Quadruplex Real-Time PCR Assay for Detection and Identification of *Vibrio cholerae* O1 and O139 Strains and Determination of Their Toxigenic Potential

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*Vibrio cholerae* is a natural inhabitant of the aquatic environment. However, its toxigenic strains can cause potentially life-threatening diarrhea. A quadruplex real-time PCR assay targeting four genes, the cholera toxin gene (*ctxA*), the hemolysin gene (*hlyA*), O1-specific *rfb*, and O139-specific *rfb*, was developed for detection and differentiation of O1, O139, and non-O1, non-O139 strains and for prediction of their toxigenic potential. The specificity of the assay was 100% when tested against 70 strains of *V. cholerae* and 31 strains of non-*V. cholerae* organisms. The analytical sensitivity for detection of toxigenic *V. cholerae* O1 and O139 was 2 CFU per reaction with cells from pure culture. When the assay was tested with inoculated water from bullfrog feeding ponds, 10 CFU/ml could reliably be detected after culture for 3 h. The assay was more sensitive than the immunochromatographic assay and culture method when tested against 89 bullfrog samples and 68 water samples from bullfrog feeding ponds. The applicability of this assay was confirmed in a case study involving 15 bullfrog samples, from which two mixtures of nontoxigenic O1 and toxigenic non-O1/non-O139 strains were detected and differentiated. These data indicate that the quadruplex real-time PCR assay can both rapidly and accurately detect/identify *V. cholerae* and reliably predict the toxigenic potential of strains detected.

Occasional outbreaks and pandemics caused by the bacterium *Vibrio cholerae* indicate that cholera is still a global threat to public health (1, 2, 6, 13, 14). The disease may become life-threatening if appropriate therapy is not undertaken quickly. Of the more than 200 serogroups of *V. cholerae* that have been identified (28), two serogroups, O1 and O139, cause epidemic and pandemic cholera (14), whereas non-O1, non-O139 serogroups are associated only with sporadic, isolated outbreaks of diarrhea (3, 23). O1 and O139 strains are also categorized as toxin-producing and non-toxin-producing strains. The toxin-producing strains cause life-threatening secretory diarrhea, while the non-toxin-producing isolates elicit only mild diarrhea. These differences among the serogroups of *V. cholerae* demand rapid diagnostic tests capable of both distinguishing O1 and O139 from other serogroups and differentiating toxin-producing from nonproducing isolates (20).

PCR has become a molecular alternative to culture, microscopy, and biochemical testing for the identification of bacterial species (27). Many PCR methods have been developed for characterization of serogroups (O1 and/or O139), biotypes, and the toxigenic potential of *V. cholerae* strains (7, 11, 15, 19, 21, 22, 24–26). However, these conventional PCR methods require gel electrophoresis for product analysis and are therefore not suitable for routine use due to the risk of carryover contamination, low throughput, and intensive labor.

Real-time PCR allows detection of amplification product accumulation through fluorescence intensity changes in a closed-tube setting, which is faster and more sensitive than conventional PCR and has become increasingly popular in clinical microbiology laboratories. Moreover, when multicolor fluorophore-labeled probes and/or melting curve analysis is used, multiplex real-time PCR can be designed to simultaneously detect many different target genes in a single reaction tube (8). So far, the majority of published real-time PCR assays for *V. cholerae* detect no more than two genes simultaneously (4, 8, 18), which precludes their use for simultaneous serogroup and toxin status determination. Recent reports show that multiplex real-time PCR greatly improves specificity and sensitivity for the detection of *V. cholerae* through either melting curve analysis (9) or using differently fluorophore-labeled probes (10).

In the present work, we report the development of a quadruplex real-time PCR assay that enables simultaneous serogroup differentiation and toxigenic potential detection. By using four different fluorophore-labeled probes, which target *hlyA*, O1-specific *rfb*, O139-specific *rfb*, and *ctxA*, the quadruplex assay can reveal whether the target is an O1, O139, or non-O1/non-O139 strain and whether the bacterium detected is capable of producing toxins. We report that by alleviating primer dimer formation by use of a homotag-assisted non-dimer system (HANDS) (5), we were able to retain the analytical sensitivity of uniplex PCR and successfully differentiated
and immune agglutination and stored at Xiamen CDC, were identified by colony morphology, biochemical properties, through routine surveillance by the Xiamen Centre of Diseases and Presentation the specificity of the quadruplex real-time PCR assay are tabulated according to

by conventional PCR, using strains of containing 25% glycerol. The identities of these reference strains were confirmed

10 00 0
30 00 0

Enterococcus faecalis
Pseudomonas aeruginosa
10 00 0
10 00 0
Proteus mirabilis
30 00 0
E. coli
Enterotoxigenic O157:H7
Serovar Typhi
Vibrio vulnificus
Vibrio mimicus
20 00 0
20 00 0
Vibrio cholerae

34 00 0
33 34
Vibrio cholerae
Vibrio parahaemolyticus
Vibrio mimicus
Vibrio fluvialis
Vibrio vulnificus
Shigella flexneri
Salmonella enterica serovar Typhi
Staphylococcus aureus
Escherichia coli O157:H7
Enteropathogenic E. coli
Enterotoxigenic E. coli
Aeromonas hydrophila
Aeromonas fæcalis
Proteus mirabilis
Proteus vulgaris
Pseudomonas aërginosa
Enteroccoccus fæcalis
Streptococcus haemolyticus

serogroups and toxigenic potentials from aquatic animal and environmental samples.

MATERIALS AND METHODS

Bacterial strains and DNA template preparation. Bacterial strains used to test the specificity of the quadruplex real-time PCR assay are tabulated according to species in Table 1. These strains, isolated from patients and food products through routine surveillance by the Xiamen Centre of Diseases and Presentation (Xiamen CDC), were identified by colony morphology, biochemical properties, and immune agglutination and stored at ~70°C in Luria-Bertani (LB) medium containing 25% glycerol. The identities of these reference strains were confirmed by conventional PCR, using strains of V. cholerae classical 569B, El Tor N16961, and O139 MO45 (ATCC 51394) as reference standards. The ctxA-positive strains were identified by a routine rabbit ileal loop assay, which detects the presence of cholera toxin by observation of fluid accumulation.

For template DNA preparation, the bacteria were first cultured in LB medium at 37°C overnight. Cells in 1-ml aliquots were harvested by centrifugation (13,000 × g for 2 min), resuspended in 100 μl of water, and lysed by heating at 100°C for 10 min. The lysed cells were snap-cooled on ice, and, after centrifugation at 13,000 × g for 8 min, the supernatant fluid for each aliquot was collected and stored at ~20°C until directly used as crude template DNA for PCR. The crude template DNA prepared as described above was used throughout this work, unless otherwise indicated. Purified template DNA was prepared using an AsyPrep bacterial genomic DNA miniprep kit (Axogen Biosciences, Union City, CA) according to the manufacturer's instructions. This DNA purification procedure was used exclusively for the analytical sensitivity study using toxigenic O1 and O139 strains.

Primers and probes. Four genes, including the cholera toxin gene (ctxA), the hemolysin gene (hlyA), O1-specific rfb, and O139-specific rfb, were chosen as targets. Primers and probes were designed using Premier Primer 5.0 (Premier Biosoft International, Palo Alto, CA). According to the principle of HANDS (5), all of the primers had a common tag sequence at their 5'-ends that served to generate a universal primer binding sequence, and the tag was used as the universal primer. The tag sequence was selected for lack of homology to any known bacterial genome by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The four probes of stem-shared molecular beacons (17) were labeled with different fluorophores, as indicated in Table 2. This arrangement allowed the detection and differentiation of general V. cholerae (ROX fluorescence only), nontoxigenic O1 V. cholerae (both ROX and 6-carboxyfluorescein [FAM] fluorescence), nontoxigenic O139 V. cholerae (both ROX and Cy5 fluorescence), toxigenic O1 V. cholerae (ROX, Cy5, and HEX fluorescence), and their mixture (ROX, FAM, HEX, and Cy5 fluorescence). All primers and modified molecular beacons were synthesized and purified, via polyacrylamide gel electrophoresis, by Sangon (Shanghai, China).

Quadruplex real-time PCR assay. Real-time PCR was performed on an Mx3005P detection system (Stratagene, La Jolla, CA). The 25-μl reaction mixture contained 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 5% glycerol, pH 8.6), 1 U Tag DNA polymerase, 3.0 mM MgCl2, a 300 μM concentration of each deoxynucleoside triphosphate, 0.12 μM universal primer identical to the common tag sequence of the HANDS primer (Table 2), 0.05 to 0.2 μM primer pairs for each amplicon, 0.05 to 0.2 μM of each differently labeled probe (Table 2), and 5 μl of template DNA, prepared by either simple heating lysis or an AsyPrep kit. The amplification procedure consisted of a preliminary denaturation step at 95°C for 3 min; 10 cycles of 95°C for 20 s (with a 1°C decrease for each cycle), 58°C for 20 s, and 72°C for 2 min.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains</th>
<th>No. of positive samples</th>
<th>O1 rfb</th>
<th>O139 rfb</th>
<th>ctxA</th>
<th>hlyA</th>
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<td>Vibrio cholerae O1</td>
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<td>Enteropathogenic E. coli</td>
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</table>

a Reference strains representing non-O1, non-O139 serogroups.

<table>
<thead>
<tr>
<th>Target and primer or probe</th>
<th>Sequence (5'→3')a</th>
<th>Conc (μM)</th>
<th>Amplicon size (bp)</th>
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<td>O1.rfb</td>
<td>Tag-CCAGGTTGTAAGGACTGC</td>
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<td>203</td>
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<td>O1-F</td>
<td>Tag-GGTCTAGAGTTCAACA</td>
<td>0.06</td>
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<td>O1-R</td>
<td>FAM-CCCCGGATTTGTAAGCCCAAATCTACCGGG-Dabcyl</td>
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<tr>
<td>O139.rfb</td>
<td>Tag-CATACCAACGCCCCTATCATCATT</td>
<td>0.02</td>
<td>160</td>
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<tr>
<td>O139-F</td>
<td>Tag-GCATGACGCTGCAATCCAAAAAT</td>
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<tr>
<td>O139-R</td>
<td>Cy5-CCGGTTGAGAAAAAGACAGCAATAACCCCG-Dabcyl</td>
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<tr>
<td>ctxA</td>
<td>ctcAG-F</td>
<td>Tag-TCCGGAGCATAGAGCTTGGA</td>
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<tr>
<td>ctcA-R</td>
<td>Tag-TCCGGAGCATAGAGCTTGGA</td>
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<tr>
<td>ctcA-P</td>
<td>HEX-CCTGCGAGATCATGCAAGCCGACCG-Dabcyl</td>
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<tr>
<td>hlyA</td>
<td>hlyA-R</td>
<td>Tag-ACCTGGATTATCTGCAGTTTG</td>
<td>0.04</td>
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<tr>
<td>hlyA-F</td>
<td>Tag-CGCTTTATTTTTCATGGACTGTTTA</td>
<td>0.06</td>
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<tr>
<td>hlyA-P</td>
<td>ROX-CCCCGATATACTGGCGAATGCATCGGGG-Dabcyl</td>
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<tr>
<td>HANDS tag</td>
<td>GCAAGCGCTACGATCCGAA</td>
<td>1.2</td>
<td></td>
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</table>

a Underlined nucleotides indicate the stem sequence of each molecular beacon. Bases in bold are foreign nucleotides introduced to the target-specific sequences.
Analysis of environmental samples. In total, 89 bullfrog samples and 68 water samples from the bullfrog feeding ponds were collected over a period of 4 months by Xiamen CDC. Fifty milliliters of 10× APW was added to 450 ml of pond water samples collected in 500-ml jars and thoroughly mixed. Bullfrogs were first anesthetized with ether, and the intestinal tracts with content inside were then recovered and cut into small pieces with sterile scissors in a 100-ml jar containing 50 ml APW. After a thorough vortex step, enrichment was carried out at 37°C overnight. The culture was first screened by an immunochromatographic assay (ICA) against serogroups O1 and O139. Those samples that tested positive were streaked on thiosulfate-citrate-bile salt-sucrose agar plates, and the suspected colonies were confirmed with serological agglutination and biochemistry testing. In parallel, each culture was detected with quadruplex real-time PCR.

RESULTS

Validation of quadruplex assay. The quadruplex real-time PCR assay was first examined with 70 strains of V. cholerae and 31 strains of other bacterial species. All V. cholerae strains were correctly identified, and no false-positive result was obtained with non-V. cholerae strains (Table 1). Among the 70 strains of V. cholerae, 25 were V. cholerae O1, including 4 (16%) ctxA-containing strains; 34 were O139 strains, of which 33 (97%) were ctxA-containing strains; and 11 were non-O1, non-O139 strains, none of which contained ctxA. Thus, the quadruplex real-time PCR assay achieved 100% specificity.

To evaluate the analytical sensitivity of the quadruplex assay, DNAs from both O1 and O139 ctxA-positive strains, ranging from 2 to 2 × 10^6 CFU equivalents per reaction, were tested. Quantitative detection was achieved in the entire range of template DNA concentrations, with the average R² being >0.999 (Fig. 1). The limit of detection was 2 CFU per reaction, which was equal to the sensitivity of the corresponding uniplex real-time PCR test (data not shown).

To determine the applicability of the quadruplex real-time PCR assay to the detection of V. cholerae in artificially made environmental samples, we added ctxA-containing O1 and O139 cells to water collected from bullfrog feeding ponds. For both toxigenic O1 and O139 strains, samples with an initial inoculation of 10^3 CFU/ml or higher were detected successfully, even without culturing. If the samples containing 10^2 CFU/ml were cultured for 2 h or longer, three of three samples were detected (data not shown). When the culture time was
shortened to 1 h, two of three samples were detected; without culturing, one of three samples scored positive. With samples of 10^3 CFU/ml, a 4-h incubation allowed 3/3 samples to be detected; detection was 2/3 samples with 2 to 3 h of culture and 1/3 samples after culture for 1 h. Samples of 10^6 CFU/ml could be detected repeatedly after culturing for 6 h or longer. This detection sensitivity was similar to that observed for detection of toxigenic O1 and O139 strains grown in pure culture media.

**Application to environmental samples.** We used our assay with actual environmental samples (89 bullfrog samples and 68 water samples from bullfrog feeding ponds). The quadruplex real-time PCR assay showed that 57/89 bullfrog samples contained nontoxigenic O1 strains, and 20/89 samples contained non-O1, non-O139 *V. cholerae*. ICA indicated that 53/89 samples were O1 strains, and culturing results showed that only 13/89 samples were O1 strains. Of the 68 water samples, the quadruplex real-time PCR assay revealed that 42/68 samples contained nontoxigenic O1 strains and 16 were positive for non-O1, non-O139 strains. The ICA test showed that 38 samples were O1 strains, and the culture test indicated that only 23/68 samples were O1 strains. All samples that were negative by the quadruplex real-time PCR assay were also negative by both ICA and culture methods. These results demonstrated that the quadruplex real-time PCR assay was more sensitive than the ICA and culture methods for detecting *V. cholerae* in environmental samples.

**Application in a case study.** In May 2008, more than 20 people suffered diarrhea after eating in a restaurant in Xiamen, China. An epidemiological investigation linked ingestion of bullfrog or shellfish to the epidemic. *Vibrio parahaemolyticus* was isolated from the patient fecal samples, and nontoxigenic *V. cholerae* O1 was detected in bullfrogs collected from the restaurant, using the quadruplex real-time PCR assay. The bullfrog samples were then traced back to a particular bullfrog feeding pond. Fifteen bullfrog samples from the pond were subjected to analysis by both quadruplex real-time PCR and the ICA. Eight of the 15 bullfrog samples were negative for *V. cholerae*, 5 were positive for nontoxigenic O1 strains (both ROX and FAM fluorescence from PCR), and 2 were positive for O1 *rfb*, ctxA, and hly (ROX, FAM, and HEX fluorescence from PCR). The results for the last two samples could be interpreted as coming from either a single clone of *V. cholerae* or mixed clones of *V. cholerae* harboring these three genes. The two samples were then streaked on thiosulfate-citrate-bile salt-sucrose agar plates after enrichment. When the colonies were analyzed individually by the quadruplex real-time PCR assay, the samples were found to be mixtures of nontoxigenic O1 and toxigenic non-O1, non-O139 strains. These observations established the usefulness of the quadruplex real-time PCR assay in both differentiating various strain types of *V. cholerae* and revealing the toxigenic potential of the strains detected.

**DISCUSSION**

We successfully developed a quadruplex real-time PCR assay for *V. cholerae* that was able to simultaneously distinguish O1 and O139 strains from non-O1, non-O139 strains and to determine whether the bacteria detected have the potential to produce toxins (e.g., contain toxin genes) in a single reaction tube. This assay robustly detected all *V. cholerae* serogroups of interest, with as little as 2 CFU per reaction, by taking advantage of primer dimer elimination using HANDS. The usefulness of this assay was confirmed by a reconstruction study in which *V. cholerae* was added to bullfrog feeding pond water and by a case study.

Rapid identification and discrimination of serogroups of *V. cholerae* are critical for treatment and prevention of the spread of cholera. Consequently, many studies have used multiplex PCR for this aim. By targeting *wbe* (O1), *wbf* (O139), and ctxA, a triplex PCR assay has differentiated O1 and O139 strains and their toxigenic potential (15). A septaplex PCR assay has been established to distinguish pathogenic, toxin-positive strains from nonpathogenic, toxin-negative *V. cholerae* serogroups, both in the environment and in clinical settings (26). A quadruplex PCR has also been developed for the simultaneous detection of genes specific for *Vibrio cholerae* O1 and/or O139 (*wbe* and/or *wbf*), the cholera toxin A subunit (ctxA), toxin-coregulated pilus (tcpA), and central regulating protein ToxR (toxR) in a single-tube reaction (16). However, these conventional PCR-based assays need to use gel electrophoresis to separate PCR products for postamplification analysis, which has a low throughput, is labor-intensive, and is susceptible to carryover contamination. By eliminating the postamplification gel electrophoresis step with real-time PCR, our quadruplex assay reduces the risk of contamination, increases assay throughput, and shortens the testing time. In our method, the four target genes were chosen to attain high specificity and sensitivity. The *V. cholerae* species-specific gene *hlyA* provides species information for taxonomic identification. The ctxA gene, encoding cholera toxin, is the most important determinant of the toxigenic potential of a *V. cholerae* strain. With O1 *rfb* and O139 *rfb* for *V. cholerae* serogroup differentiation, the four target genes proved to be a suitable combination for simultaneous differentiation of the two major outbreak serogroups (O1 and O139) from other types of *V. cholerae* and for toxigenic potential determination. For routine clinical screening, we are considering the addition of an internal positive control to indicate inhibition in the reaction. Such a system, however, requires the use of a five-color-channel instrument (e.g., ABI 7500 or Stratagene 3005P instrument) that is not yet widely available in microbiology laboratories.

It is feasible to upgrade any conventional multiplex PCR to its corresponding multiplex real-time PCR once a multicolor-channel PCR instrument is available. Such an upgrade requires overcoming problems such as decreased amplification efficiency caused by primer dimer formation. To this end, tedious experimental optimizations are often needed, without the assurance of success. In our previous work on an octuplex real-time PCR assay, we observed a 100- to 1,000-fold lower sensitivity than that seen with uniplex PCR (12). By taking advantage of HANDS (5), we achieved the same high sensitivity in the quadruplex real-time PCR as in the uniplex PCR for each target gene. HANDS was originally developed to alleviate primer dimer formation in multiplex PCR. According to the principle of HANDS, a common tag sequence is added to the 5′ ends of all primers, and the same tag is also used as a universal primer in the multiplex PCR system. Thus, any primer dimer generated at the beginning of PCR is converted into a stable hairpin structure, which prevents further replication by the universal tag primer during PCR. Adoption of
HANDS should facilitate the setup of other multiplex real-time PCR systems.

For validation of the quadruplex real-time PCR, we chose bullfrog samples and water samples from bullfrog feeding ponds due to the long and sustained high prevalence of *V. cholerae* in bullfrogs in Xiamen. Since the first isolation of *V. cholerae* from bullfrogs in 1996, we have continuously monitored *V. cholerae* in both bullfrogs and bullfrog feeding ponds. Surveillance showed that the positive rates of *V. cholerae* O1 in these samples were significantly higher than in other environmental niches. For example, an annual surveillance for *V. cholerae* O1 in three bullfrog feeding ponds between May 2007 and April 2008 showed positive rates of 28.5% and 20.8% for the bullfrogs and the pond water, respectively. These values are much higher than those for any other environmental samples in the same local area. So far, no toxigenic *V. cholerae* strain has been isolated.

The finding of mixtures of nontoxigenic O1 and toxigenic non-O1, non-O139 strains in the case study demonstrated the advantage of combining the quadruplex real-time PCR detection system with single-colony isolation. While the quadruplex real-time PCR assay was robust in differentiating serogroups of *V. cholerae* and their toxigenic potential, it could not firmly establish whether the target genes were in one bacterial strain or in a mixture of strains. As shown in the case study, after single-colony isolation, the two toxigenic O1-positive samples proved to be mixtures of nontoxigenic O1 and toxigenic non-O1, non-O139 strains. Therefore, when enriched samples are positive for more than one gene, additional single-colony-based quadruplex real-time PCR assays are needed for clarification. The combined assays provided unequivocal conclusions.

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