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A Novel Marker for the Species-Specific Detection and Quantitation of *Vibrio cholerae* by Targeting an Outer Membrane Lipoprotein *lolB* Gene

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***Vibrio cholerae* O1 and O139 are the major serotypes associated with illness, and some *V. cholera* non-O1 and non-O139 isolates produce cholera toxin. The present study describes a quantitative polymerase chain reaction (qPCR) assay for the species-specific detection and quantitation of *V. cholera* using a primer pair based on an outer membrane lipoprotein *lolB* gene for the amplification of a 195 bp DNA fragment. The qPCR primer set for the accurate diagnosis of *V. cholera* was developed from publically available genome sequences. This quantitative PCR-based method will potentially simplify and facilitate the diagnosis of this pathogen and guide disease management.**

Key words: Detection, diagnosis, quantitation, outer membrane lipoprotein *lolB*, *Vibrio cholera*

Cholera is responsible for an estimated 3–5 million cases, and over 120,000 deaths occur each year around the world [3, 12]. Many *V. cholerae* O1 strains isolated from the environment do not produce cholera toxin (CT), nor do they possess the genetic potential to produce CT, whereas some *V. cholerae* non-O1 strains produce CT [13]. *V. cholerae* O1 and O139 are the major seroytpes associated with illness, and some *V. cholerae* non-O1 and non-O139 isolates produce CT [10]. These findings require regular inspection of *V. cholerae* isolates for their ability to produce CT in order to assess their clinical significance [15]. They also need the preparation for the emergence of *V. cholerae* as a new serogroup as a result of horizontal gene transfer among serogroups.

Currently, serological and molecular assays based on the genes of hemolysin (*hlyA*), toxin co-regulated pilus (*tcpA*), cholera enterotoxin A subunit (*ctx*A), chaperonin GroEL (*groEL*), cholera toxin transcriptional activator (*toxR*), RTX toxins and related Ca^{2+}-binding proteins (*rtxA*), and heat-stable enterotoxin (*st*) are widely used for the detection of *V. cholera* strains, but there have been critical defects in the diagnosis and identification of all isolates of *V. cholera*, in that these assays also detect other *Vibrio* species [4–9, 11, 12, 14, 15] (Table 1). Therefore, it is essential to establish an accurate, rapid, sensitive, and practical method for the specific detection and quantification of the pathogen.

In recent years, there has been great increase in the number of microbial genome sequences available, as a consequence of the rapid development of technology in the area of high-throughput sequencing. These sequencing efforts have resulted in complete genome sequences for hundreds of microorganisms that represent a wide range of taxonomic diversity, allowing for a sound signature prediction capability [1].

In this study, we compared the use of a bioinformatics analysis approach to search the available *V. cholerae* genome database in the NCBI (http://www.ncbi.nlm.nih.gov/). Furthermore, we found the specific sequence region by sequence alignment search of the outer membrane lipoprotein (*lolB*) gene of *V. cholerae* using BLAST and e-PCR analysis. The BLASTN searches showed similarity to the outer membrane lipoprotein *lolB* sequences (GenBank Accession No. CP002377, region 1192922–1193536) from *V. furnissii* NCTC 11218, and the *lolB* sequences (GenBank

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Table 1. The primer specificity test results by nucleotide BLAST search.

Organism/Name	Detection of target gene							
	hlyA[a]	*tcpA*[b]	*ctxA*[c]	*groEL*[d]	*toxR*[e]	*rtxA*[f]	*st*[g]	This study[h]
Vibrio cholerae IEC224	+[i]	+	+	+	+	+	-	+
Vibrio cholerae MJ-1236	+	+	+	+	+	+	-	+
Vibrio cholerae O1 biovar El Tor str. N16961	+	+	+	+	+	+	-	+
Vibrio cholerae O1 str. 2010EL-1786	+	+	+	+	+	+	-	+
Vibrio cholerae O395	+	+	+	+	+	+	-	+
Vibrio cholerae M66-2	+	+	-	+	+	+	-	+
Vibrio cholerae LMA3984-4	-	-	-	-	-	+	-	+

[a]Reference: [4, 14]. [b]Reference: [7]. [c]Reference: [8, 15]. [d]Reference: [11]. [e]Reference: [9]. [f]Reference: [5]. [g]Reference: [6].
[h]Outer membrane lipoprotein *lolB* gene of *V. cholerae* O1 biovar eltor str. N16961. Positions VC195F/R correspond to GenBank Accession No. AE003852.1, region 2326288–2326923, protein ID AAF95326.1.
[i]*In silico* +, gene detected; -, not detected.

Accession No. CP002284; Region 1049039–1049671) from *V. anguillarum* 775. BLAST searches with the predicted protein sequence (BLASTX) revealed the closest similarity to the outer membrane lipoprotein LolB protein from *V. mimicus* SX-4 (GenBank Accession No. EGU21125.1). However, the region to be amplified with the designed primer pair revealed no significant match with either of the BLASTN searches.

The specificity of the primer set based on the outer membrane lipoprotein *lolB* gene of *V. cholerae* O1 biovar eltor str. N16961 (GenBank Accession No. AE003852.1, region 2326288-2326923, protein ID AAF95326.1) was analyzed *in silico* by a similarity search against the NCBI BLAST sequence database (http://www.ncbi.nlm.nih.gov/). There were no significant matches with previously determined sequences. The primer set was VC195F (5'-CCGTTGAGG CGAGTTTGGTGAGA-3') and VC195R (5'-GTGCGCGGG TCGAAACTTATGAT-3'), which generated specific amplicons of 195 bp.

The specificity of the VC195F/R primer set in conventional and qPCR assays was demonstrated by testing a total of 34 reference bacterial species (Table 2). All of the bacterial strains were obtained from the American Type Culture Collection in the United States, the Belgian Coordinated Collections of Micro-organisms in Belgium, the Korean Culture Center of Microorganisms, the Korean Collection for Type Cultures, and the National Culture Collection for Pathogens in the Republic of Korea.

Each of the culture media and incubation conditions used were in accordance with the *Handbook of Microbiological Media* [2]. The genomic DNA from bacterial strains was prepared using a DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. All of the amplifications were performed with approximately 50 ng of genomic DNA, the VC195F/R primers (0.5 μM final concentration), and GoTaq DNA polymerase (1.25 U final concentration; Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. The amplifications were performed using a PTC-225 thermocycler (MJ Research, Watertown, MA, USA) with the following cycling conditions: an initial denaturation of 5 min at 95°C; 35 cycles of 1 min at 95°C, 30 s at 57°C, and 1 min at 72°C; and a final extension of 7 min at 72°C. Each amplified product was separated on 1.5% agarose gel by electrophoresis at 100 V for 60 min, in 1× TAE buffer, and then stained with ethidium bromide and visualized on an UV transilluminator and imaged using a VersaDoc 1000 gel imaging system (Bio-Rad Laboratories, Inc., USA).

Amplifications were performed with approximately 5 ng of purified DNA of each sample, the primer set, and the SYBR Premix *Ex Taq* (Takara Bio, Inc., Japan), according to the manufacturer's instructions. The qPCR amplifications were performed using a CFX96 real-time PCR system (Bio-Rad) and the following cycling conditions: an initial denaturation of 3 min at 95°C, 45 cycles of 10 s at 95°C and 20 s at 57°C, and a melting curve at 65°C to 95°C, with an increment of 0.5°C per cycle. The determination of the cycle threshold (C_T) and the data analysis were automatically performed by the CFX Manager Software system (ver. 1.6; Bio-Rad). The specificity of the VC195F/R primer set was determined with both conventional and SYBR Green qPCR analyses (Fig. 1 and Table 2). As expected, a 195 bp DNA fragment was amplified with conventional PCR (Fig. 1), and the amplification plot and a unique dissociation peak at 81.50°C were observed with the qPCR assay (data not shown).

For the analysis of the limit of quantification (LOQ) and the limit of detection (LOD), cloned DNA, genomic DNA, and a cell suspension of *V. cholerae* ATCC 16961 were serially diluted 10-fold and tested with SYBR Green qPCR (Table 3). The PCR fragment of 195 bp was ligated into pGEM-T easy cloning vector by TA cloning (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The copy number of the cloned DNA was

Table 2. Bacterial strains used in the PCR specificity test.

No.	Bacterial strains	Source	This study[a]
1	*Vibrio cholerae*[T]	ATCC 16961	+[b]
2	*Vibrio cholerae*	CO-018[a]	+
3	*Vibrio parahaemolyticus*	KCCM 41664	-
4	*Vibrio parahaemolyticus*	ATCC 33844	-
5	*Vibrio vulnificus*[T]	KCCM 41665	-
6	*Vibrio vulnificus*	ATCC 33815	-
7	*Vibrio aestuarianus*[T]	KCCM 40863	-
8	*Vibrio alginolyticus*[T]	KCCM 40513	-
9	*Vibrio algoinfesta*	KCCM 40861	-
10	*Vibrio carchariae*[T]	KCCM 40865	-
11	*Vibrio campbellii*[T]	KCCM 41986	-
12	*Vibrio diazotrophicus*[T]	KCCM 41666	-
13	*Vibrio fluvialis*[T]	KCCM 40827	-
14	*Vibrio harveyi*[T]	KCCM 40866	-
15	*Vibrio mediterranei*[T]	KCCM 40867	-
16	*Vibrio mimicus*[T]	KCCM 42257	-
17	*Vibrio natriegens*[T]	KCCM 40868	-
18	*Vibrio ordalii*[T]	KCCM 41669	-
19	*Vibrio proteolyticus*[T]	KCCM 11992	-
20	*Vibrio salmonicida*[T]	KCCM 41663	-
21	*Shigella sonnei*[T]	KCCM 40949	-
22	*Shigella flexneri*[T]	KCCM 40948	-
23	*Shigella boydii*[T]	KCCM 41649	-
24	*Shigella dysenteriae*	NCTC 9952	-
25	*Escherichia coli* O1:K1:H7[T]	LMG 2092	-
26	*Escherichia coli* O157:H42	NCCP 14034	-
27	*Escherichia coli* O157:H7	LMG 21756	-
28	*Salmonella enterica* subsp. *enterica* Typhimurium[T]	KCTC 12456	-
29	*Salmonella enterica* subsp. *enterica* Paratyphi A	ATCC 9150	-
30	*Salmonella enterica* subsp. *enterica* Paratyphi B	ATCC 8759	-
31	*Salmonella enterica* subsp. *arizonae*	ATCC 13314	-
32	*Salmonella enterica* subsp. *diarizonae*	ATCC 43973	-
33	*Salmonella enterica* subsp. *indica*	ATCC 43976	-
34	*Legionella pneumophila* subsp. *pneumophila*[T]	KCCM 41777	-

ATCC, American Type Culture Collection, USA KCCM, Korean Culture Center of Microorganisms, Republic of Korea; KCTC, Korean Collection for Type Cultures, Republic of Korea; LMG, The Belgian Co-ordinated Collections of Microorganisms (BCCM), Belgium; NCCP, National Culture Collection for Pathogens, Republic of Korea.

[T]Type strain.

[a]Experiments to assess the specificity of real-time PCR assays.

[b]+, Detected; -, not detected.

calculated using the following equation [16]: copies/μl = [6.022 × 10^{23} (copy/mol) × amount (g)]/[length (bp) × 660 (g/mol/bp)]. All reactions were performed in triplicate.

The LOQ showed a good linear response and a high correlation coefficient (cloned DNA, $R^2 = 0.993$; genomic DNA, $R^2 = 0.950$), with a linear response in the concentration range for cloned and genomic DNA of 5 ng to 5 pg and the LOD of 5 fg/μl (fg per μl reaction mix) and 4.6 ×10^0 CFU/ml (CFU per ml reaction mix) of *V. cholerae*, respectively.

A standard curve analysis of the linear part of the slope resulted in a coefficient of -3.374, which yielded a PCR efficiency of 97.9%. Analysis of the melting temperature and melting peaks of *V. cholerae* with SYBR Green qPCR revealed a reproducible melting temperature of 83.50°C and specific peaks (data not shown).

In this study, the qPCR assay of *V. cholerae* showed excellent quantification characteristics and accurate detection. Considering the sensitivity, specificity, rapidity, and cost-

Fig. 1. Specific PCR amplification of the outer membrane lipoprotein *lolB* gene fragment of *Vibrio cholerae* using the VC195F/R primer set.
Ethidium bromide-stained agarose gel electrophoresis of PCR products. Lane M, size marker (1 kb DNA plus ladder; Gibco BRL); lanes 1–34 are described in Table 2; lane 35, distilled water.

Table 3. Mean C_T end-point fluorescence of 10-fold serial dilutions of *Vibrio cholerae* cloned DNA, genomic DNA, and a cell suspension determined with a real-time PCR assay.

Cloned DNA		Genomic DNA		Cell suspension	
Plasmid copies/µl reaction mix	$C_T \pm$ SD (n = 3)	Weight/µl reaction mix	$C_T \pm$ SD (n = 3)	CFU/ml reaction mix	$C_T \pm$ SD (n = 3)
1.42×10^9	17.22 ± 0.16	5 ng	16.99 ± 0.21	4.6×10^6	16.94 ± 0.46
1.42×10^8	20.42 ± 0.23	500 pg	20.22 ± 0.09	4.6×10^5	19.15 ± 0.13
1.42×10^7	23.71± 0.14	50 pg	23.46 ± 0.10	4.6×10^4	22.23 ± 0.12
1.42×10^6	27.20 ± 0.07	5 pg	26.83 ± 0.05	4.6×10^3	25.84 ± 0.16
1.42×10^5	30.57 ± 0.36	500 fg	30.32 ± 0.31	4.6×10^2	29.51 ± 0.10
1.42×10^4	34.07 ± 0.55	50 fg	34.17 ± 1.00	4.6×10^1	34.28 ± 1.11
1.42×10^3	37.40 ± 1.57	5 fg	39.77 ± 3.44	4.6×10^0	39.64 ± 3.62

effectiveness, this noble marker and qPCR method could simplify diagnoses and monitoring in the food, medical, and environmental industries.

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