**:1–78.
- Orlando, C., P. Pinzani, and M. Pazzagli. 1998. Developments in quantitative PCR. *Clin. Chem. Lab. Med.* **36**:255–269.
- Pourshafie, M. R., F. Grimont, M. Saifi, and P. A. Grimont. 2000. Molecular epidemiological study of *Vibrio cholerae* isolates from infected patients in Teheran, Iran. *J. Med. Microbiol.* **49**:1085–1090.
- Rippey, S. R. 1994. Infectious diseases associated with molluscan shellfish consumption. *Clin. Microbiol. Rev.* **7**:419–425.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sanchez-Vizcaino, J. M., and M. Cambro-Alvarez. 1987. Execution of the ELISA technique, 2nd ed. Office International des Epizooties, Paris, France.
- Shangkuan, Y. H., Y. S. Show, and T. M. Wang. 1995. Multiplex polymerase chain reaction to detect toxigenic *Vibrio cholerae* and to biotype *Vibrio cholerae* O1. *J. Appl. Bacteriol.* **79**:264–273.
- Shangkuan, Y. H., C. M. Tsao, and H. C. Lin. 1997. Comparison of *Vibrio cholerae* O1 isolates by polymerase chain reaction fingerprinting and ribotyping. *J. Med. Microbiol.* **46**:941–948.
- Singh, D. V., M. H. Matte, G. R. Matte, S. Jiang, F. Sabeena, B. N. Shukla, S. C. Sanyal, A. Huq, and R. R. Colwell. 2001. Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: clonal relationships between clinical and environmental isolates. *Appl. Environ. Microbiol.* **67**:910–921.
- Taylor, T. B., E. S. Winn-Deen, E. Picozza, T. M. Woudenberg, and M. Albin. 1999. Optimization of the performance in silicon-based microstructures. *Nucleic Acids Res.* **25**:3164–3168.
- Theron, J., J. Cilliers, M. Du Preez, V. S. Brozel, and S. N. Venter. 2000. Detection of toxigenic *Vibrio cholerae* from environmental water samples by an enrichment broth cultivation-pit-stop semi-nested PCR procedure. *J. Appl. Microbiol.* **89**:539–546.
- Vishnubhatla, A., D. Y. C. Fung, R. D. Oberst, M. P. Hays, T. G. Nagaraja, and S. J. A. Flood. 2000. Rapid 5'-nuclease (TaqMan) assay for detection of virulent strains of *Yersinia enterocolitica*. *Appl. Environ. Microbiol.* **66**:4131–4135.
- Wang, R. F., W. W. Cao, and C. E. Cerniglia. 1997. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *J. Appl. Microbiol.* **83**:727–736.
- Whitesides, M. D., and J. D. Oliver. 1997. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. *Appl. Environ. Microbiol.* **63**:1002–1005.
- Yoh, M., T. Honda, and T. Miwatani. 1985. Production of non-O1 *Vibrio cholerae* hemolysin related to thermostable direct hemolysin of *Vibrio parahaemolyticus*. *FEMS Microbiol. Lett.* **29**:197–200.