DETECTION OF HUMAN FECAL CONTAMINATION IN CORPUS CHRISTI BAY

TGLO Contract Number 10-122-000-3994

With

BACTERIA MONITORING AND SOURCE TRACKING IN CORPUS CHRISTI BAY AT COLE AND ROPES PARKS (pilot)

CBBEP Contract Number 1010.

FINAL SUMMARY REPORT OF RESULTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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EXECUTIVE SUMMARY

The determination of contamination sources in recreational waters is key in efforts to remediate impaired waters. Although traditional surface water quality monitoring identifies the presence of bacterial contamination, such testing does not provide information on specific sources of the pollution. The Texas Beach Watch Program, funded through the US Environmental Protection Agency (EPA) in compliance with the 2000 Beaches Environmental Assessment and Coastal Health (BEACH) Act, and administered through the Texas General Land Office, monitors recreational beach waters in Texas using *Enterococcus* as an indicator of fecal contamination and the health risk associated with using the waters. However, source identification is currently not a component of the program.

Two recreational parks along Corpus Christi Bay, Cole and Ropes Parks, which are monitored at six sites through the Texas Beach Watch Program, have a history of water exceeding the EPA recommended enterococci concentration limit and the parks have been identified as impaired and included on the Texas 303 (d) list. In order to facilitate pollution remediation, bacterial source tracking is needed to assess whether the high concentrations of enterococci being found at these sites are due to human fecal contamination. These parks are located in a residential area of the City of Corpus Christi with storm water outfalls at each park. Thus the potential for human contamination is high, and confirmation of this source provides a first step in planning remediation efforts.

There are a number of bacterial source tracking techniques available for determining sources of contamination. One method that has been used in previous studies to detect human source contamination is molecular analysis for the presence of the enterococcal surface protein (Esp) found in species of enterococci associated with the human intestinal tract (Scott et al., 2005; McQuaig et al., 2006). The method relies on a Polymerase Chain Reaction (PCR)-based detection of the gene for Esp, which is a putative virulence factor found in human associated *Enterococcus faecium* (Scott et al., 2005).

In this study water samples from the six Texas Beach Watch stations at Ropes and Cole Parks were analyzed to detect the *esp* marker as an indicator of human contamination at these locations. Additionally, data on three other human-specific markers--*Bacteriodales*, Human

Polyoma Viruses (HPyVs), and *Methanobrevibacter.smithii*—from another study, are included in this report for comparison with the e*sp* analysis results.

The objectives of this study were:

1. Detection of the *esp* gene as an indicator of human contamination in ambient water samples from Corpus Christi Bay (Ropes and Cole Parks).

2. Detection of the *esp* gene as an indicator of human contamination in water samples from Corpus Christi Bay (Ropes and Cole Parks) following rainfall.

Eight months of field sampling was conducted at the two Ropes (NUE028, NUE029) and four Cole Parks (NUE031, NUE032, NUE033, NUE035) stations currently monitored through the Texas Beach Watch Program, to collect water samples for detection of the *esp* gene. Six sampling events were conducted on a monthly basis (dry weather), and four were conducted following rainfall. Routine field parameters were measured and field observations documented. Three samples from each site were analyzed following the approved Texas Beach Watch Program Quality Assurance Project Plan (QAPP) for field sampling and membrane filtration and for *esp* analysis following analytical procedures in a QAPP approved by the Texas State Soil and Water Conservation Board and U.S. EPA (Mott and Hay, 2009)**.** Additionally, a subset of isolates collected at the stations following rainfall on 09/22/10 was speciated using the MicroLog^{TM} Microbial Identification System (MIS) (Biolog, Inc.).

The gene for the Esp protein was not detected in samples collected during ambient sampling events from February through July. However, *Enterococcus* levels were relatively low at each station for these events, with the exception of 06/03/10 at NUE031 and NUE032, and thus levels of *E. faecium* carrying the *esp* gene would also be expected to be low. Additionally some inhibition of PCR occurred in samples from April and May and following additional investigation and incorporation of additional controls for the June and July samples, in September, inhibitEX tablets (Qiagen) were used during DNA extraction and this treatment was effective in elimination of inhibition.

For the rainfall events, enterococci levels were less than 500 CFU/100ml except for 1 Cole Park site (NUE033) on 5/26/10 and for the 9/22/10 event where levels exceeded 1500 CFU/100ml at the two Ropes Park sites. The *esp* gene was detected in one sample from one of the Ropes Park sites (NUE028) and two from the other (NUE029), indicating human source contamination. Speciation of a subset of isolates from each site for this event showed *E. faecium* was present at most sites at ~30-40% of the *Enterococcus* isolates. However, it must be noted that not all *E. faecium* is of human origin, carrying the *esp* gene.

Results of the separate study using three other human-specific markers showed some human contribution to the water as at least one marker was detected in at least one sample at each site during the study period, although they were not detected at each site for every sampling event. For three events at least one marker was detected at four sites. Detection occurred when enterococci levels were low, and none of the human markers were detected in the samples which contained enterococci levels exceeding 1000 CFU/100 ml, collected following rainfall (05/16/10) and on 06/3/10 at site NUE031.

The results of this preliminary study suggest there is some human contribution to the fecal contamination at Ropes and Cole Parks, although detection limits and inhibition presented some initial challenges in using the molecular markers. Following rainfall and higher enterococci numbers in September 2010 the *esp* gene was detected at the two Ropes Park sites indicating human source contribution at this location.

The use of molecular markers shows promise as a rapid method for use at contaminated sites along the Texas coast, although preliminary studies to identify and address issues such as inhibition and detection levels are needed to optimize results. In addition to human-specific markers, markers for other sources are becoming more available and provide a rapid method to identify sources of contamination for use in identifying the cause and location of pollution contributing to poor water quality and allowing for remediation and implementation of best management practices.

With the modification of the *esp* procedure to remediate the effects of inhibitory substances it is suggested that this method holds promise for future studies, particularly when enterococci levels are high, for example following rainfall. Multiple markers provide an additional degree of confidence in results and in future should be included in any study using this approach to determining sources of contamination. As new markers become available, and with improved protocols, their use is recommended as a tool in the identification of sources of contamination in Texas coastal waters.

For Ropes and Cole Parks a broader bacteria source tracking project is recommended to examine not only human, but other sources of contamination which may or may not be controllable by best management practices (for example, dog source contamination can be reduced by education of the public, whereas bird source contamination is not controllable). *Bacteroides* markers specific for cow, dog and some other animals are available and could be incorporated into such a study.

Source tracking studies provide data on human pollution as well as other sources contributing to contamination that can be used to implement best management practices to remediate water quality in Texas coastal waters.

INTRODUCTION

The determination of contamination sources in recreational waters is key in efforts to remediate impaired waters. Although traditional surface water quality monitoring identifies the presence of bacterial contamination, such testing does not provide information on specific sources of the pollution. To further investigate the sources of fecal contamination, tools such as bacterial source tracking must be used. Source tracking techniques include both molecular and non-molecular methods, such as polymerase chain reaction (PCR) and antibiotic resistance analysis, which differentiate between human and various non-human sources of fecal contamination.

The Texas Beach Watch Program monitors recreational beach waters in Texas using *Enterococcus* as an indicator of fecal contamination. This program is funded through the US Environmental Protection Agency (EPA) in compliance with the 2000 Beaches Environmental Assessment and Coastal Health (BEACH) Act. Sites are monitored weekly during the summer bathing season (May-September) and bimonthly during the off-season (October to April). The concentration of indicator organisms gives an indication of the health risk associated with using these waters. The results from the lab's water analysis are uploaded to the Texas Beach Watch website so the public know whether it is safe to use a particular beach on a given day before going, but also lets local government know whether to post advisories at a particular beach. However, source identification is currently not a component of the program.

Two recreational parks along Corpus Christi Bay, Cole and Ropes Parks, which are monitored through the Texas Beach Watch Program, have a history of water exceeding the EPA recommended enterococci concentration limit and the parks have been identified as impaired and included on the Texas 303 (d) list. For the 2008 swimming season, due to exceedances, there were advisories posted at Ropes Park for 21 beach days and Cole Park for 26 beach days. These two sites represented the two highest number of beach day advisories of any monitored locations in the state. The next highest number of beach advisory postings was at Appfel Park in Galveston County with 13 days (EPA Beach Monitoring Notification 2009). In order to facilitate pollution remediation, bacterial source tracking is needed to assess whether the high concentrations of enterococci being found at these sites are due to human fecal contamination. These parks are located in a residential area of the City of Corpus Christi with storm water outfalls at each park. Thus the potential for human contamination is high, and confirmation of this source provides a first step in planning remediation efforts.

There are a number of bacterial source tracking techniques available for determining sources of contamination. The method selected may depend on a number of factors including speed, target and cost of method. One method that has been used in previous studies to detect human source contamination is molecular analysis for the presence of the enterococcal surface protein (Esp) found in species of Enterococci associated with the human intestinal tract (Scott et al., 2005; McQuaig et al., 2006). The method relies on a PCR-based detection of the gene for Esp, which is a putative virulence factor found in human associated *Enterococcus faecium* (Scott et al., 2005). This method has been used in a number of bacterial source tracking studies (McDonald et al., 2006; Brownell et al., 2007; Ahmed et al., 2008; Korajkic et al., 2009; Abdelzaher et al., 2010).

Additional PCR-based assays have been developed to detect other markers of human-associated contamination. *Bacteriodales*, human polyomaviruses, and *Methanobrevibacter smithii* are three primary microbiological targets linked with humans, used to trace contamination. *Bacteriodales* as a group are widespread in intestinal tracts of animals but use of 16S rRNA can detect human-specific types and have been used as a bacterial target in several studies since its first published use as a marker in 2000 (Bernhard and Field, 2000a; Boehm et al., 2003; Seurinck et al., 2005). Human polyomaviruses (HPyVs), common in a large portion of the population, first were utilized as a target by McQuaig et al. (2006) and have since proven to be highly-specific to human sources when detected with a PCR-based method (Harwood et al. 2009) and have been recently used as a target in several studies (McQuaig et al., 2009; Abdelzaher et al., 2010; Ahmed et al., 2010). *Methanobrevibacter smithii* has been found in both the human intestinal tract and sewage and has been utilized as a target for molecular analysis of bacterial contamination (Harwood et al., 2009).

In this study water samples from the six Texas Beach Watch stations at Ropes and Cole Parks were analyzed to detect the *esp* marker as an indicator of human contamination at these locations. Additionally, data on three other markers--*Bacteriodales*, HPyVs, and *M. smithii*—from another study, are included in this report for comparison with the e*sp* analysis results.

Objectives (GLO Contract subtasks)

1. Detection of the *esp* gene as an indicator of human contamination in ambient water samples from Corpus Christi Bay (Ropes and Cole Parks).

2. Detection of the *esp* gene as an indicator of human contamination in water samples from Corpus Christi Bay (Ropes and Cole Parks) following rainfall.

METHODS

Collection/Isolation of Enterococci from Water Samples

Sample collection and analysis followed the subtask descriptions in the GLO contract.

Water samples were collected over an eight month period, from February through September 2010. Six ambient sampling events were conducted and four sampling events followed rainfall. Three water samples per site were collected for each sampling event from the six Texas Beach Watch stations at Cole and Ropes Parks (Figures 1-4) in accordance with guidelines specified in the current Texas Beach Watch Quality Assurance Project Plan (QAPP). Larger bottles than used for routine monitoring were employed for collection due to the volumes needed to conduct the molecular assays. Routine field observations were documented and environmental parameters additional to those specified by the Texas Beach Watch program, such as pH and salinity, were measured in the field with a YSI multiprobe instrument. Water samples were transported to the lab within six hours of collection. Turbidity of water samples was tested prior to water sample analysis using a turbidimeter model DRT-15CE (HF Scientific). Enterococci were isolated from water samples following US EPA Method 1600, consistent with procedures from the Texas Beach Watch QAPP. Colony counts were performed after the standard 24 hour incubation. The plates were then incubated an additional day for a total of 48 hours of incubation for *esp* analysis. Procedures for *esp* analysis followed analytical procedures in a QAPP approved by the Texas State Soil and Water Conservation Board and U.S. EPA (Mott and Hay, 2009). Filters for *esp* analysis were next enriched in an azide dextrose broth for three hours prior to PCR analysis. Water samples for additional human makers (*Bacteroidales*, HPyVs, and *M. smithii*) were concentrated by membrane filtration with 0.45µm nitrocellulose filters. These filters were placed into PowerBead tubes from the PowerSoil™ DNA kit (MoBio), and stored at -80°C for further analysis.

Table 1. List of Sampling Stations and GPS Coordinates

Sampling Site	GPS Coordinates (N,W)
NUE028	27 45 11, 97 22 33.4
NUE029	27 45 17, 97 22 34.4
NUE031	27 46 04, 97 23 05.2
NUE032	27 46 12, 97 23 14.0
NUE033	27 46 18, 97 23 18.0
NUE035	27 46 35, 97 23 28.1

Figure 1. Map of Sampling Sites at Ropes Park – NUE028 and NUE029

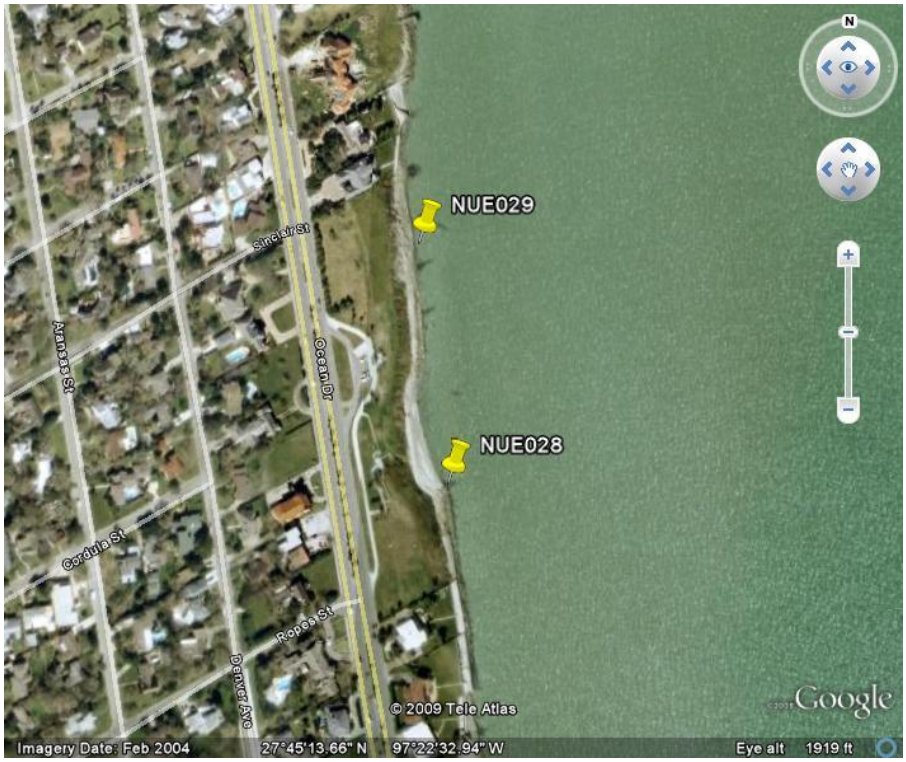

Figure 2. Map of Sampling Site at Cole Park-NUE031

Figure 3. Map of Sampling Sites at Cole Park-NUE032 and NUE033

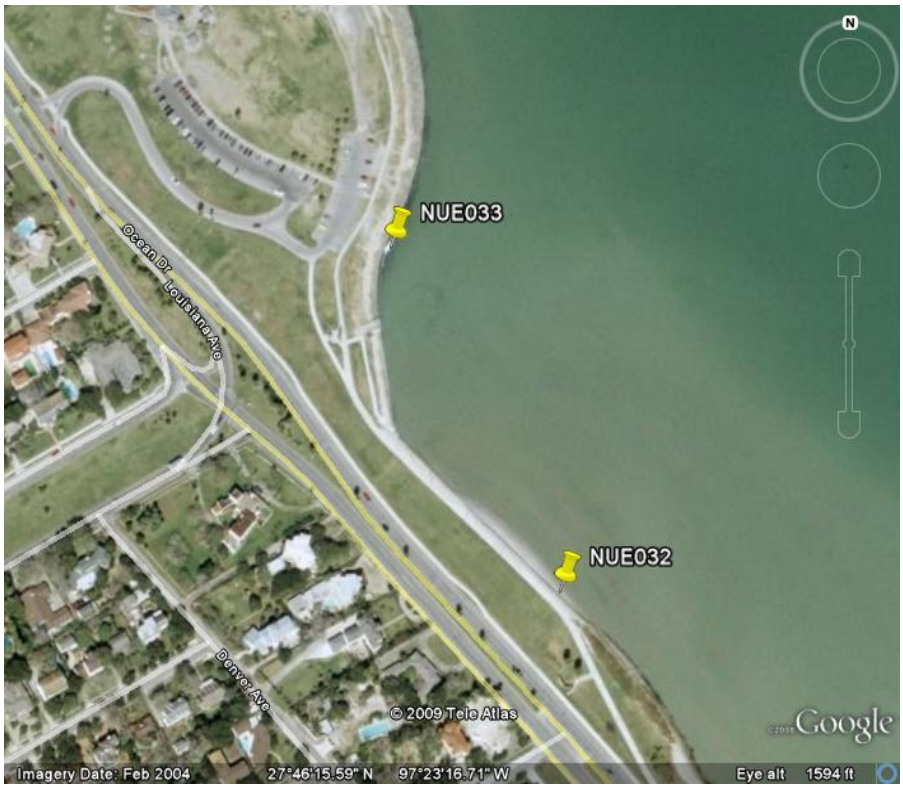

Figure 4. Map of Sampling Site at Cole Park-NUE035

Enterococcus esp Extraction and Assay

Procedures for *esp* analysis followed analytical procedures in a QAPP approved by the Texas State Soil and Water Conservation Board and U.S. EPA (Mott and Hay, 2009). Details of the procedure are also included in Appendix B. Following enrichment in the azide dextrose broth, DNA extraction was performed with Qiagen DNeasy Blood and Tissue Kit and ASL buffer (Qiagen, Inc.). Extractions for samples from the final two sampling events (September 10 and 22, 2010) were performed utilizing InhibitEX tablets (Qiagen, Inc.) to minimize inhibition of PCR analysis.

Following extraction of DNA, PCR reaction mix was prepared using GoTaq Green mix (Promega) according to the manufacturer's instruction, with *esp* forward primer (5'-TAT GAA AGC AAC AGC ACA AGT T-3') (Scott et al. 2005) and *esp* reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3') (Hammerum and Jensen 2002). Each PCR contained a total of 24 reaction tubes: six controls and the three samples from each of the six sites. After PCR cycles were completed according to procedures in Table 3, results were visualized using agarose gel electrophoresis with a Promega ladder with the expected product at the 680 bp mark.

Controls included the following, at various steps in the process:

1. a Method Blank (MB) (300ml sterile buffered dilution water) carried in the field and processed in the same manner as the water samples.

2. a method spike, SP2, (300 ml sterile buffered dilution water spiked with 100 μl of an overnight culture of *Enterococcus faecium* C68 which contains the *esp* gene) filtered through a 0.45 μm nitrocellulose filter.

3. an inhibition spike, SP1, (300 ml composite sample (all environmental samples mixed together) spiked with 100 μl of an overnight culture of *Enterococcus faecium* C68, which contains the *esp* gene) filtered through a 0.45 μm nitrocellulose filter.

SP1, SP2, and MB were carried through the entire analysis, from sample processing to electrophoresis. The purpose of SP1 and SP2 was to check for PCR inhibitors present in the environmental samples. A positive result for both SP1 and SP2 indicated no inhibition. If SP2 was positive and SP1 was negative, inhibition was suspected.

4. an Extraction Blank (EB): DNA extraction performed without adding any additional culture or sample water, thus serving as a negative control for the extraction process.

5. +PCR: positive control for PCR using *Enterococcus faecium* C68 DNA.

6. -PCR: negative control for PCR; no DNA added to reaction.

The PCR controls provided assurance that the PCR protocol had been followed and detection of the *esp* gene was possible.

PCR Analysis for *Bacteroidales*, human polyomaviruses, *Methanobrevibacter smithii*

DNA extraction was performed according to manufacturer's instructions with modifications suggested by Harwood et al. (2009). A 25 µl PCR reaction mix was prepared using GoTaq Green (Promega Inc) master mix containing deoxy nucleotide phosphates, taq polymerase and a loading dye as half the volume. Two µl of DNA template and one µl of forward and reverse primers (Table 2) were added to each reaction. The remaining volume of the reaction consisted of PCR grade nuclease free water. PCR reactions were carried out under specified conditions (Table 3), and PCR products (expected sizes listed in Table 2) were compared to a size standard on 2x agarose gels stained with ethidium bromide by electrophoresis.

Table 2. Primers for *Bacteroidales*, HPyVs, and *M. smithii*

Target Organism	Primer Sequence	Size	Reference
HPyVs	SM2: 5' AGT CTT TAG GGT CTT CTA CCT TT 3' P6: 5' GGT GCC AAC CTA TGG AAC AG 3'	172 bp	McQuaig et al., 2009
Bacteroidales	HF183f: 5' ATC ATG AGT TCA CAT GTC CG 3' Bac708r: 5' CAA TCG GAG TTC TTC GTG 3'	525 bp	Bernhard and Field, 2000b
M. smithii	Mnif342f: 5' AAC AGA AAA CCC AGT GAA GAG 3' Mnif363r: 5' ACG TAA AGG CAC TGA AAA ACC 3'	222 bp	Ufnar et al., 2006

Table 3. PCR Conditions (time and temperature settings and number of cycles for each protocol)

	esp	HPyVs	*M. smithii* & Bacteroidales
Initial denaturing	94°C 2 min	94°C 2 min	94°C 3.5 min
Denaturing	94°C 1 min	94°C 20s	94°C 45s
Anneal	58°C 1 min	55°C 20s	55°C 45s
Extension	72°C 1 min	72°C 20s	72°C 30s
Number of cycles	30	45	30
Final extension	72°C 5 min	72°C 2 min	72°C 5 min

Note: The *M. smithii* & Bacteroidales protocol has an additional touch down step between initial denaturing and denaturing in which the reaction is run at 94°C for 45 sec then 45 sec at 65–55°C (step down 1°/2 cycles from 65 to 62°C & 1°/cycle from 62 to 55°C) then 72°C 30 sec before beginning the cycling.

Identification of *Enterococcus* spp.

A subset of 140 isolates from membrane filters on mEI, collected following rainfall (9/22/10), were speciated using the MicroLog™ Microbial Identification System (MIS) (Biolog, Inc.) following the MicroLog™ System Release 4.0 User Guide (Biolog, 1999). Ten colonies from each site were spread on mEI plates, and the rest of the colonies were grown for six or more hours in tryptic soy broth (TSB). Of the 140 colonies, 58 were able to grow on MEI or in TSB. Each isolate was transferred to a Biolog™ Universal Growth (BUG B) plate with 5% sheep's blood agar (Biolog, 1999). The plates were incubated at 35°C for 16 hrs. Growth from the plates was transferred to inoculating fluid (0.4% NaCl, 0.03% Pluronic F-68, 0.01% Phytagel™) to reach a transmittance level of 20% (±2%) at 600nm. This inoculum was pipetted into a 96 well GP2 Microplate™, and the plates were incubated for 17 hrs at 35°C. After the incubation period the plates were read using the Micro Log System™, Release 4.20.04 (Biolog, 2004).

Quality Assurance/Quality Control

The YSI multiprobe instrument was calibrated both before and after field sampling to ensure proper measurements were taken. Method 1600 analysis was performed according to method and National Environmental Laboratory Accreditation Program (NELAP) standards, including provisions for positive and negative controls for media preparation, method blanks for membrane filtration, and checks on all equipment used to prepare media and supplies for use in the method. A negative control composed of unspiked dilution water and a positive control of dilution water (500mL) spiked with 10mL of raw sewage were utilized in all DNA extractions. This same positive control was used for all PCR analyses except for *esp* analysis, which utilized a sample spiked with C68 DNA, which is positive for *esp*. An additional negative control containing preparations without samples (DNA) was utilized for both extraction and PCR. An inhibition spike for each site was made using C68 DNA. The following bacteria were used as controls for the MicroLog™ Microbial Identification System (MIS) (Biolog, Inc.): *Corynebacterium minutissimum* ATCC strain 23348 *Rhodococcus equi* ATCC strain 6929 *Staphylococcus aureus* ATCC strain 12600 and *Enterococcus faecalis* ATCC strain 19433.

RESULTS

Three samples of water were collected monthly from each site and analyzed for *esp*, *Bacteroidales*, human polyomaviruses (HPyVs), and *Methanobrevibacter smithii* from February 2010 through July 2010. Results are tabulated in Tables 4-9. Additional *esp* analysis was conducted on water samples collected following rainfall in May, June and September 2010 (Tables 10-13). Field data may be found in Appendix A (Tables A1 and A2).

The gene for the Esp protein was not detected in samples collected during the monthly sampling events 1-6 (Tables 4-9). However, *Enterococcus* levels were relatively low at each station for these events, with the exception of 06/3/10 at NUE031, and thus levels of *E. faecium* carrying the *esp* gene would also be expected to be low. Some inhibition of PCR occurred in samples from April and May (based on controls). To further identify which sites' results were affected by inhibition, an additional reaction for each site was spiked with C68 DNA as recommended in the literature (Ahmed et al. 2008). In June, July and September, one sample from each site was analyzed with and without the addition of C68 DNA to the PCR chamber and inhibition was evident for each site (Tables in Appendix C). To avoid inhibition in September, inhibitEX tablets (Qiagen) were used during DNA extraction, according to the protocol for inhibitory sites (Appendix B) and results of controls showed that this treatment was effective in elimination of inhibition (Appendix C, Table C3).

For the rainfall events (Tables 10-13) enterococci levels at each site were less than 500 CFU/100ml except for NUE033 on 05/26/10 and the 9/22/10 event where levels exceeded 1500 CFU/100ml at two sites (Ropes Park). The *esp* gene was detected in one sample from NUE028 and two from NUE029, indicating human source contamination at Ropes Park.

At least one of the additional human markers was detected in at least one sample at each site during the study period (Tables 4-9) although they were not detected at each site for every sampling event. For three events at least one marker was detected at four sites. Detection occurred when enterococci levels were low and none of the human markers were detected in the samples which contained enterococci levels exceeding 1000 CFU/100 ml collected following rainfall (05/16/10) at NUE033 and on 06/3/10 at site NUE031.

Table 4. Results from Event 1 (2/17/10)

Sample Number	Ent[a] CFU/ 100ml	*esp*	HPyVs	*Re-run*[b] *with 5µl template*	human *Bacteroidales*	*Re-run*[b] *with 5µl template*	*M. smithii*	*Re-run*[b] *with 5µl template*
NUE028-1		-	-/-		-/-		-/-	
NUE028-2	4.3	-	-/-		-/-		-/-	
NUE028-3		-	-/-		-/-		-/-	
NUE029-1		-	-/-		-/-		-/-	
NUE029-2	3.3	-	+/-	-/-	**+/+**		-/-	
NUE029-3		-	-/-		+/-	-/-	-/-	
NUE031-1		-	+/-	-/-	+/-	-/-	-/-	
NUE031-2	17	-	-/-		+/-	-/-	-/-	
NUE031-3		-	-/-		-/-		+/-	**+/-**
NUE032-1		-	-/-		-/-		-/-	
NUE032-2	5	-	-/+	-/-	-/-		-/+	**+/-**
NUE032-3		-	-/-		-/+	-/-	-/-	
NUE033-1		-	-/-		-/+	-/-	-/-	
NUE033-2	4.7	-	-/-		+/-	-/-	-/-	
NUE033-3		-	-/-		-/+	-/-	-/-	
NUE035-1		-	-/+	-/-	-/-		**+/+**	
NUE035-2	3.7	-	-/-		-/-		-/-	
NUE035-3		-	-/+	-/-	-/-		-/-	

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.
[b] In the event that the two replicate PCR reactions gave different results two additional PCR reactions were performed with an increased volume (5µl) of DNA template. A positive on one or both of these re-runs was considered a positive for the corresponding marker.

Table 5. Results from Event 2 (3/24/10)

Sample Number	Ent[a] CFU/ 100 ml	*esp*	HPyVs	*Re-run*[b] *with 5µl template*	human *Bacteroidales*	*Re-run*[b] *with 5µl template*	*M. smithii*	*Re-run*[b] *with 5µl template*
NUE028-1	323.3	-	**+/+**	-/-	-/-		-/-	
NUE028-2		-	-/-		-/-		-/-	
NUE028-3		-	-/-		-/-		-/-	
NUE029-1	238	-	+/-	-/-	-/-		-/-	
NUE029-2		-	-/-		-/-		-/-	
NUE029-3		-	-/-		-/-		-/-	
NUE031-1	81.3	-	-/-		-/-		**+/+**	
NUE031-2		-	-/-		-/-		-/+	**-/+**
NUE031-3		-	-/-		-/-		-/+	**-/+**
NUE032-1	97	-	**+/+**	-/-	-/-		-/-	
NUE032-2		-	-/+		-/-		-/+	**-/+**
NUE032-3		-	**+/+**		-/-		**+/+**	
NUE033-1	27.7	-	-/-		-/-		-/-	
NUE033-2		-	-/-		-/-		-/-	
NUE033-3		-	-/-		-/-		-/-	
NUE035-1	112.3	-	-/-		-/-		-/-	
NUE035-2		-	**+/+**		-/-		-/+	-/-
NUE035-3		-	-/-		-/-		-/+	-/-

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.
[b] In the event that the two replicate PCR reactions gave different results two additional PCR reactions were performed with an increased volume (5µl) of DNA template. A positive on one or both of these re-runs was considered a positive for the corresponding marker.

Table 6. Results from Event 3 (4/28/10)

Sample Number	Ent[a] CFU/ 100 ml	*esp*	HPyVs	*Re-run*[b] *with 5µl template*	human *Bacteroidales*	*Re-run*[b] *with 5µl template*	*M. smithii*	*Re-run*[b] *with 5µl template*
NUE028-1	26.7	-	-/-		-/-		-/-	
NUE028-2		-	-/-		-/-		-/-	
NUE028-3		-	-/-		-/-		-/-	
NUE029-1	38.3	-	-/-		-/-	-/-	-/-	**-/-**
NUE029-2		-	-/-		+/-		-/+	
NUE029-3		-	-/-		-/-		-/-	
NUE031-1	6.3	-	+/-	**-/-**	-/-		-/-	
NUE031-2		-	-/-		-/-		-/-	
NUE031-3		-	-/-		-/-		-/-	
NUE032-1	2.7	-	+/-	**-/-**	-/-		-/-	
NUE032-2		-	-/-		-/-		-/-	
NUE032-3		-	-/-		-/-		-/-	
NUE033-1	1.7	-	-/-		-/-		-/-	
NUE033-2		-	-/-		-/-		-/-	
NUE033-3		-	-/-		-/-		+/-	**+/+**
NUE035-1	25.3	-	-/-		-/-		-/-	
NUE035-2		-	-/+	**-/-**	-/-		-/-	
NUE035-3		-	-/-		-/-		-/-	

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.
[b] In the event that the two replicate PCR reactions gave different results two additional PCR reactions were performed with an increased volume (5µl) of DNA template. A positive on one or both of these re-runs was considered a positive for the corresponding marker.

Table 7. Results from Event 4 (5/26/10)

Sample Number	Ent[a] CFU/100ml	*esp*
NUE028-1		-
NUE028-2	10.3	-
NUE028-3		-
NUE029-1		-
NUE029-2	14.3	-
NUE029-3		-
NUE031-1		-
NUE031-2	3	-
NUE031-3		-
NUE032-1		-
NUE032-2	1.3	-
NUE032-3		-
NUE033-1		-
NUE033-2	51.7	-
NUE033-3		-
NUE035-1		-
NUE035-2	3.7	-
NUE035-3		-

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.

Table 8. Results from Event 5 (6/3/10)

Sample Number	Ent[a] CFU/ 100 ml	*esp*	HPyVs	*Re-run*[b] *with 5µl template*	human *Bacteroidales*	*Re-run*[b] *with 5µl template*	*M. smithii*	*Re-run*[b] *with 5µl template*
NUE028-1		-	-/-		-/-		-/-	
NUE028-2	566.7	-	-/-		-/-		-/-	
NUE028-3		-	**+/+**		-/-		-/-	
NUE029-1		-	-/-		-/-		-/-	
NUE029-2	213.3	-	-/-		-/-		+/-	-/-
NUE029-3		-	-/+	-/-	-/-		-/-	
NUE031-1		-	-/-		-/-		-/-	
NUE031-2	1166.7	-	-/-		-/-		-/-	
NUE031-3		-	-/+	-/-	-/-		-/+	-/-
NUE032-1		-	-/-		-/-		+/-	**-/+**
NUE032-2	900	-	-/-		-/-		-/-	
NUE032-3		-	+/-	-/-	-/-		-/-	
NUE033-1		-	-/-		-/-		+/-	-/-
NUE033-2	66	-	-/-		-/-		-/+	-/-
NUE033-3		-	-/-		-/-		-/-	
NUE035-1		-	-/-		-/-		-/-	
NUE035-2	112	-	-/-		-/-		-/-	
NUE035-3		-	-/-		-/-		-/-	

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.
[b] In the event that the two replicate PCR reactions gave different results two additional PCR reactions were performed with an increased volume (5µl) of DNA template. A positive on one or both of these re-runs was considered a positive for the corresponding marker.

Table 9. Results from Event 6 (7/6/10)

Sample Number	Ent[a] CFU/ 100ml	esp	HPyVs	*Re-run*[b] *with 5µl template*	human *Bacteroidales*	*Re-run*[b] *with 5µl template*	*M. smithii*	*Re-run*[b] *with 5µl template*
NUE028-1		-	-/+	-/-	-/+	-/-	-/-	
NUE028-2	6	-	-/-		-/-		-/-	
NUE028-3		-	-/+	-/-	-/-		-/-	
NUE029-1		-	**+/+**		-/-		-/-	
NUE029-2	5.7	-	-/-		-/-		-/-	
NUE029-3		-	-/-		-/+	-/-	-/-	
NUE031-1		-	-/-		-/-		-/-	
NUE031-2	13.7	-	-/-		-/-		-/-	
NUE031-3		-	-/-		-/-		+/-	-/-
NUE032-1		-	-/+	-/-	-/-		-/-	
NUE032-2	1	-	-/-		-/-		+/-	-/-
NUE032-3		-	-/+	**-/+**	-/-		-/-	
NUE033-1		-	**+/+**		-/-		+/-	-/-
NUE033-2	12	-	-/-		-/-		-/+	**+/-**
NUE033-3		-	-/-		-/-		-/-	
NUE035-1		-	-/-		-/-		**+/+**	
NUE035-2	10.7	-	-/+	-/-	-/-		**+/+**	
NUE035-3		-	-/-		-/-		-/-	

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.
[b] In the event that the two replicate PCR reactions gave different results two additional PCR reactions were performed with an increased volume (5µl) of DNA template. A positive on one or both of these re-runs was considered a positive for the corresponding marker.

Table 10. Results from Rainfall Event 1 (5/26/10)

Sample Number	Ent[a] CFU/ 100ml	*esp*	HPyVs	*Re-run*[b] *with 5µl template*	human *Bacteroidales*	*Re-run*[b] *with 5µl template*	*M. smithii*	*Re-run*[b] *with 5µl template*
NUE028-1		-	**+/+**		-/-		+/-	-/-
NUE028-2	58.3	-	-/-		-/-		-/-	
NUE028-3		-	-/+	-/-	-/-		-/-	
NUE029-1		-	-/-		-/-		-/-	
NUE029-2	132.8	-	-/-		-/-		+/-	-/-
NUE029-3		-	-/+	-/-	-/-		-/-	
NUE031-1		-	-/-		-/-		+/-	-/-
NUE031-2	5	-	-/-		-/-		-/-	
NUE031-3		-	-/-		-/-		-/-	
NUE032-1		-	+/-	-/-	-/-		-/-	
NUE032-2	5.7	-	-/-		-/-		-/+	-/-
NUE032-3		-	+/-	-/-	-/-		-/-	
NUE033-1		-	-/-		-/-		-/-	
NUE033-2	2313.1	-	-/-		-/-		-/-	
NUE033-3		-	-/-		-/-		-/-	
NUE035-1		-	-/-		-/-		-/-	
NUE035-2	30	-	-/-		-/-		+/-	-/-
NUE035-3		-	+/-	-/-	-/-		-/-	

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.
[b] In the event that the two replicate PCR reactions gave different results two additional PCR reactions were performed with an increased volume (5µl) of DNA template. A positive on one or both of these re-runs was considered a positive for the corresponding marker.

Table 11. Results from Rainfall Event 2 (6/9/10)

Sample Number	Ent_a CFU/ 100ml	*esp*
NUE028-1		-
NUE028-2	115.7	-
NUE028-3		-
NUE029-1		-
NUE029-2	286.3	-
NUE029-3		-
NUE031-1		-
NUE031-2	232.3	-
NUE031-3		-
NUE032-1		-
NUE032-2	116.6	-
NUE032-3		-
NUE033-1		-
NUE033-2	131.3	-
NUE033-3		-
NUE035-1		-
NUE035-2	67	-
NUE035-3		-

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.

Table 12. Results from Rainfall Event 3 (9/10/10)

Sample Number	Ent CFU_a	*esp*
NUE028-1		-
NUE028-2	45.7	-
NUE028-3		-
NUE029-1		-
NUE029-2	38	-
NUE029-3		-
NUE031-1		-
NUE031-2	70.3	-
NUE031-3		-
NUE032-1		-
NUE032-2	37.7	-
NUE032-3		-
NUE033-1		-
NUE033-2	176.7	-
NUE033-3		-
NUE035-1		-
NUE035-2	389	-
NUE035-3		-

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.

Table 13. Results from Rainfall Event 4 (9/22/10)

Sample Number	Ent_a CFU/ 100ml	*esp*
NUE028-1		+
NUE028-2	1603.3	-
NUE028-3		-
NUE029-1		+
NUE029-2	2313.3	+
NUE029-3		-
NUE031-1		-
NUE031-2	863.3	-
NUE031-3		-
NUE032-1		-
NUE032-2	620	-
NUE032-3		-
NUE033-1		-
NUE033-2	710	-
NUE033-3		-
NUE035-1		-
NUE035-2	756.7	-
NUE035-3		-

[a] Enterococcus CFU/100 ml values are the average of the CFU counts for the three subsamples.

Speciation of *Enterococcus* isolates

Of the 58 isolates inoculated onto 96 well GP2 Microplates™, 47 were identified to at least genus level, and 36 isolates were identified to the species level. Other genera present included *Pediococcus, Alloiococcus,* and *Streptococcus.* A list of identified species can be found in Table 14 below. While ~36% of the isolates identified to genus were *E. faecium*, this species is often found in non-human sources; for example Mott et al.(2003) found *E. faecium* in bird and particularly dog (~10% of Enterococcus isolates) and in a current local study ~14% of human source *Enterococcus* were found to be *E. faecium* with higher proportions found in dog, bird and horse.

Table 14. Results of speciation of isolates collected on 9/22/10 following rainfall.

Sample Site	# Identified Isolates	# Identified as *Enterococcus*	# Identified as *E. faecium*	Other genera identified	Other species identified
NUE028	11	9	2	*Pediococcus* *Streptococcus*	*E. mundtii* *E. casseliflavus* *S. criceti*
NUE029	7	6	4	*Pediococcus*	*E. mundtii* *P. acidilactici/parvulus* *E. hirae*
NUE031	10	7	4	*Pediococcus* *Alloiococcus* *Streptococcus*	*E. dispar* *A. otitis* *E. faecalis* *S. hyointestinalis* *E. gallinarum*
NUE032	5	5	0	none	*E. casseliflavus* *E. solitarius*
NUE033	10	6	2	*Pediococcus* *Alloiococcus*	*E. faecalis* *P. pentosaceus* *P. acidilactici/parvulus* *A. otitis* *E. casseliflavus*
NUE035	4	3	1	*Pediococcus*	*P. pentosaceus* *E. casseliflavus* *E. gallinarum*
TOTAL	47	36	13	3	12

DISCUSSION

All sites were sampled and analyzed for the human contamination markers *esp*, *Bacteroidales*, human polyomaviruses (HPyVs), and *Methanobrevibacter smithii*. No consistent trend could be identified with regards to presence of markers and *Enterococcus* counts performed using USEPA Method 1600. The highest colony forming unit (CFU/100 ml) count obtained during ambient sampling was 1167 cfu/100mL (NUE031 on 06/3/10) and no human markers, including the *esp* gene, were detected within the sample. Additionally, all human markers, with the exception of *esp*, were detected in samples with very low bacterial counts, well below the EPA single sample criterion of 104 cfu/100 mL. When this level is exceeded, Texas Beach Watch Program issues an advisory. This suggests that there may be human source contamination even in ambient water samples (i.e. not reflective of run-off) that could pose a risk to human health.

The lack of detection of the *esp* gene which is found in a subset of *Enterooccus* (primarily *E. faecium*) is not unexpected when low numbers of *Enterococcus* are present in the water. Inhibition of PCR occurred with several of the samples, but the *esp* marker was detected In the final rainfall event in September when higher numbers of enterococci were present at the two Ropes Park sites (>1500 CFU/100 ml) and additional treatment of the samples for inhibition was incorporated into the procedure. Whether the detection was due to the higher levels of enterococci, or the lack of detection when lower levels of enterococci were present was partially due to inhibition, cannot be determined, although in the first few sampling events inhibition was not found, enterococci levels were low and the *esp* gene was not detected. The presence of the *esp* gene at Ropes Park following rainfall does suggest a human contribution at these sites.

The use of molecular markers shows promise for use at contaminated sites along the Texas coast, although preliminary studies to identify and address issues such as inhibition and detection levels are needed to optimize results. In addition to human-specific markers, markers for other sources are becoming more available and provide a rapid method to identify sources of contamination for use in identifying the cause and location of pollution contributing to poor water quality and allowing for remediation and implementation of best management practices.

CONCLUSIONS AND RECOMMENDATIONS

- Human source contamination was detected at Ropes and Cole Park stations under ambient weather conditions as measured by several human-specific markers, although the *esp* gene was not detected in any of these low enterococci containing samples.

- The *esp* gene was detected when levels of enterococci at Ropes Park were higher following rainfall and suggest a human contribution at this location presumably either from storm drain outflow or non-point source run-off.

- The approach used in this study was able to provide a rapid method for detection of human source contamination, and with modification of the *esp* procedure to remediate the effects of inhibitory substances it is suggested that this method can be used for future studies, particularly when enterococci levels are high, for example following rainfall.

- Multiple markers provide an additional degree of confidence in results and in future should be included in any study using this approach to determining sources of contamination

- As new markers become available and with improved protocols their use is recommended as a tool in the identification of sources of contamination in Texas coastal waters

- For Ropes and Cole Parks, a broader bacteria source tracking project is recommended to examine not only human, but other sources of contamination which may or may not be controllable by best management practices (for example, dog source contamination can be reduced by education of the public, whereas bird source contamination is not controllable). *Bacteroides* markers specific for cow, dog and some other animals are available and could be incorporated into such a study.

- Source tracking studies provide data on human pollution as well as other sources contributing to contamination that can be used to implement best management practices to remediate water quality in Texas coastal waters.

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Appendix A

Table A1. Field Data for Sampling (Non-Rainfall Events)

Sample Site	Date	Temp (°C)	Salinity (ppt)	pH	Turbidity (NTU)	DO (mg/L)	Rainfall prior to sampling (inch) (cumulative) 24 hrs	3 days	7 days
NUE028	2/17/10	12.0	27.0	8.2	1.5	9.8	0.0	0.0	0.9
NUE029	2/17/10	12.0	27.0	8.2	1.5	9.8	0.0	0.0	0.9
NUE031	2/17/10	12.4	26.9	8.2	1.6	10.1	0.0	0.0	0.9
NUE032	2/17/10	12.4	26.9	8.2	1.6	10.1	0.0	0.0	0.9
NUE033	2/17/10	12.4	26.9	8.2	1.6	10.1	0.0	0.0	0.9
NUE035	2/17/10	12.4	26.9	8.2	1.6	10.1	0.0	0.0	0.9
NUE028	3/24/10	18.8	26.2	7.9	14.1	4.4	0.0	0.0	0.7
NUE029	3/24/10	18.8	26.6	8.0	12.5	4.5	0.0	0.0	0.7
NUE031	3/24/10	18.8	26.9	7.9	15.8	4.4	0.0	0.0	0.7
NUE032	3/24/10	18.8	27.0	7.9	10.8	5.1	0.0	0.0	0.7
NUE033	3/24/10	18.7	27.0	8.1	9.4	4.4	0.0	0.0	0.7
NUE035	3/24/10	18.8	27.0	8.0	12.2	4.1	0.0	0.0	0.7
NUE028	4/28/10	21.7	30.1	6.5	20.5	4.8	0.0	0.0	0.0
NUE029	4/28/10	21.7	30.0	7.4	19.3	5.1	0.0	0.0	0.0
NUE031	4/28/10	21.8	30.1	7.6	6.6	5.7	0.0	0.0	0.0
NUE032	4/28/10	21.8	30.1	7.7	8.6	4.8	0.0	0.0	0.0
NUE033	4/28/10	21.8	30.2	7.7	9.4	4.8	0.0	0.0	0.0
NUE035	4/28/10	22.1	30.1	7.9	14.1	5.2	0.0	0.0	0.0
NUE028	5/26/10	26.5	33.4	8.1	13.6	7.7	0.0	0.0	0.0
NUE029	5/26/10	26.6	33.5	8.1	12.7	7.6	0.0	0.0	0.0
NUE031	5/26/10	27.0	33.3	8.1	19.7	7.2	0.0	0.0	0.0
NUE032	5/26/10	26.9	33.4	8.1	19.6	7.6	0.0	0.0	0.0
NUE033	5/26/10	27.3	32.4	8.1	12.5	8.4	0.0	0.0	0.0
NUE035	5/26/10	27.6	32.0	8.1	11.0	6.9	0.0	0.0	0.0
NUE028	6/3/10	28.4	30.4	7.9	14.2	na	0.0	1.4	1.4
NUE029	6/3/10	28.1	30.4	8.0	11.1	na	0.0	1.4	1.4
NUE031	6/3/10	28.8	30.8	8.0	30.6	na	0.0	1.4	1.4
NUE032	6/3/10	30.1	30.8	8.0	33.9	na	0.0	1.4	1.4
NUE033	6/3/10	29.4	31.2	8.1	19.9	na	0.0	1.4	1.4
NUE035	6/3/10	29.8	31.1	8.0	8.0	na	0.0	1.4	1.4
NUE028	7/6/10	30.0	32.8	8.1	24.5	7.2	0.0	0.0	2.3
NUE029	7/6/10	30.4	32.8	8.1	22.9	7.7	0.0	0.0	2.3
NUE031	7/6/10	30.4	33.0	8.1	27.1	7.3	0.0	0.0	2.3
NUE032	7/6/10	30.3	32.8	8.1	9.8	7.3	0.0	0.0	2.3
NUE033	7/6/10	30.5	32.5	8.0	11.1	7.8	0.0	0.0	2.3
NUE035	7/6/10	31.4	32.2	8.1	13.3	7.8	0.0	0.0	2.3

Table A2. Field Data for Sampling (Rainfall Events)

Sample Site	Date	Temp (°C)	Salinity (ppt)	pH	Turbidity (NTU)	DO (mg/L)	Rainfall prior to sampling (inch) (cumulative) 24 hrs	3 days	7 days
NUE028	5/16/10	27.9	29.5	7.9	17.2	na	1.0	1.0	1.0
NUE029	5/16/10	27.5	29.7	7.9	20.3	na	1.0	1.0	1.0
NUE031	5/16/10	28.5	30.0	7.9	11.4	na	1.0	1.0	1.0
NUE032	5/16/10	27.9	30.0	7.9	15.2	na	1.0	1.0	1.0
NUE033	5/16/10	28.0	29.2	7.8	12.4	na	1.0	1.0	1.0
NUE035	5/16/10	30.6	30.0	7.9	19.4	na	1.0	1.0	1.0
NUE028	6/9/10	29.2	32.0	8.0	29.9	6.3	1.4	1.4	3.9
NUE029	6/9/10	29.4	32.1	8.0	31.1	6.5	1.4	1.4	3.9
NUE031	6/9/10	29.6	31.4	8.0	53.3	6.2	1.4	1.4	3.9
NUE032	6/9/10	29.6	31.4	8.0	48.5	6.1	1.4	1.4	3.9
NUE033	6/9/10	28.3	31.2	7.8	25.8	6.1	1.4	1.4	3.9
NUE035	6/9/10	29.3	31.1	8.0	25.9	6.5	1.4	1.4	3.9
NUE028	9/10/10	29.0	33.7	8.0	19.3	5.7	0.0	2.3	3.6
NUE029	9/10/10	29.0	33.7	8.0	25.5	5.6	0.0	2.3	3.6
NUE031	9/10/10	28.9	33.4	8.0	30.7	5.8	0.0	2.3	3.6
NUE032	9/10/10	29.4	33.5	8.0	29.0	5.7	0.0	2.3	3.6
NUE033	9/10/10	29.5	33.0	7.9	22.3	5.0	0.0	2.3	3.6
NUE035	9/10/10	29.4	33.3	8.0	24.0	6.0	0.0	2.3	3.6
NUE028	9/22/10	27.0	26.8	7.9	38.3	6.3	0.1	5.3	8.3
NUE029	9/22/10	27.1	26.6	8.0	50.1	6.1	0.1	5.3	8.3
NUE031	9/22/10	27.1	26.4	8.0	42.4	6.8	0.1	5.3	8.3
NUE032	9/22/10	27.3	26.4	8.1	41.7	6.3	0.1	5.3	8.3
NUE033	9/22/10	27.1	26.2	8.0	40.3	6.3	0.1	5.3	8.3
NUE035	9/22/10	27.1	26.2	8.0	26.3	6.7	0.1	5.3	8.3

Appendix B

Standard Operating Procedure (Courtesy Dr. K. Gordon)

Enterococcus esp **Assay Protocol for non-inhibitory samples**

Scope of Application

This protocol applies to the extraction of *Enterococcus* spp. DNA from colonies growing on membrane filters incubated on mEI plates from sites where there is no PCR inhibition. DNA is then use to determine the presence of the esp gene at the site(s) in question

Summary

This protocol outlines the correct procedure to handle membranes containing enterococci colonies, as well as how to perform DNA extraction and PCR amplification of the esp gene.

Apparatus/Supplies

- Qiagen DNeasy Blood and Tissue Kit and ASL buffer (purchased separately, Cat #s 69506 and 19082 resp) or
- Qiamp DNA Stool mini kit (not as cost effective as above option)
- Ice cold 100% Ethanol (VWR Cat# EMD-4450)
- 15ml or 50ml centrifuge tube (VWR Cat# 89039-666 and 89039-658)
- 2X GoTaq® Green Master Mix (VWR Cat# PAM7122 Or PAM7123)
- 10 mM esp forward and esp reverse primer
- Nuclease free/ PCR grade water- comes with GoTaq mix or can be purchased separately (Fisher Sci cat # bp2484-50)
- 0.2 ml Thin walled PCR tubes (USA Sci Cat# 1402-8100)
- 0.5 ml Tube (USA Sci Cat# 1605-0099)
- DNA away (VWR cat# 53509-506)
- Thermocycler
- mEI plates
- 0.45 µm nitrocellulose filter
- Azide Dextrose broth (VWR cat# 90003-102)

Safety

Always wear a lab coat and use nitrile gloves. The chemical ingredients of the DNA away are proprietary but some users have observed a burning sensation with through latex gloves and with inhalation of fumes.

Procedures

When handling filters, it is easy to cross-contaminate samples with the forceps. When possible, a new pair of forceps should be used for each sample. Each pair should be decontaminated by dipping them into reagent alcohol and flaming then dipping into or spraying ends with DNAaway. The DNAaway must then be wiped off with a paper towel. The forceps can then be flamed again prior to use or for added precautions the forceps can be cross-linked in the cross linker, twice at an energy setting of 600.0 (~2 minutes each) before being used to transfer filters. It is especially important that this procedure be followed while transferring the filters from the mEI plates to the azide dextrose tubes.

The spike and method blank (MB) help keep track of cross-contamination therefore, the MB should always be processed after the spike. The spike should be processed either as the very first 'sample' or after all the samples and before the MB. Samples should be treated in the same order for the whole procedure.

I. Sample Processing (McQuaig *et al.* 2006)

- Filter 300ml of each sample through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

II. Controls

- Prepare two positive controls, each of which will be spiked with 100µl of a 10^{-5} dilution of an overnight culture of *Enterococcus faecium* C68, which contains the esp gene. These controls are termed spike 1 (SP1) and spike 2 (SP2). SP1 is a 300 ml composite sample of each of the sites sampled while SP2 is 300 ml of buffered dilution water.
- Prepare a method blank (MB) consisting of 300ml buffered dilution water filtered through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

III. Enrichment Step (McQuaig *et al.* 2006)

Have ready 15 mL screw-cap tubes (1 per sample), each containing 5 ml azide dextrose broth (Difco) [Azide Dextrose broth= 34.7g/L water]. Lift filters containing enterococci colonies from mEI plates with sterile tweezers which have been treated with DNAaway, crumble/fold with the aid of

another pair of forceps or a sterile swab, and place into the top of the tube. Push the filter down with a sterile swab. Vortex vigorously and incubate for 3 hours at 41^{o}C with vigorous shaking to wash bacteria from the filters and enrich the culture.

IMPORTANT NOTE: CHANGE GLOVES FOLLOWING <u>ANY</u> VISIBLE CONTACT WITH LIQUID!! USE BARRIER PIPET TIPS THROUGHOUT!

IV. Preparation for Extraction (with Qiagen DNeasy Blood and Tissue Kit and ASL or the Qiamp DNA Stool mini kit)

- Spray bench with (1) 70% ethanol and wipe, and (2) DNA Away and wipe
- Pre-heat the heating block to 95°C and ensure the 70°C is on and at 70°C.
- Set out and label 1 filter spin column per sample and 1 for extraction control.
- Label 2 sets of 2 ml microcentrifuge tubes for (a) initial centrifugation, and (b) final DNA elution step. These tubes should be crosslinked twice while open in a microcentrifuge tube tray. The tubes should then be closed while still in the crosslinker. You will need one extra tube in each set for the extraction blank.
- Aliquot reagents into 15 ml or 50 ml sterile, screwcap tubes (ASL, AL, ethanol, proteinase K, AW1, AW2, AE) or microcentrifuge tube (proteinase K).

V. Extraction for non-inhibitory sites (Modified from Manufacturers instructions)

- From each sample/control, pipet 1.8 ml into a 2 ml microcentrifuge tube. Excess culture can be stored at 4ºC in case of problems with extraction.
- Centrifuge culture tubes at high speed in microcentrifuge[c] 2-3 min. to pellet.
- Decant the supernatant and resuspend each pellet in 200 µl ASL lysis buffer (Qiagen, Inc.). Also set up the extraction blank (ASL buffer only). Transfer tubes to heating block and incubate at 95ºC for 5 min. Supernatant should be treated as biohazardous waste.
- Add 15 µl of Proteinase K (Qiagen, Inc.) to each sample followed by 200 µl Buffer AL (Qiagen, Inc.).
- Vortex the sample immediately and transfer to 70°C incubator for 10 minutes.
- Add 200 µl ice cold absolute ethanol and vortex the samples immediately.
- Transfer the resulting suspension to filter spin columns, followed by centrifugation at 10,000 × g for 1 min. Remove tubes from centrifuge SLOWLY to avoid wetting the column (this caveat applies to next steps). If the column does get wet just re-centrifuge the sample(s). Discard the collection tube.
- Place columns into new collection tubes and wash each column with 500 µl buffer AW1 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min. Discard collection tube.
- Place columns into new collection tubes and wash each column with 500 µl buffer AW2 (Qiagen, Inc.) by centrifugation at 14,000 × g for 3 min. VERY CAREFULLY remove

from centrifuge and ensure none of the filtrate has splashed back on the column. It is crucial that the column is dry before starting the next step. Discard collection tube.

- Place each column in its CORRESPONDINGLY LABELED MICROCENTRIFUGE TUBE. Add 200 µl buffer AE (Qiagen, Inc.) to the column. Let buffer sit in column 5 min. before centrifugation to maximize yield. Elute DNA by centrifugation at 10,000 × g for 1 min. Store the eluate at -20ºC until used as PCR template.

VI. PCR

- Add template to tubes on bench (NOT under hood) that has been cleaned with DNA Away. Always run a no-DNA PCR negative control in addition to a positive control reaction with *Enterococcus faecium* C68 DNA as the template.

Recipe per reaction using GoTaq Green Mix (Promega; Taq, dNTPs, buffer and gel loading dye included)

- 25 □l GoTaq Green Mix
- 15 □l H_2O
- 2.5 □l *esp* forward primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-TAT GAA AGC AAC AGC ACA AGT T-3'(Scott *et al.* 2005)
- 2.5 □l *esp* reverse primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-ACG TCG AAA GTT CGA TTT CC-3'- (Hammerum and Jensen 2002)
- 5 □l template

PCR Cycle:

- Initial 94°C for 2 min.

30 cycles of:

- 94°C 1 min
- 58°C 1 min
- 72°C 1 min

1 cycle of

- Final 72°C for 5 min

Hold at 4°C

VII. Electrophoresis

- Have ready a 2.0% agarose gel
- Ethidium bromide to stain the DNA can either be added directly to the gel (1ul of 1% EtBR per 50ml of gel) or to the running buffer (20ul of 1% EtBR added to 1L 1X TAE). 1% EtBr solution is a 1%w/v solution in water (eg. 0.1g in 10ml water).
- Load the Promega 100 bp ladder in the first lane (6ul per lane).
- Load 10ul of sample into each lane .
- Run the gel at 90 V for ~ 45 min.
- The expected product is 680 bp.

References

Hammerum, A.M. and Jensen, L.B. (2002) Prevalence of esp, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark. *J Clin Microbiol* **40**, 4396.

McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R. and Lukasik, J.O. (2006) Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. *Appl Environ Microbiol* **72**, 7567-7574.

Scott, T.M., Jenkins, T.M., Lukasik, J. and Rose, J.B. (2005) Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. *Environ Sci Technol* **39**, 283-287.

Standard Operating Procedure (Courtesy Dr. K. Gordon)

<u>*Enterococcus esp* Assay Protocol for inhibitory samples</u>

Scope of Application

This protocol applies to the extraction of *Enterococcus* spp. DNA from colonies growing on membrane filters incubated on mEI plates from sites where there is PCR inhibition. DNA is then use to determine the presence of the esp gene at the site(s) in question

Summary

This protocol outlines the correct procedure to handle membranes containing enterococci colonies, as well as how to perform DNA extraction and PCR amplification of the esp gene.

Apparatus/Supplies

- Qiagen DNeasy Blood and Tissue Kit and ASL buffer (purchased separately, Cat #s 69506 and 19082 resp) or
- Qiamp DNA Stool mini kit (not as cost effective as above option)
- InhibitEX tablets (Qiagen, Cat # 19590)
- Ice cold 100% Ethanol (VWR Cat# EMD-4450)
- 15ml or 50ml centrifuge tube (VWR Cat# 89039-666 and 89039-658)
- 2X GoTaq® Green Master Mix (VWR Cat# PAM7122 Or PAM7123)
- 10 mM esp forward and esp reverse primer
- Nuclease free/ PCR grade water- comes with GoTaq mix or can be purchased separately (Fisher Sci cat # bp2484-50)
- 0.2 ml Thin walled PCR tubes (USA Sci Cat# 1402-8100)
- 0.5 ml Tube (USA Sci Cat# 1605-0099)
- DNA away (VWR cat# 53509-506)
- Thermocycler
- mEI plates
- 0.45 µm nitrocellulose filter
- Azide Dextrose broth (VWR cat# 90003-102)

Safety

Always wear a lab coat and use nitrile gloves. The chemical ingredients of the DNA away are proprietary but some users have observed a burning sensation with through latex gloves and with inhalation of fumes.

Procedures

When handling filters, it is easy to cross-contaminate samples with the forceps. When possible, a new pair of forceps should be used for each sample. Each pair should be decontaminated by dipping them into reagent alcohol and flaming then dipping into or spraying ends with DNAaway. The DNAaway must then be wiped off with a paper towel. The forceps can then be flamed again prior to use or for added precautions the forceps can be cross-linked in the cross linker, twice at an energy setting of 600.0 (~2 minutes each) before being used to transfer filters. It is especially important that this procedure be followed while transferring the filters from the mEI plates to the azide dextrose tubes.

The spike and method blank (MB) help keep track of cross-contamination therefore, the MB should always be processed after the spike. The spike should be processed either as the very first 'sample' or after all the samples and before the MB. Samples should be treated in the same order for the whole procedure.

I. Sample Processing (McQuaig *et al.* 2006)

- Filter 300ml of each sample through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

II. Controls

- Prepare two positive controls, each of which will be spiked with 100µl of a 10^{-5} dilution of an overnight culture of *Enterococcus faecium* C68, which contains the esp gene. These controls are termed spike 1 (SP1) and spike 2 (SP2). SP1 is a 300 ml composite sample of each of the sites sampled while SP2 is 300 ml of buffered dilution water.
- Prepare a method blank (MB) consisting of 300ml buffered dilution water filtered through a 0.45 µm nitrocellulose filter.
- Incubate filters on mEI agar for 48h at 41°C.

III. Enrichment Step (McQuaig *et al.* 2006)

Have ready 15 mL screw-cap tubes (1 per sample), each containing 5 ml azide dextrose broth (Difco) [Azide Dextrose broth= 34.7g/L water]. Lift filters containing enterococci colonies from mEI plates with sterile tweezers which have been treated with DNAaway, crumble/fold with the aid of another pair of forceps or a sterile swab, and place into the top of the tube. Push the filter down with a sterile swab. Vortex vigorously and incubate for 3 hours at 41^{o}C with vigorous shaking to wash bacteria from the filters and enrich the culture.

IMPORTANT NOTE: CHANGE GLOVES FOLLOWING ANY VISIBLE CONTACT WITH LIQUID!! USE BARRIER PIPET TIPS THROUGHOUT!

IV. Preparation for Extraction (with Qiagen DNeasy Blood and Tissue Kit and ASL or the Qiamp DNA Stool mini kit)

- Spray bench with (1) 70% ethanol and wipe, and (2) DNA Away and wipe
- Pre-heat the heating block to 95°C and ensure the 70°C is on and at 70°C.
- Set out and label 1 filter spin column per sample and 1 for extraction control.
- Label 3 sets of 2 ml microcentrifuge tubes for (a) initial centrifugation, ASL and addition of InhibitEX tablet (b) Proteinase K and then transfer of inhibitEX supernatant and (c) final DNA elution step. These tubes should be crosslinked twice for two minutes while open in a microcentrifuge tube tray. The tubes should then be closed while still in the crosslinker.
- You will need one extra tube in each set for the extraction blank.
- Aliquot reagents into 15 ml or 50 ml sterile, screwcap tubes (ASL, AL, ethanol, proteinase K, AW1, AW2, AE) or microcentrifuge tube (proteinase K).

V. Extraction for non-inhibitory sites (Modified from Manufacturers instructions)

- From each sample/control, pipet 1.8 ml into a 2 ml microcentrifuge tube. Excess culture can be stored at 4ºC in case of problems with extraction.
- Centrifuge culture tubes at high speed in microcentrifuge[c] 2-3 min. to pellet.
- Decant the supernatant and resuspend the pellet in 1.2 ml ASL lysis buffer (Qiagen, Inc.).
- Vortex for 1 min or until homogenized. Also set up the extraction blank (ASL buffer only). Transfer tubes to heating block and incubate at 95ºC for 5 min.
- Add 1 InhibitEX tablet (Qiagen, Inc.) to each sample and vortex immediately and continuously for 1 min until tablet is completely suspended. Incubate for 1 min at room temperature to allow inhibitors to absorb to the InhibitEX.
- Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX.
- Pipet 200 μl of supernatant (be sure not to get any of the pellet) into a new microcentrifuge tube and add 15 μl of Proteinase K (Qiagen, Inc.). Vortex.
- Add 200 μl Buffer AL (Qiagen, Inc.) and vortex for 15 s.
- Transfer tubes to 70°C incubator for 10 minutes.
- Add 200 μl ice cold absolute ethanol and vortex the samples immediately.
- Transfer the resulting suspension to filter spin columns, followed by centrifugation at 10,000 × g for 1 min. Remove tubes from centrifuge SLOWLY to avoid wetting the column (this caveat applies to next steps). If the column does get wet just re-centrifuge the sample(s). Discard the collection tube.
- Place columns into new collection tubes and wash each column with 500 μl buffer AW1 (Qiagen, Inc.) by centrifugation at 10,000 × g for 1 min. Discard collection tube.

- Place columns into new collection tubes and wash each column with 500 µl **buffer AW2** (Qiagen, Inc.) by centrifugation at 14,000 × g for 3 min. VERY CAREFULLY remove from centrifuge and ensure none of the filtrate has splashed back on the column. It is crucial that the column is dry before starting the next step. Discard collection tube.
- Place each column in its CORRESPONDINGLY LABELED MICROCENTRIFUGE TUBE. Add 200 µl buffer AE (Qiagen, Inc.) to the column. Let buffer sit in column 5 min. before centrifugation to maximize yield. Elute DNA by centrifugation at 10,000 × g for 1 min. Store the eluate at -20ºC until used as PCR template.

VI. PCR

- Add template to tubes on bench (NOT under hood) that has been cleaned with DNA Away. Always run a no-DNA PCR negative control in addition to a positive control reaction with *Enterococcus faecium* C68 DNA as the template.

Recipe per reaction using GoTaq Green Mix (Promega; Taq, dNTPs, buffer and gel loading dye included)

- 25 □l GoTaq Green Mix
- 15 □l H_2O
- 2.5 □l *esp* forward primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-TAT GAA AGC AAC AGC ACA AGT T-3'(Scott *et al.* 2005)
- 2.5 □l *esp* reverse primer (working concentration 10 mM; diluted 1:10 from 100 mM stock); 5'-ACG TCG AAA GTT CGA TTT CC-3'- (Hammerum and Jensen 2002)
- 5 □l template

PCR Cycle:

- Initial 94°C for 2 min.

30 cycles of:

- 94°C 1 min
- 58°C 1 min
- 72°C 1 min

1 cycle of

- Final 72°C for 5 min

Hold at 4°C

VII. Electrophoresis

- Have ready a 2.0% agarose gel
- Ethidium bromide to stain the DNA can either be added directly to the gel (1ul of 1% EtBR per 50ml of gel) or to the running buffer (20ul of 1% EtBR added to 1L 1X TAE). 1% EtBr solution is a 1%w/v solution in water (eg. 0.1g in 10ml water).
- Load the Promega 100 bp ladder in the first lane (6ul per lane).
- Load 10ul of sample into each lane .
- Run the gel at 90 V for ~ 45 min.
- The expected product is 680 bp.

References

Hammerum, A.M. and Jensen, L.B. (2002) Prevalence of esp, encoding the enterococcal surface protein, in *Enterococcus faecalis* and *Enterococcus faecium* isolates from hospital patients, poultry, and pigs in Denmark. *J Clin Microbiol* **40**, 4396.

McQuaig, S.M., Scott, T.M., Harwood, V.J., Farrah, S.R. and Lukasik, J.O. (2006) Detection of human-derived fecal pollution in environmental waters by use of a PCR-based human polyomavirus assay. *Appl Environ Microbiol* **72**, 7567-7574.

Scott, T.M., Jenkins, T.M., Lukasik, J. and Rose, J.B. (2005) Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. *Environ Sci Technol* **39**, 283-287.

Appendix C

Table C.1 Results for *esp* controls for events from 2/17/10 through 5/26/10 showing PCR inhibition for some samples (SP1 no *esp* detection), but PCR controls meeting requirements

	2/17/10	3/24/10	4/28/10	5/16/10*	5/26/10
+ PCR	+	+	+	+	+
- PCR	-	-	-	-	-
SP1	+	-	-	+	-
SP2	+	-	+	-	+
MB	-	-	-	-	+
EB	-	-	-	-	+

*rainfall event

Table C.2 Results for *esp* controls for events from 6/02/10 through 9/22/10 showing PCR inhibition for some samples (SP1 no *esp* detection), but PCR controls meeting requirements

	6/03/10	6/09/10*	7/06/10	9/10/10*	9/22/10*
+ PCR	+	+	+	+	+
- PCR	-	-	-	-	-
SP1	-	-	-	+	+
SP2	+	+	+	+	+
MB	-	-	-	-	-
EB	-	-	-	-	-

*rainfall event

C3. Results of spiking samples with C68 (positive control for *esp*), showing inhibition for most samples collected in June and July but no inhibition for September when the inhibitEX tablets (Qiagen) were used during DNA extraction, according to the protocol for inhibitory sites (Appendix B)

	6/03/10	6/09/10*	7/06/10	9/10/10*	9/22/10*
NUE028-1+C68	-	-	-	+	+
NUE029-1+C68	-	-	-	+	+
NUE031A-1+C68	-	-	-	+	+
NUE032A-1+C68	-	-	-	+	+
NUE033A-1+C68	+	-	-	+	+
NUE035A-1+C68	-	-	-	+	+

*rainfall event