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Molecular-Beacon Multiplex Real-Time PCR Assay for Detection of *Vibrio cholerae*

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A multiplex real-time PCR assay was developed using molecular beacons for the detection of *Vibrio cholerae* by targeting four important virulence and regulatory genes. The specificity and sensitivity of this assay, when tested with pure culture and spiked environmental water samples, were high, surpassing those of currently published PCR assays for the detection of this organism.

The continual wave of outbreaks and pandemics all over the world caused by the bacterium *Vibrio cholerae* is a steady reminder of the immense importance of cholera as a global threat and a major public health problem (28). The disease may become life-threatening if appropriate therapy is not undertaken quickly; hence, fast, accurate, and sensitive detection of this organism is of foremost importance.

The use of PCR as a reliable molecular-biology-based technology has been reported for the detection of a variety of organisms. Although a significant number of PCR detection assays have been reported for *V. cholerae*, these reports mostly describe conventional, time-consuming, and laborious methods of PCR product characterization (1, 2, 4–6, 8, 11–15, 17, 19, 21–26). Real-time PCR analysis enables the detection of reaction products through fluorescence, which is faster and more sensitive. However, published real-time PCR assays for *V. cholerae* are few (9, 10, 16) and have limitations in sensitivity or detect no more than two genes simultaneously. Molecular beacons (MB), due to their stable stem-and-loop structure, have been demonstrated to be significantly more specific than dyes such as SYBR green I and other types of probes. The assay described here utilizes MB for the highly sensitive detection of four important *V. cholerae* genes by multiplex real-time PCR.

This assay was developed through significant modification of our previously developed fourplex real-time PCR assay, which used SYBR green I for detection (10). Three of the four targets were taken from the previously described assay: *rtxA*, *epsM*, and *tcpA* (10). The fourth gene target, *ompW*, was incorporated to replace the *mshA* target. It has been proposed that all *V. cholerae* strains, both toxigenic strains and nontoxigenic environmental isolates, contain this conserved gene sequence (19). As previously reported, the exploitation of a 68-bp deletion in *tcpA* within classical biotypes could give an indication of the presence of the El Tor/O139 biotype (10). Collectively, the four unique gene targets cover a range of gene sequences essential for the virulence and survival of *V. cholerae*.

The 51 bacterial strains used in this study (Table 1) were grown, and the DNA template was prepared, as described previously (10). Tenfold serial dilutions with the equivalent of 1 to 1×10^5 CFU of *V. cholerae* and 1×10^5 CFU of all the other bacterial species were then added directly to the PCR mixtures in order to determine the sensitivity and specificity of the assay.

The primers for the newly incorporated *ompW* target (forward, AACATCCGTGGATTTGGCATCTG; reverse, GCTG GTTCCTCAACGCTTCTG) produced an amplicon of 89 bp and were used at a final concentration of 0.40 μM. The design and optimization of the other three primer pairs have been described previously (10). To enable simultaneous detection, each of the beacons was labeled with a different fluorophore (Table 2). Initially, each of the four primer pairs and molecular beacons was individually assessed. Following this, each individual assay was incorporated stepwise to form a single, optimized multiplex assay capable of the simultaneous real-time PCR detection of all four target sequences in a single reaction.

The results obtained for the analysis of all 51 strains using the developed multiplex PCR assay indicated 100% specificity for all of the *V. cholerae* strains examined (Table 1). The only exception was the presence of a weak fluorescent signal, indicating the presence of small amounts of amplified product, for the *ompW* sequence with the two *V. mimicus* strains. This signal, however, appeared late in the amplification protocol, and upon the addition of fewer cells (1×10^3 CFU), the signal was no longer detected, indicating that the amplified product was not specific. Since limited genetic sequence data are publicly available for *V. mimicus*, it is not possible to preclude the presence of a similar gene in this organism. The *rtxA*, *epsM*, and *ompW* gene targets were detected in all of the *V. cholerae* strains, and the El Tor-type *tcpA* gene target, as previously reported, was correctly detected only for the O1 El Tor and O139 strains (10). PCR analysis of the non-O1 isolate failed to generate a product for the El Tor-type *tcpA* target. However, this lack of detection could be due to the fact that this strain contained a different allele of the gene (3, 7, 18, 20).

The limit of detection of this fourplex assay, when tested by the addition of 10-fold serial dilutions of heat-lysed *V. cholerae* cells, was very low: the assay routinely detected as few as 5 CFU per reaction (Fig. 1). This sensitivity was good and in most cases significantly better than other described PCR detection limits for *V. cholerae* (1, 8, 11, 12, 16, 17, 24–26).

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TABLE 1. Bacterial strains assayed by molecular-beacon real-time PCR

Serogroup and strain	Source[a] (origin)	Detection[b] of the indicated gene by:							
		Single PCR (1×10^3 CFU)				Multiplex PCR (1×10^5 to 5 CFU)[c]			
		rtxA	*epsM*	*ompW*	*tcpA*	*rtxA*	*epsM*	*ompW*	*tcpA*
V. cholerae O1 classical									
11966	A (Bangladesh, 1987)	+	+	+	−	+	+	+	−
AA14041	A (Bangladesh, 1985)	+	+	+	−	+	+	+	−
Z17561	A (Bangladesh, 1985)	+	+	+	−	+	+	+	−
Z17561 *tcpA*::*kan* (*tcpA* mutant)	A (University of Adelaide)	+	+	+	−	+	+	+	−
0162	C (India)					+	+	+	−
162	C (India)					+	+	+	−
569B	C (Unknown)					+	+	+	−
569B-685RNM	C (Unknown)					+	+	+	−
35A3	C (Unknown)					+	+	+	−
111-V585R	C (Unknown)					+	+	+	−
95	C (India)					+	+	+	−
V. cholerae El Tor									
N16961	A (Unknown)	+	+	+	+	+	+	+	+
AA13993	A (Bangladesh, 1985)	+	+	+	+	+	+	+	+
H1	A (India, 1985)	+	+	+	+	+	+	+	+
H1 *tcpA*::*kan* (*tcpA* mutant)	A (University of Adelaide)	+	+	+	−	+	+	+	−
HP-51-1	C (Thailand, 1972)					+	+	+	+
BRL 7738	C (1966)					+	+	+	+
N107	C (Unknown)					+	+	+	+
VB1961	C (Unknown)					+	+	+	+
I-816	C (Unknown)					+	+	+	+
V. cholerae O139									
AI-1838	A (Bangladesh, 1993)	+	+	+	+	+	+	+	+
AI-1854	A (Bangladesh, 1993)	+	+	+	+	+	+	+	+
AI-1855	A (Bangladesh, 1993)	+	+	+	+	+	+	+	+
V. cholerae non-O1									
V. cholerae non-O1	B (Fairfield Hospital, Australia)					+	+	+	−
H II (nonagglutinating)[d]	C (Hong Kong, 1963)					+	+	+	−
Other *Vibrio* spp.									
V. anguillarum ATCC 19246[e]	ATCC					−	−	−	−
V. alginolyticus	B (MDU, Australia)					−	−	−	−
V. alginolyticus ATCC 17749	ATCC					−	−	−	−
V. campbellii ATCC 25920[e]	ATCC					−	−	−	−
V. fischeri[e]	B (Microtox kit strain 2000)					−	−	−	−
V. fluvialis	A (Unknown)					−	−	−	−
V. harveyi 179[e] (tentative name)	C (Unknown)					−	−	−	−
V. mimicus	A (Unknown)			+/−		−	−	+/−	−
V. mimicus	B (Fairfield Hospital, Australia)			+/−		−	−	+/−	−
V. natriegens NCMB 857[f]	NCMB					−	−	−	−
V. pagrus MIC-2[e]	C (*Pagrus auratus*)					−	−	−	−
V. parahaemolyticus ATCC 17802	ATCC					−	−	−	−
V. parahaemolyticus NCTC10884	NCTC					−	−	−	−
V. parahaemolyticus NCTC10885	NCTC					−	−	−	−
V. vulnificus	B (RCPA, QAP 1995:8:4)					−	−	−	−
V. vulnificus C7184	CDC					−	−	−	−
V. vulnificus C7184T	CDC					−	−	−	−
Other bacteria									
Bacillus subtilis ATCC 6051[f]	ATCC					−	−	−	−
Enterococcus faecalis 159905660	Pathcentre, Perth, Australia					−	−	−	−
Escherichia coli PA03M55679	Pathcentre, Perth, Australia					−	−	−	−
Klebsiella pneumoniae 106156559	Pathcentre, Perth, Australia					−	−	−	−
Pseudomonas aeruginosa PA03M2615	Pathcentre, Perth, Australia					−	−	−	−
Serratia marcescens 13023[f]	Pathcentre, Perth, Australia					−	−	−	−
Shigella sonnei ATCC 9290	ATCC					−	−	−	−
Staphylococcus aureus ATCC 9144	ATCC					−	−	−	−
Yersinia enterocolitica W22703	Melbourne University					−	−	−	−

[a] A, Stephen Attridge, University of Adelaide; B, Celia McKenzie, Royal Melbourne Institute of Technology; C, University of New South Wales; MDU, Microbiological Diagnostics Unit; RCPA, Royal College of Pathologists of Australasia Quality Assurance Program.
[b] Blank, not tested; +, specific amplified product detected; −, no amplified product detected; +/−, limited product detected.
[c] All *Vibrio* spp. other than *V. cholerae* and all other bacterial species were assessed using 10^6 CFU.
[d] Serotype unknown.
[e] Grown at 25°C.
[f] Grown at 30°C.

To determine the applicability of the multiplex assay to the detection of *V. cholerae* from a model environmental niche, five different environmental water samples were collected and analyzed by the multiplex PCR assay (10). Initial PCR analysis performed directly on the collected water samples indicated that no detectable levels of naturally occurring *V. cholerae* were present in these samples. PCR analysis was performed directly on spiked water samples containing 10, 10^2, or 10^3 CFU of *V.*

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TABLE 2. Molecular beacon probes used in this study

Target gene	Beacon	Sequence (5′–3′)[a]	Size (bp)	Fluorophore	Quencher	Concn[b] (μM) multiplex
rtxA	MBrtxA	CGCGATCACCAGAGCGCCAAGAAGTGACTCGTAGATCGCG	40	FAM[c]	Dabcyl	0.25
epsM	MBepsM	CGCGATGCCACCGACATCGTAACGCTCCGATCGCG	35	Texas Red	BHQ2	0.25
ompW	MBompW	CCGAAGAAACAACGGCAACCTACAAAGCTTCGG	33	Cy5	BHQ3	0.25
tcpA	MBtcpA	CGCGACGCTGAAACCTTACCAAGGCTGACCAAGTCGCG	38	Cy3	BHQ2	0.50

[a] Molecular beacons were designed using Beacon Designer (version 2.12) software from Premier Biosoft (Palo Alto, CA). Underlined nucleotides indicate the stem sequence of each molecular beacon. MBrtxA, MBepsM, and MBompW were synthesized by TIB MOLBIOL (Berlin, Germany). MBtcpA was synthesized by Proligo (Helios, Singapore).

[b] Remaining PCR constituents were 2 U of FastStart *Taq* DNA polymerase, 1× PCR buffer, 4 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (all from Roche Diagnostics, Laval, Quebec, Canada), and 2 μl of template DNA in a 25-μl final volume.

[c] FAM, 6-carboxyfluorescein.

cholerae or 10^5 CFU of the other *Vibrio* spp. (Table 3). Analysis of water samples spiked with the mixture of non-*V. cholerae Vibrio* spp. resulted in the detection of a weak amplification signal, indicating small amounts of the *ompW* amplified product, synonymous with the findings obtained when the assay was tested using pure heat-lysed *V. mimicus* cells. Analysis of the samples spiked with *V. cholerae* resulted in the detection of the bacteria at 10^3 CFU per reaction, except for the seawater sample, which possibly inhibited the reaction due to its high salt content. In comparison, this was a 10-fold improvement

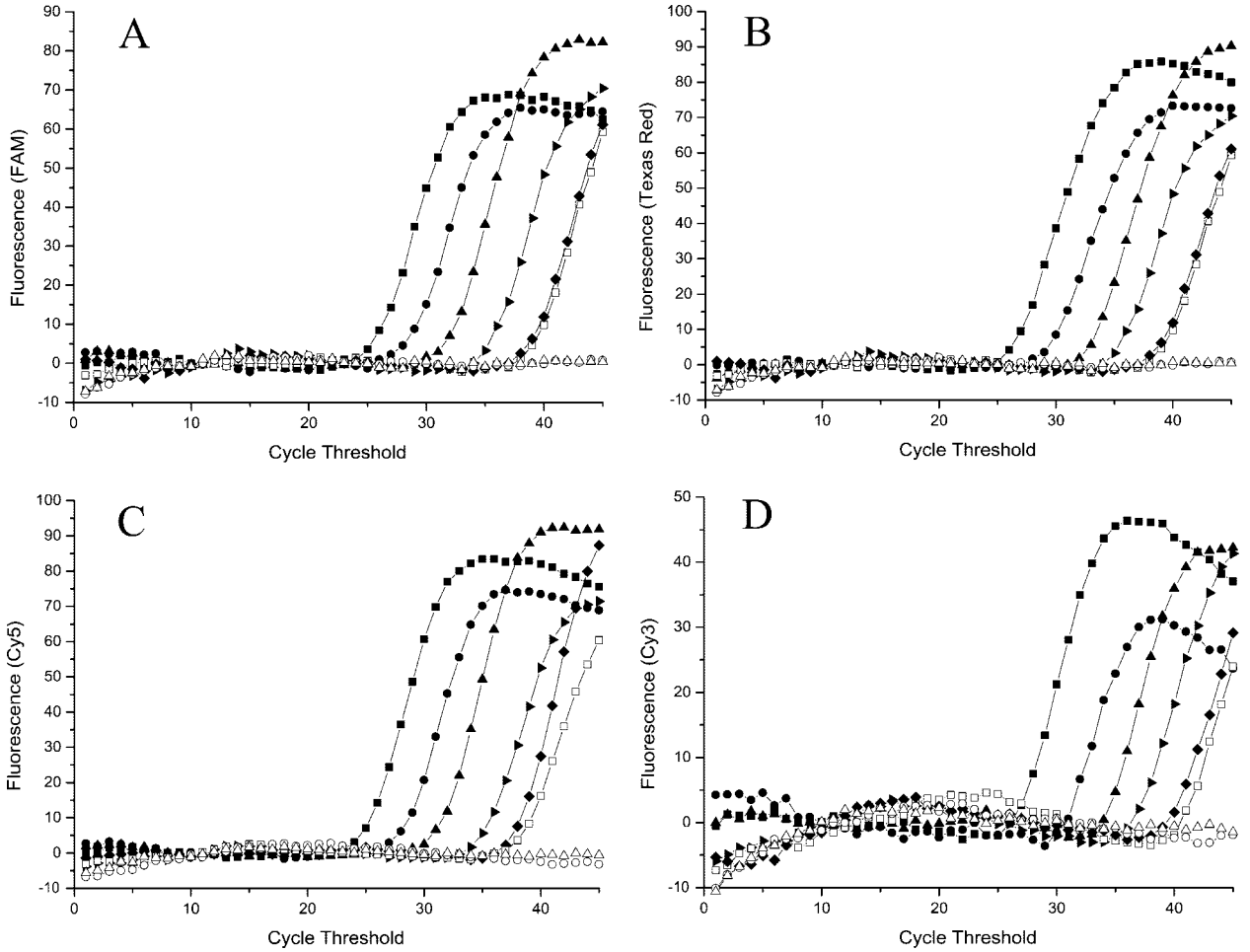

FIG. 1. Representative PCR amplification profile obtained from the fourplex real-time PCR analysis of products amplified from serially diluted heat-lysed *V. cholerae* AI-1838. All four targets—*rtxA* (A), *epsM* (B), *ompW* (C), and *tcpA* (D)—were detected simultaneously by the four different molecular beacons. To determine the limit of detection of the assay, the dilutions contained the following CFU of *V. cholerae*: 1×10^5 (■), 1×10^4 (●), 1×10^3 (▲), 1×10^2 (▶), 10 (◆), 5 (□), and 1 (○). △, negative control. The optimized multiplex PCR amplification profile consisted of 150 s at 95°C, followed by 45 cycles of three steps consisting of 30 s at 95°C, 60 s at 60°C, and 30 s at 72°C using the Smart Cycler (Cepheid, Sunnyvale, Calif.). Fluorescence signals emitted from the molecular beacon were measured at the end of each annealing step. Each analysis was repeated multiple times to ensure the reproducibility of results.

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TABLE 3. Fourplex detection of *V. cholerae* from spiked water samples

Source	Detection of *V. cholerae*[a] in:									
	Unprocessed samples (LOD, 10^3 CFU/reaction)					Isolated DNA (LOD, 10 CFU/reaction)				
	11966	N16961	AI-1839	*Vibrio*[b]	− C	11966	N16961	AI-1839	*Vibrio*	− C
Sea (Port Phillip Bay, Melbourne, Australia)	−	−	−	+/−	−	+	+	+	+/−	−
Estuarine (Yarra River, Melbourne, Australia)	+	+	+	+/−	−	+	+	+	+/−	−
River (Plenty River, Victoria, Australia)	+	+	+	+/−	−	+	+	+	+/−	−
Dam (South Morang, Victoria, Australia)	+	+	+	+/−	−	+	+	+	+/−	−
Commercial spring water	+	+	+	+/−	−	+	+	+	+/−	−

[a] LOD, limit of detection; − C, negative control (unspiked water sample); +, specific amplified product detected; −, no amplified product detected; +/−, limited product detected (*ompW* only).
[b] *Vibrio*, a mixture of *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, and *V. vulnificus* at 1×10^5 CFU of each strain per PCR.

over the 10^4-CFU limit of detection for the previously described SYBR green I assay (10). Upon the addition of 100 and 10 CFU of *V. cholerae*, the multiplex MB assay was capable of detecting the organism, although with some variability.

With the aim of increasing the sensitivity, DNA was extracted from the spiked water samples to remove inhibitory substances by using InstaGene Matrix (Bio-Rad) (10). Fourplex PCR analysis of this semipurified DNA resulted in the routine detection of as few as 10 *V. cholerae* CFU (lower dilutions were not assessed). This was a significant improvement over the previously described SYBR green I assay, which had a detection limit of 10^3 CFU per reaction (10). Several groups have similarly employed a DNA extraction step prior to PCR analysis of environmental water samples for *V. cholerae* (12, 15, 16, 23, 27). In comparison, a major advantage of the DNA extraction method used in this study is that it can be easily adapted to filter very large volumes of water. This can effectively provide an even greater capacity to detect low numbers of *V. cholerae* in large volumes of water.

Through the use of molecular beacons for the simultaneous detection of four target genes, the specificity and sensitivity of this assay surpass those of the published PCR assays for the detection of *V. cholerae*. The application of the assay to environmental water samples suggests that the assay could be used for the sensitive and cost-effective monitoring of environmental and drinking water samples. Importantly, this assay is the first to apply molecular beacons for the detection of *V. cholerae* and is the first fourplex molecular-beacon real-time PCR assay published for the detection of a single bacterial species.

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