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Short Communications

Analysis of Coral Mucus as an Improved Medium for Detection of Enteric Microbes and for Determining Patterns of Sewage Contamination in Reef Environments

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Abstract: Traditional fecal indicator bacteria are often subject to a high degree of die-off and dilution in tropical marine waters, particularly in offshore areas such as coral reefs. Furthermore, these microbes are often not associated with human waste, and their presence may not be indicative of health risk. To address the offshore extent of wastewater contamination in the Florida Keys reef tract, we assayed coral surfaces for the presence of human-specific enteric viruses. The overlying water column and surface mucopolysaccharide (mucus) layers from scleractinian corals were sampled from three stations along a nearshore-to-offshore transect beginning at Long Key in the middle Florida Keys, USA. Samples were assayed for standard bacterial water quality indicators (fecal coliform bacteria and enterococci) and for human enteroviruses by direct reverse transcriptase-polymerase chain reaction (RT-PCR). The concentration of the bacterial indicators was greatest at the nearshore station in both the water column and corals, and decreased with distance from shore; no indicator bacteria were detected at the offshore station. Whereas human enteroviruses were not detected in any of the water column samples, they were detected in 50–80% of coral mucus samples at each station. These data provide evidence that human sewage is impacting the reef tract up to ~6.5 km from shore in the middle Florida Keys and that coral mucus is an efficient trap for viral markers associated with anthropogenic pollution.

Key words: enteric virus, pollution, coral, reef, Florida Keys, fecal indicator

Introduction

Coral reefs are valuable ecosystems that provide habitat to a highly diverse group of marine organisms and support a variety of human recreational and commercial activities (Bryant et al., 1998; Kayanne, 1996; Mayer, 1903; Pennisi, 1997; Shinn, 1988, 2001; Warren-Rhodes et al., 2003). However, surveys suggest that coral reefs are in decline, particularly popular recreational reefs or those in the vicinity of high population centers (Green and Bruckner, 2000). Among the various sources of reef stress is contamination with human wastewater due to direct dumping from live-aboard boats, septic systems, leaking sewer lines, and storm-water runoff (Bruno et al., 2003; Grigg, 1994; Hallock and Schlager, 1986; Lapointe et al., 1990; Nobles et al., 2000; Paul et al., 1995; Shinn et al., 1994). In the Florida Keys, wastewater issues have prompted concern over public

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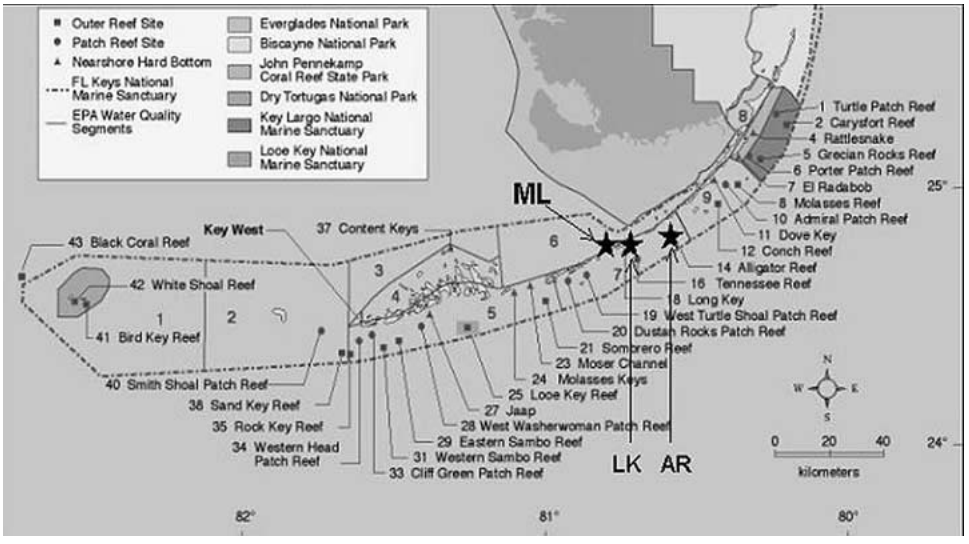

Figure 1. Map of sampling stations in the middle Florida Keys using the Florida Marine Research Institute Coral Reef Monitoring Program survey sites as reference (adapted from Porter et al., 2001). Arrows denote sampling stations for this project.

health risks, as well as increased rates of coral disease and stony coral loss (Nobles et al., 2000; Patterson et al., 2002; Porter et al., 2001). Much of the human wastewater is disposed of via ~30,000 septic systems, 5000–10,000 cesspits, and >600 Class IV injection wells, which are known to contribute to elevated nutrients and enteric microbes in surface waters near shore (Lapointe et al., 1990; Paul et al. 1995, 1997; Shinn et al., 1994); however, because of problems with traditional fecal indicators in tropical marine waters (e.g., Fujioka et al., 1988; Garcia-Lara et al., 1991; Griffin et al., 2001), little is known about distribution of these contaminants offshore and in the coral reef tract.

Recently, human-specific enteric viruses have been used to document wastewater contamination in a number of nearshore marine environments (Griffin, 1999; Jiang et al., 2001; Lipp et al., 2001a,b, 2002; Noble and Fuhrman, 2001). In 2002, we reported that coral mucus offered a surface for accumulation of both enteric bacteria and human viruses relative to the overlying water column (Lipp et al., 2002). Here we demonstrate the utility of this technique in tracking human wastewater in offshore corals and reefs and propose that the analysis of mucus may be a useful tool to document extent and distribution of microbes, including coral pathogens, derived from human sewage.

Methods

Field Sampling

Samples were collected by hand from three stations in the middle Florida Keys in July 2002 (Fig. 1). Nearshore samples were collected just offshore at the Florida Keys Marine Lab (ML) within 50 m of the small beach due west of the boat basin, bayside. Mid-offshore samples were taken from a Long Key hardbottom community (LK; Coral Reef/Hardbottom Monitoring Project, station 7H2 [Wheaton et al., 2001]). Offshore samples were collected from Alligator Reef (AR), which is located approximately 6.5 km south-southeast of Islamorada. At each station, water was collected in sterile 1-liter polypropylene bottles from approximately 0.5-m depth. At the nearshore station (ML), the mucus layers from four distinct, randomly selected coral colonies were collected with sterile 60-ml syringes and transferred to sterile 50-ml conical bottom tubes. At the remaining two sites (LK and AR), 10 colonies were sampled at each station in a similar manner. All corals were photographed and identified (Colin, 1988). Water column temperature, pH, and salinity were recorded for each site.

Microbiological Analyses

Samples were vigorously mixed and filtered in duplicate onto sterile 47-mm, 0.45-μm-pore-size mixed cellulose ester membranes (up to 100 ml for water and 10 ml for mucus). Filters were placed on agar media for the specific detection of fecal coliform bacteria (mFC [American Public Health Association, 1998]) and enterococci (mEI; [USEPA, 1997]). mFC plates were incubated at 44.5°C for 24 ± 4 hour in a water bath, and blue colonies were enumerated as fecal coliforms. mEI plates were incubated at 41°C for 24 ± 4 hours, and colonies exhibiting a blue halo were counted as enterococci. Bacterial counts were reported as colony forming units (CFU) 100 ml^{-1}.

Table 1. Description of Corals Sampled[a]

Station	Sample ID	Species	Sample depth (m)
Florida Keys Marine Lab	ML1	*Siderastrea radians*	0.9
	ML2	*Siderastrea radians*	0.9
	ML3	*Siderastrea radians*	0.9
	ML4	*Siderastrea radians*	0.9
Long Key hardbottom	LK1	*Siderastrea siderea*	1.5
	LK2	*Montastraea annularis* complex	1.5
	LK3	*Siderastrea siderea*	1.6
	LK4	*Siderastrea siderea*	2.0
	LK5	*Siderastrea siderea*	2.0
	LK6	*Montastraea annularis* complex	1.5
	LK7	*Siderastrea siderea*	1.5
	LK8	*Siderastrea siderea*	1.3
	LK9	*Siderastrea siderea*	1.3
	LK10	*Dichocoenia stokesii*	1.3
Alligator Reef	AR1	*Colpophyllia natans*	4.6
	AR2	*Siderastrea siderea*	4.6
	AR3	*Palythoa caribbea*[b]	4.6
	AR4	*Palythoa caribbea*[b]	4.6
	AR5	*Siderastrea radians*	6.1
	AR6	*Siderastrea radians*	6.1
	AR7	*Siderastrea radians*	6.1
	AR8	*Siderastrea siderea*	6.1
	AR9	*Dichocoenia stokesi*	6.1
	AR10	*Dichocoenia stokesi*	6.1

[a]Florida Keys Marine Lab (ML) was the nearest to shore, Long Key hardbottom (LK) was farther offshore, and Alligator Reef (AR) was on the outer reef tract, approximately 6.5 km from shore. All water column samples were collected from a depth of ~ 0.5 m.
[b]Zoanthid.

Viruses were concentrated by adsorption-elution according to the method described by Katayama et al. (2002) with slight modifications. For each sample, 2 liters of water or 20 ml of well-mixed mucus were acidified with 1 N acetic acid to reduce the pH to ~4.0 (the isoelectric point of most enteroviruses [Lukasik et al., 2000]). The acidified samples were filtered through sterile HA membranes (Millipore, Billerica, MA) and rinsed with 100 ml of 0.5 mM H_2SO_4. Viruses were eluted from the membranes with 10 ml of 1 mM NaOH into a sterile 15-ml conical tube containing a 100 × TE and 50 mM H_2SO_4 solution for neutralization. Eluent was further concentrated by ultrafiltration to ~2 ml (Centriprep-50, Millipore). Total RNA was extracted and purified from 200 µl of concentrated viral samples and eluted in 50 µl of RNase free water using the RNeasy Kit (Qiagen, Valencia, CA). RNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) specific for human enteroviruses using previously described primers and reaction conditions (DeLeon et al., 1990; Griffin et al., 1999). Products were confirmed by gel electrophoresis and dot-blot hybridization using an oligonucleotide probe internal to the amplified region (Griffin et al., 1999). Sterile 0.02-µm-filtered Ultrapure water (US Filter, Warrandale, PA) was used as a no-template negative control. Poliovirus Lsc-1 (courteously provided by Dr. C.P. Gerba) was used as a positive control.

Results

Water temperature (28.7°C offshore to 31.8°C nearshore), pH (7.9–8.0) and salinity (36 parts per thousand) varied slightly between stations, but values were typical for summertime levels in the Florida Keys. Among the three sta-

Table 2. Indicator Bacteria and Human Enteric Viruses Detected from Water and Coral Mucus Samples at Each Station[a]

Station	Sample ID	Fecal coliform bacteria (CFU/100 ml)	Enterococci (CFU/100 ml)	Enterovirus[b]
Florida Keys Marine Lab	ML SW	63	75	−
	ML1	220	330	−
	ML2	245	280	+
	ML3	230	175	+
	ML4	235	370	−
Long Key hardbottom	LK SW	BDL[c]	BDL[c]	−
	LK1	45	150	+
	LK2	35	60	++
	LK3	5	35	+
	LK4	BDL[d]	15	−
	LK5	BDL[d]	15	+
	LK6	BDL[d]	5	+
	LK7	BDL[d]	10	−
	LK8	BDL[d]	BDL[d]	+
	LK9	BDL[d]	BDL[d]	+
	LK10	30	85	++
Alligator Reef	AR SW	BDL[c]	BDL[c]	−
	AR1	BDL[d]	BDL[d]	−
	AR2	BDL[d]	BDL[d]	++
	AR3	BDL[d]	BDL[d]	+
	AR4	BDL[d]	BDL[d]	−
	AR5	BDL[d]	BDL[d]	++
	AR6	BDL[d]	BDL[d]	+
	AR7	BDL[d]	BDL[d]	+
	AR8	BDL[d]	BDL[d]	+
	AR9	BDL[d]	BDL[d]	−
	AR10	BDL[d]	BDL[d]	++

SW, surface water; BDL, below detection limits; CFU, colony forming units.

[a]Numbered samples = coral mucus (see Table 1 for species identification).

[b]Presence/absence based on dot blot hybridization of polymerase chain reaction (PCR) product. Relative intensity of signal is designated by the number of plus signs.

[c]Detection limit for water was 2 CFU 100 ml^{-1}.

[d]Detection limit for coral mucus was 5 CFU 100 ml^{-1}.

tions, five scleractinian coral species and one zoanthid were represented (Table 1).

Fecal coliform bacteria and enterococci were detected in the water column only at the nearshore station (ML), at 63 CFU 100 ml^{-1} and 75 CFU 100 ml^{-1}, respectively. Levels averaged 3.69- to 3.85-fold greater than in the overlying water column for fecal coliform bacteria and enterococci, respectively (Table 2). All of the bacterial levels decreased with distance from shore. At the mid-offshore station (LK), 80% of corals were positive for fecal coliform bacteria or enterococci, at 5 to 45 CFU fecal coliform bacteria 100 ml^{-1} and 5 to 150 CFU enterococci 100 ml^{-1}. In the offshore station (AR), bacterial indicators could not be recovered from any sample (Table 2).

While human enteroviruses were not detected in the water column from any station, they were consistently detected from coral mucus at all sites; 50% (2 of 4) of the mucus samples from the nearshore station (ML), 80% (8 of 10) at the mid-offshore station (LK), and 70% (7 of 10) of the samples at the offshore station (AR) were positive (Table 2). There was no apparent relationship between coral species and detection rate. There was also no significant relationship between the concentration of bacterial indicators and the presence of enteric viruses (Table 2).

Discussion

Results from this study demonstrated a trend in increased detection rates of both bacterial indicators and human enteroviruses in coral mucus relative to the overlying water column, supporting previous reports on nearshore corals (Lipp et al, 2002). The increased distance from shore and the resulting elevated exposure of fecal microorganisms to environmental stressors (e.g., UV, salinity, and high temperatures) may explain the reduction in indicator concentrations offshore compared to the nearshore site (Bordalo et al., 2002; Fujioka and Yoneyama, 2002).

Enteric viruses were as prevalent in the offshore stations as they were at stations closer to shore, independent of the concentration of indicator bacteria. These results are consistent with previous work that has demonstrated that enteric viruses are more stable in marine water than indicator bacteria (e.g., Gerba et al., 1979; Fujioka and Yoneyama, 2002). Additionally, results from this study, as well as those of Lipp et al. (2002), demonstrated that viruses were more likely to be detected in coral mucus than in the overlying water column. In these analyses, viruses were concentrated 1000-fold (2 liters to 2 ml) from water samples but only 10-fold (20 ml to 2 ml) from coral mucus; assuming detection efficiencies are similar, the high prevalence of positive samples from corals compared to water suggests that enteroviruses are enriched in coral mucus. It is unclear if viruses are merely trapped in the coral mucus or if this substance offers a protective environment relative to the water column. Factors such as UV radiation, temperature, and grazing are known to impact viral stability in environmental waters (Bitton, 1980; Le Guyader et al., 1994; Suttle and Chen, 1992; Wetz et al., 2004); the presence of coral mucus may reduce these pressures. To address this issue, ongoing research is investigating the stability of enteric viruses in coral mucus versus seawater over time.

As residential and tourist populations increase in tropical and subtropical areas, the volume of wastewater disposed is also rising. While increasing the potential for contamination, the influx of people also translates into increased risk for exposure to sewage-associated pathogens during recreational activities such as snorkeling and Scuba diving. Additionally, recent reports suggest that human activities and sewage contamination might affect reef health through the introduction of sediments associated with runoff and construction, possible coral pathogens (Patterson et al., 2002), opportunistic enteric heterotrophs (Frias-Lopez et al., 2002), or nutrients, which may exacerbate certain coral diseases (Bruno et al., 2003). Research is needed to determine if the sources of these agents are of human (fecal) origin; therefore, targeted methods to determine the distribution and extent of human wastewater contamination are needed both to protect public health and to facilitate the management of reef resources.

Conclusions

Anthropogenic contaminants in coastal environments can adversely impact ecosystem as well as human health. Several reports have demonstrated that nearshore waters of the Florida Keys are impacted by septic systems and injection wells, and contaminants may present a risk to recreational swimmers (Griffin et al., 1999; Paul et al., 1995, 1997). In 2002, Lipp et al. published research that demonstrated that coral mucus might concentrate enteric bacteria and viruses from the overlying water column in nearshore environments, but no studies, to date, have shown that human pathogens or enteric bacteria might reach offshore recreational areas or the popular outer reefs in the Florida Keys. Here we show for the first time that human sewage markers (human enteric viruses) can be detected consistently along a nearshore-to-offshore transect in the middle Florida Keys. Furthermore, the high rate of detection of viruses in coral mucus rather than in the water column suggests that the coral mucus can be used as part of a biomarker analysis to determine exposure of offshore environments to human sewage.

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