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A Preliminary Investigation of Fecal Indicator Bacteria, Human Pathogens, and Source Tracking Markers in Beach Water and Sand

Kelly D. Goodwin[1,*], **Lisa Matragrano**[2,†], **David Wanless**[2], **Christopher D. Sinigalliano**[2], **Michael J. LaGier**[2,‡]

[1]National Oceanographic and Atmospheric Administration, Atlantic Oceanographic and Meteorological Laboratories, 4301 Rickenbacker Causeway, Miami, FL, 33149, stationed at the Southwest Fisheries Science Center, 8600 La Jolla Shores Drive, San Diego, CA 92137, USA

[2]Cooperative Institute of Marine and Atmospheric Studies, University of Miami, 4600 Rickenbacker Causeway, Miami, FL, 33149, USA

Abstract

Data suggesting that fecal indicating bacteria may persist and/or regrow in sand has raised concerns that fecal indicators may become uncoupled from sources of human fecal pollution. To investigate this possibility, wet and dry beach sand, beach water, riverine water, canal water, and raw sewage samples were screened by PCR for certain pathogenic microbes and molecular markers of human fecal pollution. The targets included in this study were human specific *Bacteroides* (HF8 marker), human-specific enterococci (esp gene), *Staphylococcus aureus*, *Escherichia coli* 0157:H7, *Campylobacter jejuni*, and adenovirus. Sewage samples were also tested for *Salmonella* species. The results were compared to concentrations of enterococci, *Escherichia coli*, and *Bacteroides* species, as determined by membrane filtration methods. Molecular analysis yielded positive results for human specific *Bacteroides*, and *S. aureus*, in samples of raw sewage. Two of the environmental samples were positive for human specific *Bacteroides* and one was positive for *S. aureus*. The PCR screen was negative for other samples and targets, despite exceedance of EPA single sample guidelines for recreational waters on several of the sample dates (5/11 dates). However, estimates of the number of cells delivered to the PCR reaction suggested that few of the samples met the detection limit of the PCR reaction due to a variety of factors. The analysis indicated a need to improve nucleic acid processing in order to enable better delivery of DNA to downstream molecular methods.

Introduction

The United States Environmental Protection Agency (EPA, 2003) and the Food and Drug Administration (FDA/ISSC, 2003) use fecal indicating bacteria to regulate the closure of

[*]Phone: 858-546-7142, kelly.goodwin@noaa.gov.

[†]Current address: Emory University, Graduate Division of Biological and Biomedical Sciences, 1462 Clifton Rd, Suite 314, Atlanta, GA 30322, USA

[‡]Current address: University of North Carolina at Chapel Hill, Department of Genetics, CB#7264, 111 Mason Farm Road, Chapel Hill, NC 27599-7264, USA

recreational and shell fish harvesting waters. Fecal indicating bacteria are not necessarily human pathogens; instead, they are bacteria whose presence is supposed to indicate the existence of sewage-associated pathogens. Fecal indicating bacteria are employed because they are abundant in comparison to the actual pathogens that cause waterborne illness. In addition, measuring all potential pathogens in a given water sample would be technically and financially unfeasible. Therefore, fecal indicators are typically used to monitor and manage coastal water quality. In practice, a fecal indicator should be: (1) a member of the intestinal flora of warm blooded animals, (2) non-pathogenic, (3) present when pathogens are present and absent when they are not, (4) present in greater numbers than the pathogen, (5) unable to multiply in the environment, (6) at least equally resistant as the pathogen to environmental factors and to disinfection in water and wastewater treatment plants, and (7) detectable by means of rapid, easy, and inexpensive protocols (Bitton, 2005; National Research Council, 2004).

The risk of gastrointestinal illness is correlated to the concentration of fecal indicating bacteria when waters receive point sources of human fecal pollution (Cabelli et al., 1979; Dufour, 1984; Wade et al., 2003), and such illnesses cause negative economic impacts (Dwight et al. 2005). However, there is question whether concentrations of fecal indicators reliably predict the presence of human pathogens in regions that do not have point sources of human fecal contamination. For example, both *E. coli* and *enterococci* have been found in areas without apparent sewage contamination (Carillo et al., 1985; Rivera et al., 1988). Furthermore, there is evidence that fecal indicating bacteria may persist outside of their hosts (Solo-Gabriele et al., 2000; Wright, 1989). If fecal indicators are able to multiply in the environment, the theory of fecal indicators (described above) would be violated.

The role that sand plays in the survival of fecal indicating bacteria is an area of growing interest (Gerba and McLeod, 1976; Hood and Ness, 1982; Davies et al., 1995; Lee et al., 2006). Research suggests that sand may act a bacterial reservoir, providing a source of fecal indicators to adjacent waters (Yamahara et al. 2007; Whitman and Nevers, 2003; Craig et al., 2002; Goyal et al., 1977). However, only fecal indicator concentrations in water are currently monitored; therefore, there is growing concern that public exposure to human pathogens may be underestimated (Clean Beaches Council, 2005).

An underestimation of risk might occur if high concentrations of fecal indicators in sand signified the presence of pathogenic microbes. Conversely, an overestimation of risk might occur if persistence or growth occurred only for the indicators but not for the pathogens. Furthermore, it is possible that risk is estimated incorrectly by not considering the concentration of nonfecal pathogens such as *Staphylococcus aureus*.

To test the hypothesis that fecal indicating concentrations can be uncoupled from the presence of pathogenic organisms, samples from a variety of coastal environments were subjected to a suite of molecular assays in conjunction with traditional methods for detecting fecal indicting bacteria. The assays were designed to detect several human pathogens (*Staphylococcus aureus*, *Campylobacter jejuni*, *E. coli* O157:H7, *Salmonella* species, and adenovirus), and two markers of human fecal pollution (the *esp* gene for human specific *Enterococcus faecium* and the HF8 cluster for human specific *Bacteroides* species).

Materials and Methods

Site Description

Samples were taken from several sites in southern Florida, USA, including: Hobie Beach, Marco Island, Wagner Creek, and the Virginia Key Waste Water Treatment Plant. Hobie Beach is a relatively shallow, semi-enclosed beach located in the southern portion of Biscayne Bay, Miami (Shibata et al., 2004). The Virginia Key Waste Water Treatment Plant is located near Hobie Beach. The outfall and the waters adjacent to Hobie Beach are not thought to have a significant hydrological connection; therefore, Hobie Beach is considered free of point sources of human fecal pollution. Wagner Creek is a tributary to the Miami River, which is an urbanized, tidally influenced river site that is located downstream from flood control gates. Marco Island (Figure 1) is located on the Gulf of Mexico in southwest Florida. Samples were collected from the following stations: Collier Bridge (N 25 56.726′, W 081 44.448′), Barfield Bridge (N 25 57.694′, W 081 43.345′), Perrine (N 25 57.943′, W 081 43.886′) JH Park (N 25 57.121′, W 081 43.830′) Hollyhock (N 25 56.607′, W 081 41.558′), Hummingbird (N 25 56.544′, W 081 42.189′), HC Center (N 25 56.263′, W 081 43.083′).

Sample Collection

Samples of sand or water were collected in sterile containers between June 2004 and June 2007. Sand was collected from Hobie Beach above the high water mark (“dry sand”) or from the surf zone at knee-deep depth (“wet sand”). All samples were transported on ice and kept cool until processed within 6 hours of collection. Raw sewage samples were collected on 17 September 2005 from the liquid sewage of the primary settling tank from the Miami-Dade Water and Sewer wastewater treatment plant on Virginia Key, Miami, Florida.

Sample Processing and Enumeration

Samples for bacterial culture analysis were filtered onto 47 mm, 0.45 μm, cellulose nitrate membrane filters (Whatman) and rinsed with 20 ml phosphate buffered saline (PBS), according to standard membrane filtration protocols (EPA, 2002). Typically 3 to 5 dilutions were plated and values for plates with countable colonies (~6 to 100) were averaged to obtain the bacterial density for that sample. Samples were processed within 6 hours of collection. The filters were placed on selective media and incubated as outlined in Table 1. Anaerobic conditions for selection of *Bacteroides* were generated using the Gas Pak EZ-Anaerobe Container System with GasPak indicators (Becton Dickenson).

Samples for nucleic acid analysis were filtered onto 47 mm, 0.2 μm, hydrophilic polyethersulfone membrane filters (Supor-200, Pall). Filters were placed in Analyslide® petri dishes (Pall) and frozen at −80 °C until used for DNA extraction.

Membrane filtration for sand samples was achieved by first vigorously hand shaking 2 g of sand into 80 ml of PBS for 2 min (Baums et al. 2007). This solution of sand and dislodged particles was vacuum filtered through a sterile, 30 μm, 47 mm nylon net filter (Millipore). Two additional 10 ml rinses with PBS were used to remove any remaining sand from the shaking container. This procedure was repeated until a sufficient volume of “sand

water" was generated to satisfy the membrane filtration needs for that site. The sample was homogenized by hand mixing prior to filtration.

The water content of the sand was determined by weighing sand aliquots before and after overnight drying at 110°C. Concentrations of bacteria were calculated in terms of colony forming units (CFU) per 100 ml of water or CFU per gram dry sand. To estimate the number of bacterial cells available for DNA extraction, the CFU values were multiplied by the amount of sand processed onto the Supor-200 membrane filters used for DNA extraction.

Viral analysis was performed on sand samples collected 1 February 2006 and on water samples collected on 14 March 2006 and 19 April 2007, as outlined in Figure 2. In these cases, samples for bacterial culture analysis were filtered through Durapore HV (Millipore HVLP04700) or through cellulose nitrate filters (Whatman 7141104) (47 mm, 0.45 μm). The filtrate was saved and combined and then filtered through a 90 mm, 0.45 μm HA filter (Millipore HAWP04700) using a custom-made filter holder (courtesy of Dr. H. Solo-Gabriele, University of Miami). The filter was rinsed with 200 ml of 0.5 mM H_2SO_4, pH 3.0. Elution was achieved with 5 ml of 1 mM NaOH, pH 10.5 (Katayama et al. 2002), and the eluant was neutralized with 50 μl of 50 mM H_2SO_4 and 100× TE buffer.

DNA Extraction

DNA was obtained from sand using the FastDNA Spin Kit for Soil (Q-BIOgene). Either the sand or the membrane filter used to process the sand was placed into the microcentrifuge tubes supplied by the kit. DNA from membrane filters used to process water or raw sewage was obtained either with the FastDNA Spin Kit for Soil or the FastDNA Spin Kit using the plant protocol (Q-BIOgene). In addition, some membrane filters were incubated on growth media to allow colony formation (Table 1). The goal was to filter enough water to achieve confluent growth; however, this was not achieved in all cases. Growth filters were placed into 50 ml conical tubes and the cells were dislodged by shaking the filters at 225 rpm for 1 hr in 25 ml 1× PBS. The solution was pelleted by centrifugation at 3000×g for 15 min. The pellets were resuspended in 600 μl of the lysis solution from the Wizard DNA Spin Kit (Promega) and the protocol for Gram (+) or Gram (−) bacteria was followed, depending on the growth medium.

Sources of Control DNA

A plasmid containing cloned DNA from the human-specific *esp* gene of *E. faecium* (Scott et al., 2005) was provided by Dr. Joan Rose (Michigan State University). A plasmid containing the PCR target for adenovirus (He and Jiang, 2005) was supplied by Dr. Sunny Jiang (University of California at Irvine). The human-specific HF8 cluster of *Bacteroides* (Bernhard and Field, 2000) was PCR amplified from human fecal DNA and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). DNA from *E. coli* (ATCC #25922) and *E. faecalis* (ATCC #29212) was isolated from cultures using the FastDNA SPIN Kit for Soil. DNA from *Campylobacter jejuni*, *Salmonella typhi*, and *E. coli* 0157:H7 were provided by Dr. Nick Cirino (New York State Department of Health). DNA from *Staphylococcus aureus* was obtained from ATCC (#700699D).

Target Amplification and Screening

The PCR primer sequences, thermal cycling conditions, amplicon sizes, and references for the procedures are given in Table 2. The PCR reactions for pathogens contained 5μl of 10x DyNAzyme II buffer (contains 1.5 mM $MgCl_2$), 1.25 μl of 0.2 mM dNTP, primers (concentrations given in Table 2), 0.75 μl of DyNAzyme II DNA polymerase (Finnzymes), 1.50 μl BSA (10 mg/ml), 5 μl of DNA sample, and nuclease free water for a total reaction volume of 50 μl.

For the PCR of *Salmonella*, each 50 μl PCR reaction also contained 2% formamide (v/v) and an additional 1U of DNA polymerase. Positive and negative controls contained all of the PCR reagents but with 5 μl of isolated genomic DNA or nuclease free water, respectively.

DNA was amplified by standard PCR with an Eppendorf Mastercycler and PCR products were visualized using standard gel electrophoresis with 1% agarose gels (w/v). All of the samples were first run with 5 μl of sample. Negative samples were tested for PCR inhibition by adding 1 μl (10 ng) of the positive control genomic DNA to the 5 μl sample being tested and reanalyzed to determine possible inhibition. If grossly inhibited, the expected band of a specific amplicon size would not be seen or would be dim relative to the positive control. Inhibited samples were re-amplified using 1 μl of sample rather than 5μl, and in some cases the DNA was first diluted 1:10 prior to amplification.

DNA Sequencing of Colonies Picked from Agar Plates

Colonies were picked from BBE and BVSA agar plates and sequenced to determine the identity of the colonies that had grown. A single colony was placed in 25 μl sterile water and subjected to 95°C for 10 min in order to release the DNA from the cells. An aliquot of the solution (1 to 5 μl) was amplified for the 16S rRNA gene using a Bacteroides forward and a universal 16S rRNA reverse primer (Bacterfor/Unirev800) to increase the concentration of Bacteroides-like sequences. Amplification reactions contained 25 μl of HotStar MasterMix (Qiagen), 80 pmol of each primer, 3 μl genomic DNA (colony solution), and nuclease-free water for a final volume of 50 μl. A PTC-100 thermocycler (MJ Research) was used to amplify the DNA with the following PCR conditions: 94°C for 10 min; 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and a final 8 min extension at 72°C. The amplicons were purified with the QIAquick PCR Purification Kit (Qiagen). Sequencing reactions were performed using the BigDye 3.1 sequencing kit (Applied Biosystems) in two directions, using forward and reverse primers. The two different reaction mixtures included the following: 1.075 × buffer, 0.32 pmol of Bacterfor or Unirev800 primer, 6 μl plasmid DNA, 1/16 dilution of BigDye 3.1 mix, and nuclease free water for a final volume of 10 μl. Reactions were sequenced on an ABI 3730 capillary sequencer (Applied Biosystems).

Forward and reverse sequences were joined using the ContigExpress program of the Vector NTI 9.1 computer software (Invitrogen). Sequences were submitted to GenBank for BLAST analysis (http://www.ncbi.nlm.nih.gov/). The most homologous genes to the submitted sequences were included in a sequence alignment using the AlignX program of the Vector NTI 9.1 software (Invitrogen). Phylogenetic trees were produced to illustrate the percentage of similarities among the sequences. The tree was generated by PAUP*

4.0 software (Sinauer Associates) using the neighbor-joining algorithm with the Kimura 2-parameter correction factor.

Results

Bacterial Concentrations and Bacteria Available for DNA Extraction

Sand Samples—Bacterial concentrations in beach sand collected from Hobie Beach ranged from 77 to 2695 CFU/g dry weight for *E. coli* and from 73 to 445 CFU g/dry weight for enterococci (Table 3). Essentially no putative *Bacteroides* spp. colonies were observed on BBE or BVSA plates from dry or wet sand. The bacterial densities obtained from culturing were used to estimate the number of cells on membrane filters used for DNA extraction. This analysis (Table 3) showed that most of the membrane filters contained approximately 100 *E. coli* cells (76 – 128). Two of the samples had over 1000 *E. coli* cells and one sample contained over 66,000 *E. coli* cells prior to DNA extraction.

The enterococci values were similar; most filters had about 100 enterococci cells (55–187), three samples had around 1000 cells (644 – 1740), and one sample had approximately 4900 cells. There did not appear to be a significant number of *Bacteroides* cells in the sand samples used for PCR analysis.

Water Samples—Lower bacterial concentrations were observed in seawater samples collected from Hobie Beach or Marco Island compared to river water from Wagner Creek (Tables 4 and 5). Concentrations of *E. coli* in seawater ranged from <1 – 83 CFU/100 ml and enterococci concentrations ranged from <1 – 61 CFU/100 ml. Few putative *Bacteroides* spp. were identified in the Hobie Beach samples and thus this parameter was not analyzed with the Marco Island samples (Table 4). In contrast, the river water samples ranged from 235 – 7600 CFU/100 ml *E. coli* and 47 – 5098 CFU/100 ml enterococci.

Unlike the other tested sites, a significant number of putative *Bacteroides* spp. colonies were obtained (62 – 850 CFU/100 ml). The estimated number of cells on the membrane filters used for DNA extraction was correspondingly higher in the river samples than for the seawater samples. As expected, the raw sewage samples contained high concentrations of fecal indicating bacteria, with concentrations ranging from 4×10^6 to 6×10^6 CFU/100 ml for *E. coli,* enterococci, and *Bacteroides* spp. (Table 6).

Molecular Detection of Source Tracking Markers and Bacterial and Viral Pathogens

The marker for human-specific *Bacteroides* spp. (HF8 human cluster) and the pathogen *S. aureus* were positively detected by PCR in the samples of raw sewage. The human-specific enterococci marker (*esp* gene) and the pathogens *E. coli* O157:H7, *Salmonella* spp., *C. jejuni*, and adenovirus were not detected (Table 7).

Two environmental samples were positive for human-specific *Bacteroides* spp. (HF8 human cluster) and one was positive for *S. aureus.* Samples 31 and 35 were positive for human specific *Bacteroides* and sample 31 was positive for *S. aureus* (Table 8). It is interesting to note that the replicate of sample 31 (sample 32) was negative for both *S. aureus* and the human *Bacteroides* marker. These samples differed only in the treatment of the filter, in that

sample 32 was cut into pieces before being placed into the microfuge tube used for DNA extraction, whereas sample 31 was left intact (Table 8).

Many of these samples contained low amounts of fecal indicating bacteria (Tables 3–5); therefore it is not surprising that source tracking markers and pathogens were not detected. However, a few samples did contain high numbers of fecal indicating bacteria (samples 3, 29, 30, 31, 32). Nonetheless, source tracking markers and pathogens were not detected in these samples (Table 8).

A two-part enrichment step is recommended to achieve detection of the *esp* gene (Scott, 2005), but it was not used here for the sewage samples or for dry sand samples (runoff ditch) collected 5 June 2007 (#36, 37, 38). Three of the environmental samples (#39, 40, 41) did receive an incubation step. Filters were incubated on MeI agar plates to select for enterococci (Table 1), and DNA was extracted from the growth filters. DNA from dry sand collected 10 February 2006 (sample #39) was extracted from 69 CFU of enterococci. DNA from river water collected 19 April 2006 (sample #40) was extracted from 24 CFU of enterococci. DNA from dry sand collected 5 June 2007 (sample #41) was extracted from 883 CFU. None of these samples tested positive for the *esp* gene (Table 8).

DNA Sequence Results

Plate culture results indicated the presence of a high number of *Bacteroides* spp. in Wagner Creek river water (Table 5) in comparison to seawater samples. Two Wagner Creek samples were positive for human specific *Bacteroides*; whereas, none of the seawater samples were positive. In contrast, other Wagner Creek samples with high *Bacteroides* counts were not positive for human-specific marker.

Bacteroides counts could have been overestimated if the media used were not selective. Colony sequencing was used to investigate the specificity of BBE and BVSA plates when used with environmental samples. A portion of the 16S rRNA gene was sequenced for colonies grown from samples collected from Hobie Beach, Wagner Creek, and sewage samples. The analysis showed that some of the picked colonies contained sequences closely related to those of known *Bacteroides* species (Figures 3 and 4), although few of the sequences obtained from environmental samples were exact matches to sequences available in Genbank.

Phylogenetic relationships were determined for partial 16S rRNA gene sequences (654 base pairs) from colonies grown from river or beach water and picked from BBE or BVSA plates designed to select for *Bacteroides* species (Figure 4). Some sequences (9/52) were related to named *Bacteroides* species or were closely related to various uncultured human intestinal flora, but few were exact matches (Figure 4).

Many colonies were not related to *Bacteroides*. Sequences related to *Cetobacterium somerae, Parabacteroides distasonis, Klebsiella oxytoca, E. coli,* and *Aeromonas hydrophila* were obtained (Figure 4). This data demonstrates that the BBE and BVSA plates were not perfectly selective for *Bacteroides* spp. when used for environmental samples. Overall,

the paucity of exact matches to species previously identified in GenBank illustrated the molecular diversity present in these samples.

Discussion

Several studies support the idea that sand acts as a reservoir for fecal indicating bacteria (Alm et al., 2006; Lee et al., 2006; Yamahara et al. 2007). Sand may protect adsorbed bacteria by reducing exposure to stressors such as ultraviolet radiation, high salinity, high temperatures, and wave action. Studies also have suggested that the persistence of bacteria in sediments may result from a balance between the rates of bacterial growth versus predation. (Davies et al., 1995). The survival of enteric bacteria on dry sand has been hypothesized to be minimal due to environmental stresses including a lack of adequate moisture and nutrients (World Health Organization, 2003). However, dry sand in this study harbored a significant number of fecal indicating bacteria (Table 3).

The possibility that fecal indicators can grow and persist in sand and become a source of these bacteria to adjacent waters raises the possibility that the fecal indicators can be independent of human feces and thus not indicative of the presence of pathogens. An alternative hypothesis is that if sand is a reservoir for fecal indicators it may also be a reservoir for pathogens. This preliminary study performed PCR screening of samples collected from rivers, canals, beach water, and beach sand in order to look at the relationship between fecal indicator concentrations and several pathogens and source tracking markers.

Raw sewage was used as a positive control to compare against sand and water results. As expected, sewage showed the most bacterial colony growth and positive hits in the PCR screen. *S. aureus* and the HF8 human marker for *Bacteroides* spp. were detected in the samples (Table 7). *Salmonella* spp., *E. coli* O157:H7, *C. jejuni*, and adenovirus were not detected. Unlike *S. aureus*, which is carried by 30–50% of the population (Youmans et al., 1985), these other pathogens are not expected in healthy individuals; therefore, lack of detection may have been due to the lack of their presence at the time of sampling. In addition, the sewage samples were not processed by the viral sorption method (Fig. 2), making it less likely to achieve viral detection in those samples.

Although only a subpopulation is expected to carry the human specific enterococci marker (Shankar et al., 1999), the lack of detection of the *esp* gene in the sewage samples was unexpected. However, this analysis did not utilize a preincubation step. The published protocol suggested a two-stage culture step in which filtered cells were grown at 41 °C on MeI agar for 48 hr and then the filter was incubated at 41 °C in tryptic soy broth for 3 hr (Scott et al., 2005). The lack of enrichment step for the sewage samples may have accounted for the lack of detection of the *esp* gene. Three samples (#39, 40, 41) were preincubated on MeI agar, and DNA was extracted from the growth filters. None of these samples tested positive for human specific enterococci (Table 8). However, two of the samples (# 39, #40) had few enterococci colonies from which to extact DNA (see results section); therefore, negative results were not surprising. For the third sample, it is possible that the PCR was inhibited due to carry over of inhibitory substances in the MeI agar.

On 45% of the sampling dates (5/11), the water exceeded EPA standards for recreational water quality (EPA, 2003) using the single sample standard of ≥104 CFU of enterococci/100 ml or ≥235 CFU of *E. coli*/100 ml (Tables 4 and 5). On 80% of the sampling dates (4/5), the sand had relatively high concentrations of fecal indicating bacteria, as defined here by ≥ 100 CFU/g dry weight sand of enterococci or *E. coli* (Table 4). How these values translated to the number of cells available for molecular analysis greatly depended on the processing procedures used (Tables 3–5). In some cases, less cells were available for molecular detection than was implied by the bacterial density because of the small amount of sample processed (Table 3). Six of the 27 samples had no cells on the DNA extraction filters, despite filtering 100 ml of canal water (Table 4). Nine of the 27 samples had <100 cells available on the DNA extraction filter, and 10 of the samples had >100 cells available. The remaining samples (3/27), had >9500 cells (Table 5), and one of those had on the order of 10^4 enterococci cells, similar to what was seen in sewage samples. Only two samples returned positive detects in the PCR screen. Sample 35 was positive for human specific *Bacteroides* and sample 31 was positive for HF8 and *S. aureus*. Both of these samples had high numbers of cells available for DNA extraction (Table 5); although other samples that were negative in the screen contained even higher numbers of cells (Table 5).

The detection limits for some of the molecular targets used in this study have been previously determined. The detection limit for the *Bacteroides* spp. human marker (HF8) was found to be 1 plasmid per PCR reaction, and the detection limit for the human specific enterococci marker (*esp* gene) was found to be 10 plasmid copies per PCR reaction (LaGier et al., 2007). Other research found that the *Bacteroides* marker could be detected by PCR in sewage samples diluted to 1:150,000 (Bower et al., 2005), which would equate to ~3 *Bacteroides* cells in the PCR reaction for the samples used in this study. For the *esp* gene, approximately 100 CFU of enterococci was needed on a growth plate to achieve detection, and this detection limit included a two-step enrichment procedure, as described above (Scott et al. 2005).

Although the detection limits mentioned above appear low, there are several reasons that concerns remain about false negative results possibly arising from an inability to meet the detection limit of the PCR assays. First, previous work and the analysis here have shown that the media were not completely selective when used with environmental samples. For *Bacteroides*, the per cent match ranged from 6–39% (Table 9) (Baums et al. 2007). In the work presented here, sequencing of putative *Bacteroides* colonies (Figures 3 and 4) confirmed that many of the sequences (40/52) were not closely related to *Bacteroides* spp.; therefore, the number of *Bacteroides* actually available for DNA detection was less than that estimated from the colony counts. In addition, the source tracking markers are expected to be present in only a subset of the *Bacteroides* or enterococci colonies because not every human is a carrier of these markers. These factors act to reduce the number of targets available for molecular detection as compared to the number of cells estimated to be available on the DNA filters (Tables 3–5).

Of even more concern with regard to meeting the detection limit of a PCR assay is the fact that few of the cells on a DNA extraction filter are likely to reach the PCR reaction. The number of cells delivered to the PCR reaction depends on several factors such as 1) the

DNA extraction efficiency, 2) the final volume of the eluant or lysate, 3) the amount of the eluant or lysate put into the PCR reaction, 4) the amount of dilution necessary to overcome PCR inhibition, and 5) the amplification efficiency. For example, if one assumes a DNA extraction efficiency of 30% (Mumy and Findlay, 2004), an eluant volume of 100 µl, the use of 1 µl of template in the PCR reaction, no dilution necessary to achieve PCR (which was not always the case here), and an amplification efficiency of 1 (although less is expected in reality), it would take 1000 cells on the DNA extraction filter in order to deliver 3 cells into the PCR reaction. Only one environmental sample is estimated to have met this criteria for *Bacteroides* (Table 5). In comparison, ~3,350 cells would be needed to deliver the 10 cells required for the *esp* assay. This criteria for enterococci was met for 4 samples, and 5 samples met this criteria for *E. coli*. Out of these samples, none returned positive detections for any of the tested targets.

This analysis points to the need to find better methods of extracting nucleic acids from environmental samples in order to reduce the possibility of false negative results. Pre-incubation is a strategy, but that does not enable rapid detection. Overall, this study could not fully support or deny the hypothesis that the concentrations of fecal indicating bacteria can be uncoupled from markers of human fecal pollution or with human pathogens. This analysis illustrated the need to improve the current standard practices of sample concentration and DNA extraction.

Conclusion

The PCR screen returned positive results for human specific *Bacteroides* and *S. aureus* for the raw sewage samples. In addition, positive results were obtained for two of the environmental samples (sample 31 and 35 for human specific *Bacteroides* and sample 31 for *S. aureus*). This preliminary investigation did not find a correlation between the concentration of fecal indicators and detection of a variety of human pathogens and source tracking markers. However, the possibility of false negative results made it difficult to properly test the hypothesis that fecal indicating bacteria can become uncoupled from human fecal pollution. This analysis suggested that most of the samples may not have been able to achieve the detection limit of the PCR reaction, despite the fact that several of the water samples exceeded single sample standards for fecal indicators and many of the sand samples contained relatively high counts of fecal indicating bacteria. Failure to meet the detection limit likely could arise from a combination of factors that effectively reduce the number of cells that reach the PCR reaction tube from the DNA extraction filter. These factors include the DNA extraction efficiency, the amount of sample dilution due to the volume of eluant or the need to remove PCR inhibition, the amount of template in the PCR reaction and the overall amplification efficiency (which will be reduced by PCR inhibitors present in the sample).

Overall, this analysis pointed to the need to find better methods of extracting nucleic acids from environmental samples in order to reduce the possibility of false negative results. High quality nucleic acids need to be consistently and efficiently delivered to the detector system if the relationship between fecal indicators and human pathogens and human source tracking markers is to be elucidated.

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Figure 1.

Satellite image showing water quality collection sites for the city of Marco Island, Florida. For this study, samples were collected from the following stations: 2) Collier Bridge, 4) Barfield Bridge, 5) Perrine, 6) JH Park, 7) Hollyhock, 8) Hummingbird, 12) HC Center. Map provided courtesy of City of Marco Island, Florida Cartography Department.

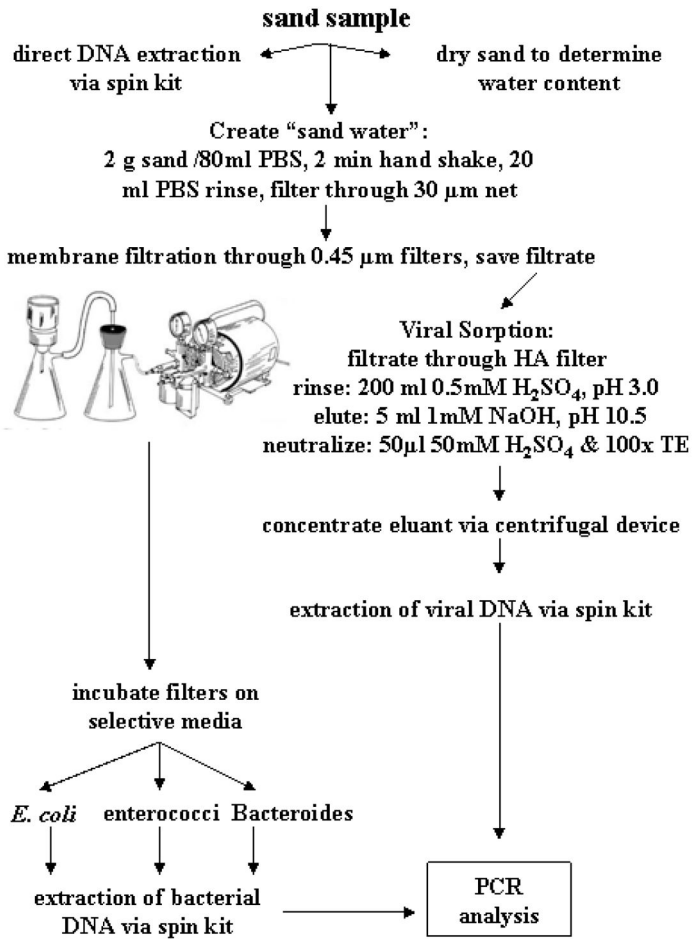

Figure 2.

Schematic of sample processing to perform PCR analyses on sand for bacterial and viral targets.

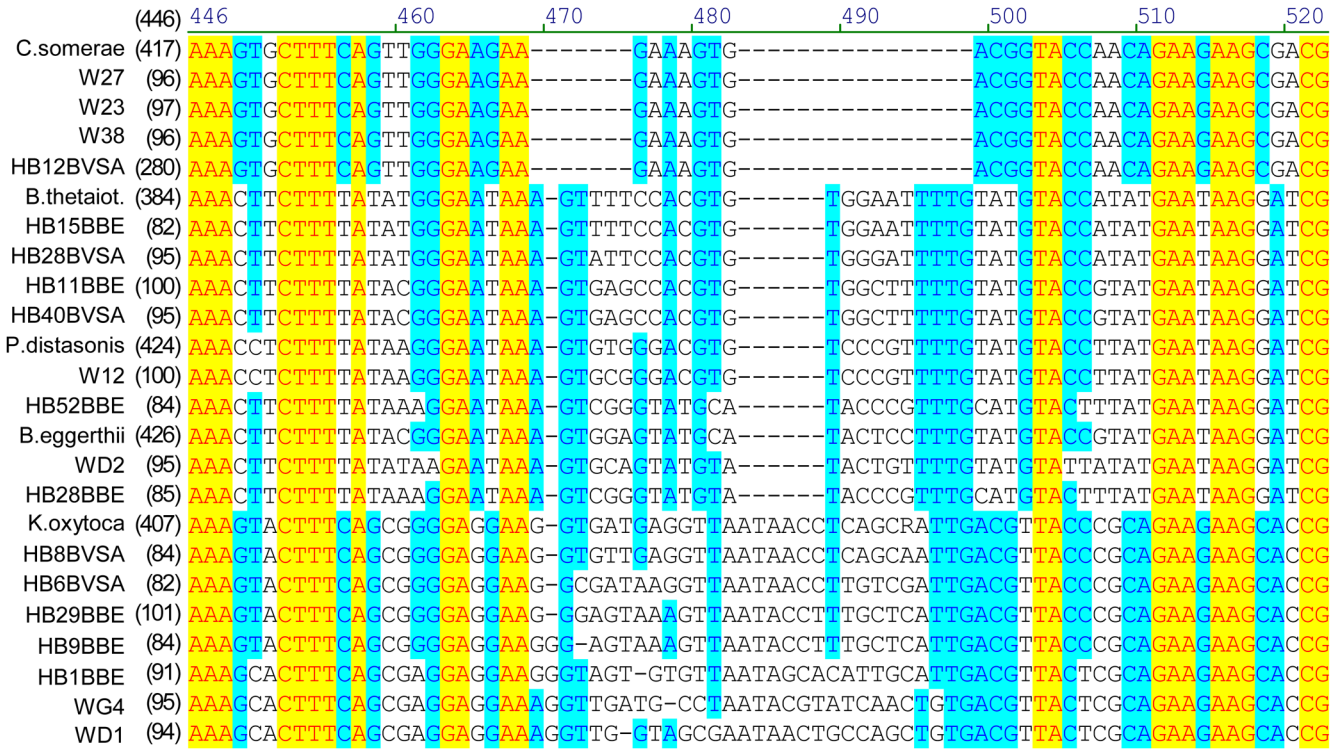

Figure 3.

Portion of an alignment of partial 16S rRNA gene sequences from colonies grown on BBE or BVSA plates from samples of river or beach water.

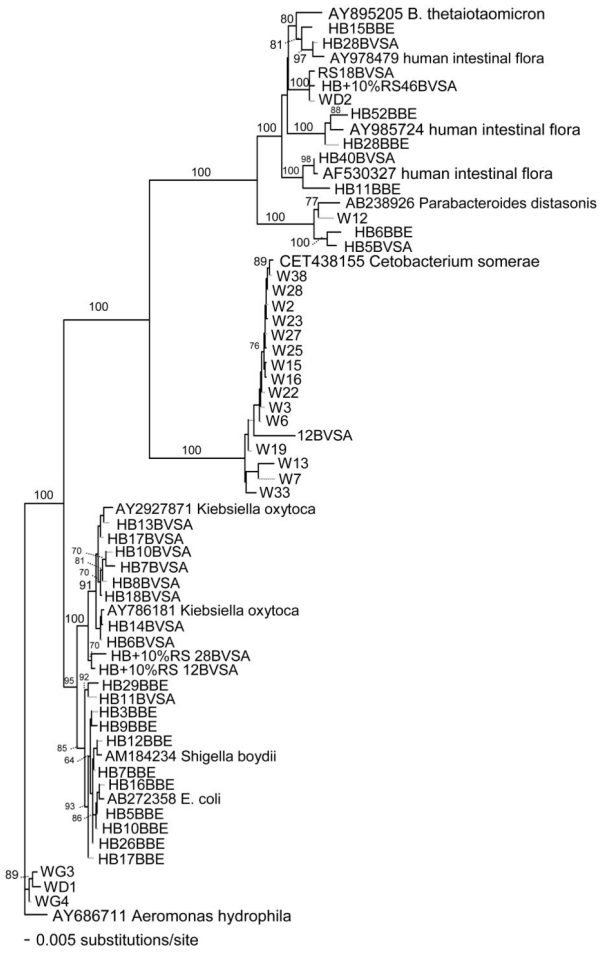

Figure 4.

Phylogenetic relationships of partial 16S rRNA gene sequences (654 bp) from putative Bacteroides colonies grown on BBE or BVSA media. Aeromonas hydrophila was used as an outgroup. Bootstrap values for 1000 trees generated by PAUP* 4.0 are shown for nodes >60.

Table 1

Summary of membrane filtration protocols used to enumerate bacteria in this study

Target	Growth medium	Incubation	Target colony description	Reference
E. coli	Modified mTEC	35 °C for 2 hr, then 44.5 °C for 22 hr	Red or magenta color	EPA method 1603 (EPA 2002)
Enterococcus	mEI	41 °C for 24 hr	Colonies with blue halo (regardless of colony color)	EPA method 1600 (EPA 2002)
Fecal coliform	mFC	44.5 °C for 24 hr	Blue colonies	Standard Method 9222D (American Water Works Association, 1999)
Bacteroides	BBE (Bacteroides Bile Esculin Agar)	Anaerobic, 35 °C for 22 – 48 hr	Brown or black colonies surrounded by a brown zone in the medium	Anaerobe Systems package insert
Bacteroides	BVSA (Bacteroides Vulgatus Selective Agar)	Anaerobic, 35 °C for 22 – 48 h	Brown or black colonies surrounded by a brown zone in the medium	Anaerobe Systems package insert
Total coliform	M-Endo-LES	35 °C for 24h	Red colony with golden metallic sheen	Standard Method 9222B (American Water Works Association, 1999)

Table 2

Summary of PCR and qPCR conditions used in this study

Target	Gene	Primer label[b]-name-sequence, 5′→3′ (μM per PCR reaction)	Cycling[a]	based on Reference
Enterococcus	23S rRNA	ECST748F-AGAAATTCCAAACGAACTTG (0.9) ENC854R-CAGTGGTCTACCTCCATCATT (0.3)	94°C 30 s; 60°C 30 s; 72°C 30 s; 30 cycles	Haugland et al., 2005
Human-specific *Enterococcus faecium*	*esp*	Biotin-espF-TATGAAAGCACAAGTT (0.3) FITC-espR-ACGTCGAAAGTTCGATTTCC (0.3)	94°C 1 min; 58°C 1 min; 72°C 1 min; 40 cycles	Scott et al., 2005
Human-specific *Bacteroides*	16S rRNA	Biotin-HF183F-ATCATGAGTTCACATGTCCG (0.4) FITC-Bac708R-CAATCGGAGTTCTTCGTG (0.4)	94°C 30 s; 59°C 30 s; 72°C 30 s; 40 cycles	Bernhard and Field, 2000
Campylobacter jejuni	*hipO*	Biotin-CjF1-TGCTAGTGAGGTTGCAAAAGAATT (0.5) FITC-CjR1-TCATTTCGCAAAAAAATCCAAA (0.5)	94°C 30 s; 60°C 30 s; 72°C 30 s; 40 cycles	LaGier et al., 2004
Salmonella spp.	*ipaB*	Biotin-IpaBF-GGACTTTTTAAAAGCGGCGG (0.3) FITC-IpaBR-GCCTCTCCCAGAGCCGTCTGG (0.3)	94°C 1 min; 62°C 1 min; 72°C 1 min; 35 cycles	Kong et al., 2002
E. coli 0157:H7	*rfb*	Biotin-0157PF8-CGTGATGATGTTGAGTTG (1.0) FITC-0157PR8-AGATTGGTTGGCATTACTG (1.0)	94°C 30 s; 55°C 30 s; 72°C 30 s; 40 cycles	Maurer et al., 1999
Human adenovirus	Hexon	Biotin-AD2F-CCCTGGTAKCCRATRTTGTA (0.3) FITC-AD3R-GACTCYTCWGTSAGYGGCC (0.3)	94°C 30 s; 60°C 30 s; 72°C 30 s; 40 cycles	He and Jiang, 2005
Staphylococcus aureus	*clfA*	Biotin-clfAF-GCAAAATCCAGCACAACAGGAAACGA (0.1) FITC-clfAR-CTTGATCTCCAGCCATAATTGGTGG (0.1)	94°C 1 min; 55°C 1 min; 72°C 1 min; 40 cycles	Mason et al., 2001

[a] In all cases, the initial heat denaturation step was 94°C for 10 min and the final extension step was 70°C for 8 min;

[b] Biotin and FITC labels were included because the PCR product also was used for other projects that required these labels. FITC = fluorescein.

Table 3

Average bacterial densities in sand (CFU/g dry wt) as determined by culturing, and the calculated cells available for DNA extraction per filter (Sample #) used for PCR analysis. NA = not available

SAND									
Bacterial density (CFU/g dry wt ± STD[a])				**Sample parameters**			**# of cells on DNA extraction filters**		
Sample date	***E. coli***	***Enterococcus***	***Bacteroides***	**Sample #**	**Sand type**	**Amount processed (g dry wt)**	***E. coli***	***Enterococcus***	***Bacteroides***
14-Jul-04	77	73 ± 23	<1	1	wet sand	0.99	76	72	0
				2	wet sand	13.20	1214	660	0
30-Jun-04	2695 ± 448	198 ± 175	<2	3	dry sand	0.37	1001	74	<1
				4	dry sand	24.8	66781	4906	<50
14-Jul-04	79	445 ± 89	<1	5	dry sand	1.45	114	644	0
1-Feb-06	270 ± 28	113 ± 40	<1	9	dry sand	0.49	131	55	0
				10	dry sand	0.49	131	55	0
				11	dry sand	0.49	131	55	0
				12	dry sand	0.49	131	55	0
5-Jun-07[b]	NA	335 ± 153	NA	36	dry sand	0.48	NA	161	NA
				37	dry sand	0.56	NA	187	NA
				38	dry sand	5.2	NA	1740	NA

[a] performed in duplicate or triplicate, when no STD given n=1 countable plate;

[b] taken from a natural drainage ditch that brings runoff to the beach during rain events.

Table 4

Average bacterial densities in seawater (CFU/100 ml) collected from Hobie Beach (knee-deep) or Marco Island (saltwater canal) as determined by growth on selective media and the calculated cells available for DNA extraction for each membrane filter (Sample #) used for PCR analysis. NA = not available

SEAWATER									
Bacterial density (CFU/100 ml ± STD[a])				Sample parameters			# of cells on DNA extraction filters		
Sample date	***E. coli***	***Enterococcus***	***Bacteroides***	**Sample #**	**Water type (site #)**	**Amount processed (ml)**	***E. coli***	***Enterococcus***	***Bacteroides***
14-Jul-04	30 ± 14	61 ± 17	<1	13	beach	700	210	427	<1
				14	beach	500	150	305	<1
14-Mar-06	<1	<1	NA	17,18[a]	canal (3)	100	<1	<1	NA
14-Mar-06	<1	<1	NA	19, 20	canal (7)	100	<1	<1	NA
14-Mar-06	<1	<1	NA	21, 22	canal (9)	100	<1	<1	NA
14-Mar-06	<1	<1	NA	23, 24	canal (10)	100	<1	<1	NA
14-Mar-06	<1	<1	NA	25, 26	canal (11)	100	<1	<1	NA
14-Mar-06	<1	<1	NA	27, 28	canal (12)	100	<1	<1	NA

[a] performed in duplicate.

Table 5

Average bacterial densities in river water (CFU/100 ml) collected from Wagner Creek as determined by growth on selective media and the calculated cells available for DNA extraction for each membrane filter (Sample #) used for PCR analysis

RIVERWATER								
Bacterial density (CFU/100 ml ± STD)				**Sample parameters**		**# of cells on DNA extraction filters**		
Sample date	***E. coli***	***Enterococcus***	***Bacteroides***	**Sample #**	**Amount processed (ml)**	***E. coli***	***Enterococcus***	***Bacteroides***
11-Feb-04	7600 ± 5657	94 ± 37	215 ± 7	29	500	3.8×10^5	470	1075
29-Mar-04	1158 ± 706	5098 ± 5958	115 ± 27	30	500	5790	2.5×10^4	575
10-Feb-05	2050 ± 919	1381 ± 680	62 ± 60	31	700	1.4×10^4	9667	434
				32	700	1.4×10^4	9667	434
19-Apr-06	235 ± 50	47 ± 33	850 ± 212	33	100	235	47	850
				34	100	235	47	850
				35	100	235	47	850

Table 6

Average bacterial densities (CFU/100 ml) in raw sewage samples collected from a wastewater treatment plant as determined by growth on selective media and the calculated cells available for DNA extraction for each membrane filter (Sample #) used for PCR analysis

RAW SEWAGE								
Bacterial density (CFU/100 ml ± STD)				**Sample parameters**		**# of cells available for DNA extraction**		
Sample date	***E. coli***	***Enterococcus***	***Bacteroides***	**Sample #**	**Amount processed (ml)**	***E. coli***	***Enterococcus***	***Bacteroides***
17-Sep-05	$6\times10^6 \pm 8\times10^5$	$1.4\times10^6 \pm 2\times10^6$	$3\times10^6 \pm 2\times10^6$	50	8	4.8×10^5	1.1×10^5	2.4×10^5
				54	12	7.2×10^5	1.7×10^5	3.6×10^5
				60	2	1.2×10^5	2.8×10^4	6.0×10^4

Table 7

Results of PCR screening of raw sewage for a variety of fecal indicators, source tracking markers, and pathogens. Sample numbers are described in Table 6. Positive (+) or negative (−) result based on visual inspection of electrophoresis gel

Sample #	*Enterococcus*	*Enterococcus* human marker	*Bacteroides* human marker	*S. aureus*	*C. jejuni*	*E. coli* 0157:H7	*Salmonella* spp.	Adenovirus
50	+	−	+	+	−	−	−	−
54	+	−	+	+	−	−	−	−
60	+	−	+	+	−	−	−	−

Table 8

Results of PCR screening of sand and water samples for a variety of fecal indicators, source tracking markers, and pathogens. Sample numbers are described in Tables 3 – 5. Positive (+) or negative (−) result based on visual inspection of electrophoresis gel

Sample #	Sample type	*E. coli*	Enterococci	*Bacteroides* human marker	enterococci human marker	*E. coli* 0157:H7	Adenovirus[b]	*S. aureus*
1 – 2	wet sand	+	+	−		−		−
3 – 4	dry sand	+	+	−		−		−
9 – 12	dry sand	+	+	−		−	−	−
36 – 38	dry sand	+	+	−	−	−		−
39, 41[G]	dry sand, enriched				−			
13 – 14	beach water	+	+	−		−		−
17 – 28	canal water		+				−	−
29	river water	+	+	−		−		−
30	river water	+	+	−		−		−
31 – 32	river water	+	+/−[c]	+		−		+/−[c]
33 – 35[a]	river water		+	+		−	−	−
40[G]	river water, enriched				−			

[G] DNA extracted from growth filter (MeI agar);

[a] these samples were also negative for *C. jejuni*;

[b] only samples 9–12, 17–28, and 33–35 were processed for viral analysis;

[c] sample 31 was positive, but sample 32 was negative. These samples were replicates except that sample 32 was cut during processing and sample 31 was left intact.