Detection and Quantification of the Coral Pathogen *Vibrio coralliilyticus* by Real-Time PCR with TaqMan Fluorescent Probes†‡

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A real-time quantitative PCR-based detection assay targeting the *dnaJ* gene (encoding heat shock protein 40) of the coral pathogen *Vibrio coralliilyticus* was developed. The assay is sensitive, detecting as little as 1 CFU per ml in seawater and 10⁶ CFU per cm² of coral tissue. Moreover, inhibition by DNA and cells derived from bacteria other than *V. coralliilyticus* was minimal. This assay represents a novel approach to coral disease diagnosis that will advance the field of coral disease research.

*Vibrio coralliilyticus* has recently emerged as a coral pathogen of concern on reefs throughout the Indo-Pacific. It was first implicated as the etiological agent responsible for bleaching of corals in several Pacific reefs (14). *WS* is a collective term describing coral diseases characterized by a spreading band of tissue loss exposing white skeleton on Indo-Pacific scleractinian corals (2). More recently, *WS* has been identified as an emerging model pathogen for understanding the mechanisms linking bacterial infection and coral disease (13) and therefore provides an ideal model for the development of diagnostic assays to detect coral disease. Current coral disease diagnostic methods, which are based primarily upon field-based observations of macroscopic disease signs, often detect disease only at the latest stages of infection, when control measures are least effective. The development of diagnostic tools targeting pathogens underlying coral disease pathologies may provide early indications of infection, aid the identification of disease vectors and reservoirs, and assist managers in developing strategies to prevent the spread of coral disease outbreaks. In this paper, we describe the development and validation of a TaqMan-based real-time quantitative PCR (qPCR) assay that targets a segment of the *V. coralliilyticus* heat shock protein 40-encoding gene (*dnaJ*).

Nucleotide sequences of the *dnaJ* gene were retrieved from relevant *Vibrio* species, including *V. coralliilyticus* (LMG 20984), using the National Center for Biotechnology Information’s (NCBI) Entrez Nucleotide Database search tool (http://www.ncbi.nlm.nih.gov/). Gene sequences of strains not available in public databases (*V. coralliilyticus* strains LMG 21348, LMG 21349, LMG 21350, LMG 10953, LMG 20538, LMG 23696, LMG 23691, LMG 23693, LMG 23692, and LMG 23694) were obtained through extraction of total DNA using a Promega Wizard Prep DNA Purification Kit (Promega, Sydney, Australia), PCR amplification, and sequencing using primers and thermal cycling parameters described by Nhung et al. (8). A 128-bp region (nucleotides 363 to 490) containing high concentrations of single nucleotide polymorphisms (SNPs), which were conserved within *V. coralliilyticus* strains but differed from non-*V. coralliilyticus* strains, was identified, and oligonucleotide primers Vc_dnaJ_F1 (5′-CCG TTC GYG GTG TTT CAA AA-3′) and Vc_dnaJ_R1 (5′-AAC CTG ACC ATG ACC GTG ACA-3′) and a TaqMan probe, Vc_dnaJ_TMP (5′-6-FAM-CAG TGG CGC GAA G-MGBNFQ-3′; 6-FAM is 6-carboxyfluorescein and MGBNFQ is molecular groove binding nonfluorescent quencher), were designed to target this region. The qPCR assay was optimized and validated using DNA extracted from *V. coralliilyticus* isolates, non-target *Vibrio* species, and other bacterial species grown in marine broth (MB) (Table 1), under the following optimal conditions: 1× TaqMan buffer A, 0.5 U of AmpliTaq Gold DNA polymerase, 200 μM deoxynucleotide triphosphates (with 400 μM dUTP replacing deoxythymidine triphosphate), 0.2 U of AmpErase uracil N-glycosylase (UNG), 3 mM MgCl₂, 0.6 μM each primer, 0.2 μM fluorophore-labeled TaqMan, 1 μl of template, and sterile MilliQ water for a total reaction volume to 20 μl. All assays were conducted on a RotorGene 300 (Corbett Research, Sydney, Australia) real-time analyzer with the following cycling parameters: 50°C for 120 s (UNG activation) and 95°C for 10 min (AmpliTaq Gold DNA polymerase activation), followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 60 s (annealing/extension). During the annealing/extension phase of each thermal cycle, fluorescence was measured in the FAM channel (470-nm excitation and 510-nm detection).

The qPCR assay specifically detected 12 out of 13 isolated *V. coralliilyticus* strains tested in this study (Table 1). The exception was one Caribbean strain (C2), which failed to give specific amplification despite repeated attempts. Positive detection of the target gene segment was determined by the increase in fluorescent signal beyond the fluorescence threshold value (normalized fluorescence, 0.010) at a specific cycle, referred to

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† Supplemental material for this article may be found at http://aem.asm.org/.

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TABLE 1. Species, strain, and threshold cycle for all bacterial strains testeda

<table>
<thead>
<tr>
<th>Species</th>
<th>Strainb</th>
<th>Origin</th>
<th>Host organism</th>
<th>C_T ± SEMc</th>
<th>dnaJ gene sequence accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio coralliilyticus</td>
<td>LMG 23696</td>
<td>Nelly Bay, Magnetic Island, Australia</td>
<td>Montipora aequituberculata</td>
<td>12.43 ± 0.20</td>
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<td>LMG 23691</td>
<td>Majuro Atoll, Republic of Marshall Islands</td>
<td>Acropora cytherea</td>
<td>14.07 ± 1.33</td>
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<td>LMG 23693</td>
<td>Nikko Bay, Palau</td>
<td>Pachysira speciosa</td>
<td>10.83 ± 2.76</td>
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<td>LMG 23692</td>
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<td>Pachysira speciosa</td>
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<td>LMG 23694</td>
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<td>LMG 10953</td>
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<td>Crassostrea gigas</td>
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<td>LMG 20538</td>
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<td>C1</td>
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<td>Caribbean Sea, La Parguera, Puerto Rico</td>
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<td>C2</td>
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<td>Pseudopterogorgia americana</td>
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</tbody>
</table>

Species Strain Origin Host organism C_T ± SEMdnaJ gene sequence accession no. Reference

a Origin, host organism, and dnaJ gene sequence accession numbers are shown for V. coralliilyticus strains. b Strain designations beginning with LMG were derived from the Belgian Coordinated Collections of Microorganisms, ATCC strains are from the American Type Culture Collection, DSM strains are from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH culture collection, AIMS strains are from the Australian Institute of Marine Science culture collection, and C1 and C2 were provided by Pamela Morris. c †, amplification in one of three reactions; ††, amplification in two of three reactions; NA, no amplification. d Isolated from seawater above coral.

as the threshold cycle (C_T). Specific detection was further confirmed by gel electrophoresis, which revealed a PCR product of the correct theoretical size (128 bp) (data not shown), and DNA sequencing, which confirmed the target amplified product to be a segment of the dnaJ gene. No amplification with the assay was detected for 13 other closely related Vibrio strains, including the closely related Vibrio neptunius and two non-Vibrio species (Table 1). A total of five other Vibrio strains and one non-Vibrio strain (Shewanella sp.) exhibited C_T values less than the cutoff of 32 cycles. However, C_T values for these strains (mean ± standard error of the mean [SEM], 27.96 ± 2.40) were all much higher than those for V. coralliilyticus strains (12.30 ± 1.52), and no amplicons were evident in post-qPCR gel electrophoresis (data not shown).

The detection limit for purified V. coralliilyticus genomic DNA was 0.1 pg of DNA, determined by performing 10-fold serial dilutions (100 ng to 0.01 pg per reaction), followed by qPCR amplification. Similarly, qPCR assays of serial dilutions of V. coralliilyticus (LMG 23696) cells cultured overnight in MB (10^6 CFU ml^{-1} to extinction) were able to detect as few as 10^4 CFU (Fig. 1). Standard curves revealed a strong linear negative correlation between C_T values and both DNA and cell
concentrations of *V. coralliilyticus* over several orders of magnitude, with $r^2$ values of 0.998 and 0.953 for DNA and cells, respectively (Fig. 1).

Little interference of the qPCR assay was observed when purified *V. coralliilyticus* (LMG 23696) DNA (10 ng) was combined with 10-fold serial dilutions (0.01 to 100 ng per reaction) of non-*V. coralliilyticus* DNA (i.e., *Vibrio campbellii* [ATCC 25920])

Over the entire range of nontarget DNA concentrations tested, the resulting $C_T$ values (mean ± SEM, 17.76 ± 0.53) were not significantly different from those of a control treatment containing 10 ng of *V. coralliilyticus* DNA and no nonspecific DNA (16.75 ± 0.18; analysis of variance [ANOVA], *P* = 0.51) (Table 2). Detection of *V. coralliilyticus* (LMG 23696) bacterial cells (10^6, 10^7, 10^8, or 10^9 CFU per ml) in a background of non-*V. coralliilyticus* cells (i.e., *V. campbellii* [ATCC 25920]) at 0, 10, 10^4, or 10^7 CFU per ml showed little reduction in assay sensitivity (see Fig. S1 in the supplemental material). For example, when *V. coralliilyticus* was seeded at 10^7 cells with similarly high concentrations of nontarget cells, little inhibition of the assay was observed.

The assay's detection limit in seawater was tested by inoculating 10-fold serial dilutions of *V. coralliilyticus* (LMG 23696) cultures (grown overnight in MB medium, pelleted at 14,000 rpm for 10 min, and washed twice with sterile phosphate-buffered saline [PBS]) into 1 liter of seawater (equivalent final concentrations were 10^6 to 1 CFU ml$^{-1}$). The entire volume of *V. coralliilyticus*-seeded seawater was filtered through a Sterivex-GP filter (Millipore), and DNA was extracted using the method described by Schauer et al. (11). The lowest detection limit for *V. coralliilyticus* cells seeded into seawater was 1 CFU ml$^{-1}$ (Fig. 2), with no detection in a 1-liter volume of an unseeded seawater negative control. Standard curves revealed a strong correlation between $C_T$ values and the concentrations of *V. coralliilyticus* bacteria seeded into the seawater over several orders of magnitude ($r^2$ of 0.986) (Fig. 2).

The detection limit in seeded coral tissue homogenate was determined by seeding 10-fold dilutions (10^10 to 10^3 CFU ml$^{-1}$) of pelleted, PBS-washed and resuspended (in 10 ml of sterile PBS) *V. coralliilyticus* cells onto healthy fragments (~10 cm$^2$) of the coral *Montipora aequituberculata* collected from Nelly Bay (Magnetic Island, Australia). Corals were collected in March 2009 and maintained in holding tanks supplied with flowthrough ambient seawater. Resuspended cells were inoculated onto M. aequituberculata fragments, each contained in an individual 3.8-liter plastic bag, allowed to sit at room temperature for 30 min, and then air brushed with compressed air until only white skeleton remained. One-milliliter aliquots of the resulting slurry (PBS, bacteria, and coral tissue) was vortexed for 10 min at 14,000 rpm, and DNA was extracted using a PowerPlant DNA Isolation Kit (Mo Bio, Carlsbad, CA). The lowest detection limits for *V. coralliilyticus* cells seeded onto coral fragments was 10^4 CFU per cm$^2$ of coral tissue (Fig. 2). Again, standard curves revealed a strong correlation between $C_T$ values and the concentrations of seeded bacteria over several orders of magnitude ($r^2$ of 0.981) (Fig. 2). When a 1-ml aliquot of the slurry was also inoculated into 25 ml of MB and enriched for 6 h at 28°C (with shaking at 170 rpm), the detection limit increased by 1 order of magnitude, to 10^3 CFU of *V. coralliilyticus* per cm$^2$ of coral tissue (Fig. 2). The slope of the standard curve reveals some inhibition, particularly at the highest *V. coralliilyticus* concentrations, which could result from lower replication rates in the cultures with the highest bacterial densities (i.e., 10^9 CFU). However, since this effect is most pronounced only at the highest bacterial concentrations, the detection limit is still valid. In all trials, unseeded coral fragments and enrichment cultures derived from uninoculated coral fragments served as negative controls.

The current study describes the first assay developed to

![FIG. 1. Standard curves delineating threshold ($C_T$) values of fluorescence for indicators of pathogen presence: (A) concentration of *V. coralliilyticus* DNA and (B) number of *V. coralliilyticus* cells in pure culture. Error bars indicate standard error of the mean for three replicate qPCRs.](http://aem.asm.org/)
detect and quantify a coral pathogen using a real-time quantitative PCR (qPCR) approach. While previous studies have utilized antibodies or fluorescent in situ hybridization (FISH) to detect coral pathogens (1, 6), the combination of high sensitivity and specificity, low contamination risk, and ease and speed of performance (5) make qPCR technology an ideal choice for rapid pathogen detection in complex hosts, such as corals. The assay developed is highly sensitive for \textit{V. coralliilyticus}, detecting as few as 1 CFU ml\(^{-1}\) of seawater and 10\(^6\) CFU cm\(^{-2}\) of coral tissue (10\(^8\) CFU cm\(^{-2}\) of coral tissue with a 6-h enrichment). These detection limits are likely to be within biologically relevant pathogen concentrations. For example, antibodies for specific detection of the coral bleaching pathogen \textit{Vibrio shiloi} showed that bacterial densities reached 8.4 \times 10^6 \text{cells cm}^{-1} \text{month} prior to maximum visual bleaching signs on the coral \textit{Oculina patagonica} (6). Each seeded seawater and coral (enriched and nonenriched) dilution assay was performed in triplicate. The linearity of the resulting standard curves indicates consistent extraction efficiencies over \textit{V. coralliilyticus} concentrations spanning 6 orders of magnitude (Fig. 2) and provides strong support for the robustness of the assay. In addition, the presence of competing, non-\textit{V. coralliilyticus} bacterial cells and DNA had a minimal impact on the detection of \textit{V. coralliilyticus}. This is an important consideration for accurate detection within the complex coral holobiont, where the target organism is present within a matrix of other microbial and host cells.

\textit{V. coralliilyticus}, like \textit{V. shiloi} (10), is becoming a model pathogen for the study of coral disease. Recent research efforts have characterized the organism’s genome (W. R. Johnson et al., submitted for publication), proteome (N. E. Kimes et al., submitted for publication), resistome (15), and metabolome (4) and enhanced our understanding of the genetic (7, 9) and physiological (7, 13) basis of its virulence. Before effective management response plans can be formulated, however, continuing research on the genetic and cellular aspects of \textit{V. coralliilyticus} must be complemented with knowledge of the epidemiology of this pathogen, including information on its distribution, incidence of infection, and rates of transmission throughout populations. The \textit{V. coralliilyticus}-specific qPCR assay developed in this study will provide important insights into the dynamics of pathogen invasion and spread within populations (6) while also aiding in the identification of disease vectors and reservoirs (12). These capabilities will play an important role in advancing the field of coral disease research and effective management of coral reefs worldwide.

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REFERENCES


