Use of Quantitative Real-Time PCR for Direct Detection of *Serratia marcescens* in Marine and Other Aquatic Environments

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Serratia marcescens is the etiological agent of acroporid serratiosis, a distinct form of white pox disease in the threatened coral *Acropora palmata*. The pathogen is commonly found in untreated human waste in the Florida Keys, which may contaminate both nearshore and offshore waters. Currently there is no direct method for detection of this bacterium in the aquatic or reef environment, and culture-based techniques may underestimate its abundance in marine waters. A quantitative real-time PCR assay was developed to detect *S. marcescens* directly from environmental samples, including marine water, coral mucus, sponge tissue, and wastewater. The assay targeted the *luxS* gene and was able to distinguish *S. marcescens* from other *Serratia* species with a reliable quantitative limit of detection of 10 cell equivalents (CE) per reaction. The method could routinely discern the presence of *S. marcescens* for as few as 3 CE per reaction, but it could not be reliably quantified at this level. The assay detected environmental *S. marcescens* in complex sewage influent samples at up to 761 CE ml⁻¹ and in septic system-impacted residential canals in the Florida Keys at up to 4.1 CE ml⁻¹. This detection assay provided rapid quantitative abilities and good sensitivity and specificity, which should offer an important tool for monitoring this ubiquitous pathogen that can potentially impact both human health and coral health.

Serratia marcescens is a ubiquitous bacterium in the environment; it is naturally found in water and soil and in association with plants and animals, often as a pathogen (1–4). *S. marcescens* is also an opportunistic pathogen for humans, commonly associated with hospital-acquired infections (5, 6). In 1999, it was found within the mucus layer of elkhorn coral (*Acropora palmata*) and later identified as an etiological agent of white pox disease (designated acroporid serratiosis only if *S. marcescens* is present) (7, 26). In the Florida Keys and broader Caribbean, multiple white pox disease outbreaks have contributed to the decline of elkhorn coral since the late 1990s (7). During the 2002–2003 Florida Keys outbreak, where acroporid serratiosis was confirmed, the dominant strain of *S. marcescens* circulating among diseased corals and reef water was concurrently detected in human sewage (strain type PDR60) but in no other potential sources; this led to the hypothesis that wastewater treatment practices may have a direct impact on coral health (8).

Current methodology to detect *S. marcescens* from aquatic samples requires a multistep process for culture, detection, and then identification of the bacterium (8–10). The protocol used for detection in marine waters and coral includes an initial culture on a selective medium (MacConkey sorbitol agar amended with colistin [MCSA]), verification on a second selective medium (DNase agar amended with toluidine blue and cephalothin [DTC]), and then PCR for a *Serratia*-specific region of the 16S rRNA gene (8). This process likely underestimates the total concentration, and without all three steps it lacks the specificity for *S. marcescens* to determine the true abundance of the bacterium in the environment; it can also take days to confirm results. The time and materials required for the culture-based assays effectively limit the number of samples that can be screened. Some assays using PCR or quantitative real-time PCR (qPCR) are available for *S. marcescens* (11, 12); however, their applications were designed for specific settings (i.e., clinical, cultured cells, and building debris) and may not be effective for environmental samples of di-

verse microbial communities (11–13). A rapid, culture-independent, and quantitative method is needed to screen large numbers of environmental samples, which is critical for determining the prevalence of this organism among diseased coral, apparently healthy corals, other organisms, and surrounding water. Efficient detection at high resolution (i.e., with large numbers of samples collected over space and time) is also required to better inform models of disease dynamics and transmission. In addition to a fast screening assay for environmental samples, any direct detection technique should also be applicable as a diagnostic tool. A rapid diagnostic assay would provide a method to accurately identify diseased lesions in corals as acroporid serratiosis versus another (as-yet-unknown) potential agent of white pox disease (14, 15, 26). Therefore, our overall objective in this study was to develop an efficient and qPCR assay to detect *S. marcescens* directly from environmental samples.

MATERIALS AND METHODS

Selection of amplification target. Multiple common genetic regions were explored *in silico* as suitable gene targets for an *S. marcescens*-specific assay, including *gyrB*, the 16S rRNA gene, the 23S rRNA gene, and *luxS* (11, 12). The *luxS* gene, associated with quorum sensing, was selected for additional consideration given its potential for higher specificity for *S. marcescens*, compared to that of other possible targets, according to submitted gene sequences within National Center for Biotechnology Information's (NCBI) (www.ncbi.nlm.nih.gov) GenBank. The *luxS* gene in *S. marc-*

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TABLE 1 *Serratia marcescens* real-time quantitative primer and probe sequences, nucleotide positions, and melting temperatures

<i>luxS</i> qPCR primer or probe	Nucleotide position	Melting temp (°C)
Forward primer, 5'-TGCCTGGAAGCGGCGATGG-3'	306–325	66.6
Reverse primer, 5'-CGCCAGCTCGTCGTTGTGGT-3'	480–461	66.6
Probe, 5'-6FAM-GTGGTACCTACCACATGCACTCGCTGGAA-BHQ1a-3'	384–413	70.3

escens diverged from other *luxS*-containing bacteria but was highly conserved among *S. marcescens* strains (see Fig. S1 in the supplemental material). A previous study by Zhu and colleagues also identified *luxS* as suitable to detect *Serratia* spp. in environmental samples by using traditional PCR (12). Finally, *luxS* has the additional benefit of having only a single copy within the *S. marcescens* genome, making specific quantification through qPCR simpler.

Primer and probe design. NCBI's Primer BLAST (16) was used to create forward and reverse primers for a region within the *luxS* gene that was highly specific to *S. marcescens* (about 516 bp in *S. marcescens* [GenBank accession numbers EF164926.1 and AJ628150.1]). In developing the candidate primer pair, the amplicon size was restricted to ≤ 300 bp in length, with primer lengths between 18 and 22 bp. Corresponding candidate sequences for a 5'-exonuclease hydrolysis probe (i.e., TaqMan probe) were designed by aligning *S. marcescens* sequences with those of other *Serratia* species and closely related bacteria using the MAFFT multiple-sequence alignment program (17). The probe was also chosen to be between 20 and 30 bp in length, with a melting temperature greater than the melting temperature of the associated primers.

Three sets of primers and two hydrolysis probes for *luxS* were evaluated. Probes were designed to increase the assay specificity by exclusively aligning *luxS* with a variety of *Serratia* species and other closely related bacteria (see Fig. S2 in the supplemental material). The final primers and probe combination (Table 1) had only minor secondary structures as confirmed using Primer Express (Applied Biosystems, Foster City, CA).

Controls. Pure cultures of known strains of *S. marcescens* (ATCC 13880 and Db11) were grown overnight in LB broth (Fisher, BP1426) at 37°C to an estimated cell density of 10^8 cells ml⁻¹. The DNeasy blood and tissue kit (Qiagen, Valencia, CA) was used to extract DNA according to the manufacturer's protocol for Gram-negative bacteria. DNA quantity and quality were checked with a NanoDrop1000 instrument (Thermo Scientific, Wilmington, DE). DNA with an $A_{260/280}$ purity ratio of 1.8 to 2.0 and ≥ 20 ng μ l⁻¹ was used. An Invitrogen TOPO TA PCR cloning kit (Life Technologies, Grand Isle, NY) was used to clone the *luxS* amplicon (the PCR assay is described below) of *S. marcescens* Db11 into a plasmid. The Invitrogen plasmid Miniprep kit was used to extract plasmid DNA, which was used as a positive control and in the development of standard curves. Plasmid DNA was checked for purity, quantified, divided into aliquots, and stored at -80°C.

Sensitivity and specificity. To optimize the qPCR protocol, an *S. marcescens* Db11 *luxS*-containing plasmid was serially diluted in 10-fold increments over a 9-log scale. This serial dilution (10 points) was used to create the standard curve, in triplicate, for quantification of environmental samples. In addition to the sequence alignments completed when designing the primers, the designed assay (developed primers, probe, and reaction conditions) was applied to four other *Serratia* species for verifying specificity and non-cross-reactivity of the primers: *S. plymuthica* (ATCC 27593), *S. liquefaciens* (ATCC 27592), *S. rubidaea* (ATCC 33670), and *S. odorifera* (ATCC 33077). Additionally, other bacteria (non-*Serratia* spp.) were screened for primer cross-reaction: *Enterococci faecalis* (ATCC 19433), *Escherichia coli* (ATCC 15597), *Vibrio cholerae* (O1 strain; ATCC 14035), and *V. parahaemolyticus* (ATCC 17803). These species were chosen because they represent other genera that carry the *luxS* gene and are found in the environment naturally or through wastewater contamination.

Primers and reaction conditions were initially screened using SYBR green-based qPCR (Bio-Rad, Hercules, CA) on a StepOne Plus platform

(Applied Biosystems, Life Technologies, Grand Isle, NY). All qPCRs were completed with duplicate technical replicates and duplicate no-template negative controls. Following successful reactions for duplicate qPCR runs with no evidence of nonspecific primer binding, reaction conditions were optimized for TaqMan-based qPCR (QuantiTect probe kit; Qiagen, Valencia, CA). A successful preliminary standard curve was created and used to further test the sensitivity and specificity for *S. marcescens* in environmental samples. Final reaction mixtures included 0.9 μ M (each) forward and reverse primers, 0.06 μ M TaqMan Black Hole Quencher probe, 1 $\times$ Taq master mix (as provided in the QuantiTect probe kit), 1 μ l of sample DNA, and PCR-grade water for a total reaction volume of 25 μ l. Using this complete reaction master mix formula, a temperature gradient was run on a StepOne real-time PCR system (Life Technologies, Grand Isle, NY) from 60°C to 67°C to determine the best primer annealing temperature of 62°C, which was also effective for extension. The completed run program was 95°C for 15 min and then 45 cycles of 95°C for 5 s and 62°C for 40 s.

Evaluation of inhibition in environmental samples. The sample matrix from environmental sources was tested for inhibition of the qPCR assay. Extracted DNA from duplicate environmental samples (coral mucus, sponge pore water, sediment, canal water, wastewater, and 1:10 diluted wastewater) (see below for extraction method) was mixed 1:1 with 10^4 cell equivalents (CE) (from plasmids) for a total of 2 μ l and added to 48 μ l of reaction master mix at the concentrations described above. The complete reaction volume was divided and used as duplicate technical replicates in 25- μ l qPCR assays. An equivalent concentration of plasmid CE, achieved by using PCR-grade water instead of sample DNA, was used in qPCR assays as a standard to evaluate the environmental extracts for inhibitory characteristics.

Application to environmental samples. Coastal canal water, sediment, sponge tissue, coral mucus, and sewage influent from the Florida Keys were collected to evaluate the performance of this qPCR for environments previously known to harbor culturable *S. marcescens* (8). Water samples were collected in 1-liter sterile polypropylene bottles from just below the surface in residential canals of the Florida Keys (September 2011 and August 2012). Sediment ($n = 3$) and marine sponge species ($n = 3$) were also collected (about 5 g each from near-shore Key Largo, FL, in August 2012), and after vigorous vortexing and settling of the sample, the supernatant fluid (2 ml) was saved for DNA extraction. Mucus was collected from the surface of the coral *Siderastrea radians* ($n = 3$) from near-shore Key Largo, FL, in August 2012 by aspirating the mucus with needleless syringes. Sewage influent (post-bar screen) was collected in 1-liter sterile polypropylene bottles with the assistance of the treatment plant staff using their established protocol for plant monitoring. Sewage samples were collected from Key West, FL, Marathon, FL, and Key Largo, FL, plants in September 2011 and August 2012. After collection, all samples were placed on ice and processed within 3 h.

In the field laboratory, water, mucus, and sewage samples were split to compare culture- and qPCR-based detection. For molecular detection, replicate 2-ml aliquots of each sample (biological replicates) were centrifuged at $\sim 13,000 \times g$ for 20 min and the supernatant fluid decanted. The pellet containing bacteria was stored at -20°C until DNA could be extracted (described below). The remaining sample was used immediately for the detection of *S. marcescens* by culture. Up to 25 ml of water and 10 ml of coral mucus were filtered onto 47-mm-diameter 0.45- μ m-pore size mixed cellulose ester membranes (Millipore, Billerica, MA). Filters were placed onto selective agar for *S. marcescens* (MCSA). Up to 100 μ l of sewage influent were spread directly onto MCSA agar plates. Sponge and

FIG 1 *Serratia marcescens* real-time quantitative PCR standard curve using plasmids of *luxS* gene extracted from the Db11 strain. There is only one copy of *luxS* per cell, and therefore each copy is indicative of one cell. The accuracy for quantifying the amount of DNA is 10 cell equivalents (CE) per reaction; however, this assay can detect as few as 3 CE per reaction. Error bars are one standard deviation from the mean ($n = 3$).

sediment samples were not cultured. MCSA plates were incubated for 19 to 24 h at 37°C, and presumptive *Serratia* colonies (pink colonies indicative of sorbitol fermentation) were transferred to DTC agar for phenotypic confirmation (indicated by red halos around colonies), as described by Sutherland and colleagues (8, 9). Isolated colonies of presumptive *S. marcescens* were saved in deep agar stabs (LB agar) following two rounds of isolation, until further genotypic confirmation to species level by PCR (or qPCR).

DNA was extracted from saved isolates by growing a subculture in 5 ml LB broth (Fisher, BP1426) for 12 to 16 h at 37°C. Cells were centrifuged ($4,000 \times g$ at 24°C for 5 to 10 min) and the pellet washed three times with $1 \times$ phosphate-buffered saline (PBS). The final pellet was resuspended in 1 ml of $1 \times$ PBS and brought to a temperature of 100°C for 10 min. The lysed cell suspensions were centrifuged for 10 min at $\sim 13,000 \times g$, and the supernatant fluid (containing DNA) was stored at -80°C or diluted and used immediately for qPCR.

The ethanol precipitation protocol of Boström and colleagues (18) was used to extract environmental DNA from frozen pellets, with slight modifications. A sterile 2-ml centrifuge tube was used as an extraction negative control. Lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA, 50 mM Tris-HCl [pH 9.0], and lysozyme (1 mg ml^{-1}) was added to the pelleted sample. Following incubation at 37°C for 30 min, proteinase K (100 $\mu\text{g ml}^{-1}$ final concentration) and SDS (1% [wt/vol] final concentration) were added and tubes incubated at 55°C for 16 to 18 h. To aid in the precipitation of DNA, tRNA (50 μg) (to act as a DNA carrier molecule), 0.1 volume sodium acetate (NaAc), and 2.5 volumes ethanol (EtOH) (99%) were added and incubated for an hour at -20°C . Samples were centrifuged ($\sim 13,000 \times g$ for 20 min) and the supernatant fluid decanted, retaining pelleted DNA in the original tube. DNA pellets were then washed with 500 μl EtOH (70%) and centrifuged ($\sim 13,000 \times g$ for 20 min) and supernatant fluid decanted. A SpeedVac (Eppendorf Concentrator 5301) was used to dry the DNA pellet, which was then resuspended in 100 μl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The final DNA suspension was stored at -80°C or used immediately for qPCR. All samples were subjected to qPCR as two technical replicates.

Additionally, runs included two no-template negative controls, extraction negative controls, and a 3-point standard curve, run in duplicate, with *luxS* plasmid standards.

Amplicons of the *luxS* qPCR from sewage ($n = 2$) and presumptive isolates of *S. marcescens* (from canal water and sewage in the Florida Keys; $n = 10$) were submitted for sequencing by primer extension (Macrogen, Rockville, MD). The sequences were screened through the NCBI BLAST search engine and aligned to the *S. marcescens* Db11 *luxS* gene sequence using the MAFFT alignment tool (using the Q-INS-i strategy, scoring matrix of 1PAM/K = 2, and the default gap-opening penalty of 1.53).

RESULTS

Sensitivity and specificity. A final standard curve for the qPCR assay was established from 10 dilutions and 3 reaction replicates. The final curve showed a y intercept of 38.842, a slope of -2.883 , and a mean efficiency of 122% (Fig. 1). Since there is only one *luxS* copy in the *S. marcescens* genome, cell equivalents (CE) and genome equivalents are the same and CE is used as the quantification unit. The assay was able to quantify *S. marcescens* to an optimized limit of detection of 10 CE per reaction, and it could regularly detect as few as 3 CE per reaction but without reliable quantification. Thus, values in the range below 10 CE per reaction should be considered “detected but not quantified” (DNQ). Subsequent qPCR runs confirmed the standard curve. The assay was also highly specific; there was no cross-reaction detected with any of the *Serratia* or non-*Serratia* strains tested (i.e., no amplification detected) (Table 2).

Inhibition analysis. Positive-control plasmid DNA (10^4 CE μl^{-1}) was seeded into all extracts of sample matrices, and quantification cycle (C_q) values were compared to those for template DNA in PCR-grade water. No inhibition (i.e., no change in C_q values) was noted for water, coral mucus, sponge, and sediment. Undiluted sewage caused significant inhibition, noted by a delayed C_q ($P = 0.0124$); a 10-fold dilution removed the inhibitory effect (Fig. 2).

Environmental application. Sewage influent and canal water were the only samples in which *S. marcescens* was detected using both culture and qPCR methods. *S. marcescens* was not detected in the coral, sponge, and sediment samples (screened using culture and qPCR). All DNA extraction controls were screened, and results for all were below the assay’s detection limits.

Among the canal samples ($n = 3$), *S. marcescens* was detected at a mean of 3.63 CE ml^{-1} , ranging from 2.8 to 4.1 CE ml^{-1} . Concentrations of *S. marcescens* using culture methods averaged 0.5 CFU ml^{-1} (ranging from 0.2 to 1 CFU ml^{-1}). Among sewage

TABLE 2 Bacterial strains tested for specificity of the *luxS* qPCR assay^a

Species	Strain	<i>luxS</i> qPCR result
<i>Serratia marcescens</i>	Db11	+
	ATCC 13880	+
<i>Serratia plymuthica</i>	ATCC 27593	—
<i>Serratia liquefaciens</i>	ATCC 27592	—
<i>Serratia rubidaea</i>	ATCC 33670	—
<i>Serratia odorifera</i>	ATCC 33077	—
<i>Enterococcus faecalis</i>	ATCC 19433	—
<i>Escherichia coli</i>	ATCC 15597	—
<i>Vibrio cholerae</i> O1	ATCC 14035	—
<i>Vibrio parahaemolyticus</i>	ATCC 17803	—

^a Only *S. marcescens* strains were positive with this assay.

FIG 2 Inhibition effects of environmental matrix on the detection of *S. marcescens*. The dashed line indicates the quantification cycle (C_q) for the seeded quantity of plasmids (10^4). The bars (with standard deviation error bars) represent each sample type with seeded plasmids ($n = 3$). Significant inhibition (*) was recorded for the undiluted sewage influent sample ($P = 0.0124$).

samples ($n = 3$), *S. marcescens* was detected at a mean of 277.3 CE ml^{-1} , ranging from 9 to 761 CE ml^{-1} . Concentrations of *S. marcescens* based on culture resulted in a mean level of 40 CFU ml^{-1} (20 to 50 CFU ml^{-1}) (Table 3).

The amplicon sequences of environmental isolates and sewage samples from September 2011 (presumptive *S. marcescens* based on the conventional culture method) were confirmed as *S. marcescens* and aligned with the *luxS* gene of *S. marcescens* strain Db11 (see Fig. S3 and Table S1 in the supplemental material). All 9 sequences from environmental isolates matched *S. marcescens luxS* sequences (NCBI accession numbers [EF164926.1](#) and [AJ628150.1](#)). With the exception of one sequence, all showed identity with *S. marcescens luxS* at greater than 91%. Environmental isolate SC42 showed 88% sequence homology to *S. marcescens luxS* and 96% sequence homology to a noncoding region of *S. liquefaciens* ATCC 27592 (NCBI accession number [CP006253.1](#), submitted June 2013). The amplicon sequences from qPCR-positive sewage samples were identified using BLAST nucleotide searching as *S. marcescens luxS* (NCBI accession numbers [EF164926.1](#) and [AJ628150.1](#)) with 98% (Key Largo) and 99% (Marathon) maximum identities.

DISCUSSION

This assay was able to specifically detect *Serratia marcescens* in marine environmental and sewage samples using qPCR directed at the single-copy *luxS* gene. The assay was not cross-reactive with other known *Serratia* strains or other *luxS*-containing bacteria tested in the laboratory. Of the 12 qPCR amplicons submitted for sequencing, 11 were confirmed for *S. marcescens* (the final one was of poor sequence quality and was not included in the analyses).

TABLE 3 *Serratia marcescens* found in Florida Keys environmental samples (collected August 2012) using culture and qPCR methods

Sample description	Location (latitude, longitude)	Culture (CFU ml ⁻¹)	qPCR (CE ml ⁻¹) ^a
Canal water, Eden Pines	24°41.463'N, 81°22.674'W	1	4
Canal water, Doctor's Arm	24°42.092'N, 81°21.124'W	0.4	2.8
Freshwater lens, Blue Hole	24°42.368'N, 81°22.837'W	0.2	4.1
Sewage influent, Key Largo	25°6.041'N, 80°25.930'W	20	8.9
Sewage influent, Marathon	24°43.855'N, 81°0.241'W	50	62
Sewage influent, Key West	24°34.115'N, 81°47.818'W	50	761

^a As calculated by the standard curve.

While one amplicon (environmental isolate SC42) also showed homology with both *S. liquefaciens* (an intergenic, unannotated region) and *S. marcescens (luxS)*, the *S. liquefaciens* control strain (ATCC 27592) was never amplified in this assay, nor was it identified in sewage samples. These results suggest that this assay is specific for *S. marcescens luxS* over other related sequences and is highly sensitive, with a detection limit as low as 3 CE per reaction.

Comparison of this qPCR method to culture-based detection in environmental samples demonstrated similar results for surface water samples, with qPCR (CE) concentrations slightly greater than concentrations determined by culture. The literature also suggests that qPCR typically results in a higher concentration than culture due to detection of both dead and viable but nonculturable cells (19, 20). In this case, qPCR concentrations were within one order of magnitude of those from culture, and results were obtained with a smaller sample volume (2 ml for qPCR versus 5 or 15 ml for culture). Among raw sewage samples, concentrations determined by qPCR were similar to those determined by culture for two of the wastewater treatment plants but were significantly greater than those determined by culture in one plant (Key West). This large difference may be due to the high level of nonspecific growth on the MCSA spread plates from sewage, which may have reduced detection of presumptive *S. marcescens* colonies. While *S. marcescens* was not detected in the few sponges and corals collected for this study, data from efficiency, specificity, sensitivity, and inhibition assays suggest that the bacteria were absent from these samples, rather than simply not detected.

No inhibition was noted for the tested canal surface waters, but sewage was likely to contain significant inhibitors; this could generally be alleviated with a 1:10 dilution of sample extract before qPCR. Inhibitors in environmental or other complex samples can increase the likelihood of false negatives by PCR and reduce the concentration estimates in qPCR. This is a common issue for PCR and qPCR detection assays and can be addressed by the development of a specific internal control to calculate the inhibition within each qPCR (21–23). In the absence of a unique internal control sequence, template can be spiked into sample extracts to estimate inhibition effects, as was done here (22).

In addition to increased sensitivity and specificity for *S. marcescens*, the qPCR detection assay significantly reduces the time to obtain results compared to that for culture-based techniques (24,

25). Furthermore, qPCR provides a platform for high-throughput detection and analysis. To date, *S. marcescens* is the only confirmed etiological agent of white pox disease (termed acroporid serratiosis when this etiological agent is confirmed in disease lesions) in the threatened elkhorn coral (7, 8, 26). Outbreaks of disease consistent with signs of white pox continue to occur in the Florida Keys and elsewhere in the Caribbean; however, in many cases efforts to assign these outbreaks as acroporid serratiosis have not been carried out due to the lack of a simple diagnostic tool. In order to better describe patterns of disease associated with occurrence and distribution of *S. marcescens* versus general white pox signs, rapid and high-throughput tools are needed to screen large numbers of samples from a variety of environments (e.g., corals, water, etc.). Such detailed observations are also needed to track potential pathogen sources or reservoirs (14, 15). Using this qPCR assay to detect *S. marcescens* within a white pox disease lesion and confirm acroporid serratiosis is a key advance in the study and management of the coral disease.

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The use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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