PCR-based Assay for Detection of Four Coral Pathogens

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Abstract. Several microorganisms have been identified as pathogenic agents responsible for various outbreaks of coral disease. Little has been learned about the exclusivity of a pathogen to given disease signs. Most pathogens have only been implicated within a subset of corals, leaving gaps in our knowledge of the host range and geographic extent of a given pathogen. PCR-based assays provide a rapid and inexpensive route for detection of pathogens. Pathogen-specific 16S rDNA primer sets were designed to target four identified coral pathogens: *Aurantimonas coralicida, Serratia marcescens, Vibrio shilonii,* and *Vibrio coralliilyticus*. Assays detected the presence of targets at concentrations of less than one cell per microliter. The assay was applied to 142 coral samples from the Florida Keys, Puerto Rico, and U.S. Virgin Islands as an *in situ* specificity test. Assays displayed a high-level of specificity, seemingly limited only by the resolution of the 16S rDNA.

Key words: coral disease, pathogen, bacteria, detection assay, PCR.

Introduction

The study of coral microbiology has been largely fueled by investigations of disease. These studies have been aimed at identification of specific pathogenic agents, followed by the classical method for confirmation of disease causation by fulfillment of Koch's Postulates (Koch 1882). In this manner, numerous putative pathogenic agents have been identified (reviewed in Rosenberg et al. 2007).

Due to the opportunistic manner in which disease samples must often be collected, many coral pathogens were identified based upon limited host, geographic, and temporal occurrences of disease. The tendency to group occurrences of coral mortality with similar gross lesion morphologies into named diseases, has often led to the assumption that all instances of a given "disease" are identical. By extension, the practice has propagated the idea that a given pathogen is responsible for all pathologies with similar lesions. Recent evidence, such as the observation of apparent white plague disease in the absence of the published pathogen (Pantos et al. 2003), has brought these assumptions under scrutiny.

The use of PCR has become very commonplace, with most research facilities having access to the required equipment and expertise. Pathogen-specific PCR assays have been extensively utilized for the detection of various human and veterinary pathogens; evolving into a relatively simple and inexpensive detection method. These assays have the advantage of selectively identifying target sequences from a population and provide a route for further confirmation through sequencing of the amplicons.

This study details construction and testing of PCR primers specifically targeting four bacteria previously implicated in coral disease, *Aurantimonas coralicida*: white plague (type II) (Richardson et al. 1998), *Serratia marcescens*: white pox (Patterson et al. 2002), *Vibrio shilonii*: bleaching (Kushmaro et al. 1997), and *Vibrio coralliilyticus*: bleaching and necrosis (Ben-Haim and Rosenberg 2002). Also presented is the application of these primer sets to healthy and diseased corals in the Florida Keys, Puerto Rico, and U.S. Virgin Islands. These assays represent cost-effective mechanisms for rapid, initial screening for the presence of the target microbes.

Materials and Methods

Bacterial Strains

Aurantimonas coralicida WP1 (DSMZ 14790) (Denner et al. 2003), *Serratia marcescens* PDL100 (ATCC BAA-632) (Patterson et al. 2002), *Vibrio shilonii* AK-1 (ATCC BAA-91) (Kushmaro et al. 2001), and *Vibrio coralliilyticus* YB (ATCC BAA-450) (Ben-Haim et al. 2003) were used as target bacteria for primer design, sensitivity, and specificity testing. *Escherichia coli* (ATCC 8739) and strains of *Vibrio* spp., *Photobacterium* spp., and *Shewanella* spp. bacteria isolated from healthy acroporid corals (Polson 2007) were used as additional control strains during specificity testing.

Extraction of DNA

Bacterial cultures were grown on glycerol artificial

sea water (GASW) agar media (Smith and Hayasaka 1982) for 18 hr (except *A. coralicida*, 36 hr). Single colonies were transferred to a PCR tube containing 150 µl of QuickExtract reagent (Epicentre). Each was incubated at 65ºC for 30 min and 98ºC for 16 min with vortexing at each step.

DNA extractions from environmental samples were performed by processing 200 µl of liquid sample (mucus, seawater, or mucus-tissue slurry) or ~0.5-1g of cryo-milled coral fragments using the FAST DNA Spin Kit for Soil (Qbiogene) according to modifications of Webster *et al.* (2003).

Primer Design and PCR Optimization
Sequence data for the 16S rDNA of a given target bacterium, sequences from closely related bacteria, and outlier sequences were aligned using Lasergene MegAlign (DNAStar). Primer sequences were chosen from regions with increased variability between target and comparison sequences. Each primer sequence was checked for specificity by BLAST against GenBank nucleotide database and analysis with the Ribosomal Database Project's (RDP) Probe Match. Candidate primers were checked for interfering secondary structure with Lasergene PrimerSelect (DNAStar).

Temperature gradient PCR was run on each candidate primer pair using DNA from the target bacterium as template. Approximately 10 ng of DNA template (optical density 260 nm) was added to PCR mixture (2mM $MgCl_2$, 0.2 mM dNTP (each), 0.2 µM primers (each), 1.25 U ExTaq Polymerase (Takara)) with the following thermal cycle: 95ºC-5min; 30 cycles: 95ºC-30s, 62ºC±10ºC-30s, 72ºC-60s; 72ºC-10min. PCR products were visualized by electrophoresis on a 1% agarose gels post-stained with ethidium bromide. Annealing temperatures were chosen based on highest annealing temperature producing a consistently visible PCR product.

Selected primer sets (Table 1) were compatible with a uniform annealing temperature of 67ºC. Final conditions for assay: 10-20 ng of DNA were added to 50 µl reactions using the standard mixture and PCR was performed: 95ºC-5min; 30 cycles: 95ºC-30s, 67ºC-30s, 72ºC-60s; 72ºC-10min. Amplicons were

Table 1. Coral pathogen targeted primer sets with expected amplicon size. Primer sequences oriented from 5' to 3'.

| Target	Name	Sequence
A. coralicida	Ac-995F	TCG ACG GTA TCC GGA GAC GGA T
(500 bp)	UB-1492R	TAC GGY TAC CTT GTT ACG ACT T
S. marcescens	Sm-456F	GGT GAG CTT AAT ACG TTC ATC A
(1040 bp)	UB-1492R	TAC GGY TAC CTT GTT ACG ACT T
V. coralliilyticus	Vc-76F	GTT RTC TGA ACC TTC GGG GAA CG
(940 bp)	Vc-1019R	CTG TCT CCA GTC TCT TCT GAG G
V. shilonii	Vs-457F	GGT ACG TTA ATA GCG TGC TCG
(570 bp)	Vs-1023R	ACC TGC GTC TCC GCT GGC
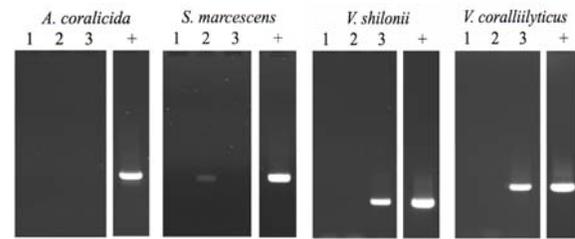
Figure 1. Representative assay results from three samples taken in Biscayne National Park, FL. +: positive control

visualized by gel electrophoresis (Fig. 1).

Primer Specificity
One hundred forty-two samples of coral mucus/tissue or seawater, collected from reefs in the U.S. Virgin Islands National Park (USVINP), St. John, U.S. Virgin Islands, Florida Keys, and southwestern Puerto Rico, were used as material for testing primer specificity. DNA extraction, PCR, and amplicon visualization were performed as described earlier. Suitable PCR bands were excised from agarose gels.

Gel purification was performed using the NucleoSpin Extraction Kit (Clontech). Amplicons were cloned by use of the TOPO TA Cloning Kit (Invitrogen). Plasmids were purified using the QIAprep 96 Turbo kit (QIAGEN) and sequenced with standard M13F and M13R primers.

Assay Sensitivity
GASW broth was inoculated with 1ml of an overnight broth culture of a target microbe or *E. coli*. Flask cultures were incubated at 25ºC with shaking until mid-logarithmic growth phase. Two 25ml volumes were centrifuged at 4ºC for 10 min at 3500 X *g*, washed in filter-sterilized artificial seawater (FASW; 35 ppt), resuspended in 25 ml of FASW, and frozen at -80ºC (mimicking sample collection protocol). Three 1 ml sub-samples of the growth culture were serially (10-fold) diluted in FASW and 100 µl of the 10^3 through 10^6 fold dilutions were spread onto GASW agar, incubated at 25ºC until sufficient colony growth and colony forming units (cfu) determined.

The 25ml volumes were thawed and diluted to 10^6 cfu per ml. Each was then diluted with washed *E. coli* cells (10^6 cfu/ml), reducing concentration of target microbes, but maintaining a background of 10^6 cfu/ml. Fifteen standards were prepared ranging from 1000 to 0.31 cells/µl (extrapolated from cfu/ml data). DNA was extracted from each sample as described for environmental samples and PCR amplifications were conducted as described for assay PCR.

Results
Primer Specificity
The 142 samples (mucus, slurry, milled, and water) screened for the presence of the four target microbes

Table 2. Environmental sample results used in primer specificity testing. Numbers denote positive results by PCR assay for each target microbe.

	Florida Keys	Virgin Islands	Puerto Rico	**Total**
# of Samples	**67**	**24**	**51**	**142**
A. coralicida	4	5	4	**22**
S. marcescens	0	7	13	**11**
V. shilonii	5	0	1	**6**
V. coralliilyticus	4	0	3	**7**

resulted in 46 positive amplifications (Table 2). All amplicons produced from *A. coralicida* primer sets resulted in clone sequences consistent with the target organism. In all cases the highest sequence homology to a characterized strain was to *A. coralicida*.

Amplicons indicating positive matches for *S. marcescens* resulted in closest homology to *S. marcescens* strains by BLAST and RDP Seqmatch. Some sequences, while 100% identical to *S. marcescens* strains, were also indistinguishable from bacteria identified in the BLAST database as *Kluyvera ascorbata*.

Vibrio shilonii amplicons displayed very close homology to *V. shilonii* strains, but it should be noted that strains of *V. mediterranei, V. aestaurianus, V. ichthyaenteri,* and *Listonella anguillarum* (=*V. anguillarum*) often show very high homology to the known *V. shilonii* sequences. Similarly, *V. coralliilyticus* primers resulted in sequences consistently displaying high identity with *V. coralliilyticus*, but often also similar to *V. neptunis, V. brasiliensis,* and *V. shilonii*.

Assay Sensitivity
Amplification of target DNA was, in all cases, possible at concentrations below 1 target cell/µl. *S. marcescens* and *V. coralliilyticus* primers sets produced easily visible bands at the minimum tested concentrations of 0.31 target cells/µl. The *A. coralicida* primer set produced easily visible bands to 5 cells/µl, with detectible product to 0.31 cells/µl. The *V. shilonii* primer set produced properly-sized amplicons (~600 bp) to 0.62 cells/µl. *V. shilonii* primers non-specifically amplified background *E. coli* DNA forming a product well above the anticipated size (~2.5 kb). This product was easily discernible from the target amplicons.

Field Testing
Sixty seven samples were assayed from healthy and diseased acroporid corals at 6 sites from Biscayne National Park, Florida Keys National Marine Sanctuary, and Drt Torugas National Park during a 2003 acroporid mortality event (Williams and Miller 2005). The presence of three of the microbes (*A. coralicida, V. shilonii*, and *V. coralliilyticus*) were indicated in the coral sample set (no instances of *S. marcescens*), while none of the targets were detected in the samples from the surrounding seawater. Sampling location, coral species, sample type (mucus or fragment), and health state did not correlate with the occurrence of any of the microbes.

Acropora spp. corals were sampled at five sites surrounding the island of St. John (USVI) from healthy corals and those exhibiting signs consistent with white pox disease or white band disease. Positive results were obtained from *A. coralicida* and *S. marcescens* (Table 3). While these microbes were present in ten and seven samples respectively, no consistent associations were noted with either white band or white pox like disease states. Sampling location and coral species were also not linked to the occurrence of any of the microbes.

Samples were collected from 51 apparently healthy *Porites astreoides* colonies at eight sites near La Paguera, Puerto Rico. Results indicate the presence of each of the four target bacteria. *S. marcescens* was the most commonly detected, with six of seven colonies at one site (17.93ºN 66.94ºW) and three of six at an adjacent site (17.96ºN 67.02ºW) testing positive for this microbe.

Discussion
Primers were initially tested against a small number of known bacterial cultures, but the

Table 3. Results of primer assay on acroporid corals near St. John, U.S Virgin Islands. Diseased corals were characterized as exhibiting signs consistent with white band disease (WBand) or white pox (WPox), based on field observations.

ID	Health	Species	*A.cor.*	*S.mar.*	*V.shi.*	*V.cor.*
4	Healthy	*A. palmata*				
7	Healthy	*A. palmata*		X		
8	Healthy	*A. palmata*				
9	Healthy	*A. palmata*	X			
3	Healthy	*A. cervicornis*			X	
5	Healthy	*A. cervicornis*			X	
6	Healthy	*A. cervicornis*	X	X		
10	Healthy	*A. cervicornis*				
16	Healthy	*A. cervicornis*				
17	Healthy	*A. cervicornis*	X	X		
11	WPox	*A. palmata*				
13	WPox	*A. palmata*	X			
14	WPox	*A. palmata*				X
15	WPox	*A. palmata*				
19	WPox	*A. palmata*	X			
18	WPox	*A. palmata*	X			
28	WPox	*A. palmata*		X		
30	WPox	*A. palmata*				
12	WBand	*A. palmata*	X	X		
23	WBand	*A. cervicornis*				
24	WBand	*A. cervicornis*	X	X		
26	WBand	*A. cervicornis*		X		
27	WBand	*A. cervicornis*	X		X	
29	WBand	*A. cervicornis*	X			

sometimes crowded phylogenies and abundance of undescribed and/or unculturable phylogenetic neighbors made exhaustive specificity testing in this manner unwieldy and potentially incomplete. The model for testing used here relied on virtual hybridizations against sequences from the RDP and NCBI nucleotide databases, followed by application of the assays to numerous coral samples from multiple locations, dates, species, and health states. All amplicons were validated by DNA sequence analysis. In this manner, the primers were subjected to evaluation against a large number of environmentally relevant microorganisms.

The ability to exhaustively validate the specificity of the *Aurantimonas coralicida* primers is limited by a lack of suitable representatives of this relatively novel and poorly characterized genus (3 members) and candidate family (2 genera). Without a more extensive database of sequences from these taxa it is hard to determine specificity precisely, but based on sequencing of environmental amplicons it appears that only sequences with near 100% identity to the pathogenic strain are amplified using this primer set.

The *S. marcescens* primer set displayed a very high level of specificity, with multiple strains of *S. marcescens* inducing a positive result while no other species of *Serratia* were detected in samples. Identical sequences are deposited in GenBank for some strains of *S. marcescens* and the related species *Kluyvera ascorbata*. The phylogenetic relationships among genera of the family Enterobacteriaceae are poorly defined and overlap between genera has been reported (Pavan et al. 2005). There are no reports of *Kluyvera* spp. associated with any marine organism and only a single *Kluyvera* sp. sequence (DQ316136; unpublished) deposited in GenBank is noted to be from the marine environment (seawater from China), thus the likelihood of a non-specific identification of *K. ascorbata* in coral is exceedingly unlikely.

The genus *Vibrio* is highly subdivided, comprised of 88 species with standing in nomenclature (Euzéby 2009). There is much contention over the criteria used to divide related strains into separate species. Many have 99 to 100% homology over portions (and occasionally the full length) of the 16S rDNA. The issue of differentiating *Vibrio* spp. is further compounded by the sheer number of sequences deposited, the GenBank database contained 5,473 16S rDNA sequences annotated "*Vibrio*" (January 2009). Many of these clones were annotated based solely on similarity of 16s rDNA, thus many are likely misidentified at the species or even genus level. In addition there are countless unannotated clones that are likely *Vibrio* spp. These combined issues make the task of determining specificity among *Vibrio* specific primers very difficult.

Amplicons from *V. shilonii* and *V. coralliilyticus* primer sets produced sequences that were difficult to precisely place phylogenetically. These sequences did however show a high level of specificity, as only slight degeneracy in primer binding would have been expected to produce amplicons from every sample due to the ubiquitous nature of V*ibrio* spp. in the reef environment. These primers only detected sequences that would be expected based on 0 or 1 bp mismatch in the primer binding. It was noted that the *V. coralliilyticus* primers would occasionally amplify sequences homologous to those annotated as *V. shilonii* from environmental samples, but never from DNA extracted from stock cultures of *V. shilonii.* This could be due to some similarity to sequences of certain *V. shilonii* strains, but is very likely due to erroneous annotation of sequences in GenBank. The *V. shilonii* primers never produced amplicons homologous to *V. coralliilyticus* sequences, providing an avenue for detection of false-positives.

Qualitative identification of target microorganisms is classically subdivided into three types of nested tests: presumptive, confirmed, and completed (in order of increasing confidence) (Eaton et al. 1995). A positive result obtained by application of the assays presented here should not be viewed as proof that the target microbe is present. It rather demonstrates that the target bacterium, or one with very close sequence homology, occurs in the sample. Thus the PCR assay should be treated as a "presumptive test", with subsequent sequencing of amplicons acting as the complementary "confirmed test". This confirmed test can provide reasonable certainty that the target is present. The only "completed test" that can be offered is to isolate and characterize the target microbe, followed by laboratory infection studies.

Sensitivity assays indicate the lower detection limits of each assay exceeded 0.62 target cells/µl (620 cells/ml) within a relevant background concentration of approximately 1×10^3 non-target cells/µl (1×10^6 cells/ml), or 0.06% of the total. These sensitivity levels exceed what would likely be necessary to detect an active pathogen. For instance, *V. shilonii* has been shown to maintain populations in the coral mucus exceeding 1×10^6 cells/ml during an infection (Banin et al. 2000). Thus these assays provide detection of target bacteria at what are likely sub-infection levels.

The three case studies presented in this paper, illustrate potential utility of these assays. The 2003 mortality of acroporid corals along the Florida Reef Tract had disease signs, which displayed both differences and similarities to two "white disease", white pox (previously reported in *A. palmata*) and white plague (reported in a large number of non-acroporid species). The application of the primer sets

quickly excluded the previously published white plague and white pox pathogens. These results were later confirmed by extensive culturing and 16S rDNA library sequencing (Polson 2007).

A survey of healthy and diseased acroporid corals from the US Virgin Islands identified a number of both healthy and diseased corals that tested positive for the target microbes. The diseased corals in this sample collection had disease signs that fit the classical descriptions for white band and white pox disease. Interestingly, the white pox samples did not show any correlation with the presence of *S. marcescens*, the reported pathogen from the Florida Keys. This observation underlines the critical point that identical coral disease signs do not necessarily equate to identical etiologies. This survey represents the first confirmation of *Acropora palmata* exhibiting disease consistent with that described as white pox without the association of *S. marcescens.*.

Surveys of *Porites astreoides* colonies along the southwest coast of Puerto Rico indicated the presence of all targeted microbes. In particular *Serratia marcescens* is noted to occur in approximately 25% of the corals sampled, with greater than 50% abundance at two of the sampling sites. *S. marcescens* is not known to produce disease in *P. astreoides*. While this observation is anecdotal, it does indicate that non-affected coral species may be a potential reservoir for this reported acroporid pathogen.

Each diagnostic primer set demonstrated potential for use in applications ranging from the diagnostic assessment of specific coral disease occurrences to more generalized surveillance of corals. Any application should be undertaken with the caveat that they represent an initial screening tool (a presumptive test) for the presence of target organisms. Despite this limitation, the assay allows for results to be obtained from a large number of samples in a relatively short period of time, quickly narrowing the number of samples on which more intensive investigation may be needed. The utilization of a standardized protocol (sampling, processing, and analysis) will allow data comparisons across laboratories, enabling the combination of disparate sample sets that would not normally be processed together, setting the stage for addressing large-scale questions.

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