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TECHNIQUE FOR DETECTING SOURCES OF HUMAN POLLUTION IN WATER USING AN *Enterococcus* BIOMARKER

presented by

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has been accepted towards fulfillment
of the requirements for the

M.S. degree in Crop and Soil Sciences

Major Professor's Signature

10/10/05

Date

TECHNIQUE FOR DETECTING SOURCES OF HUMAN POLLUTION IN WATER USING AN *Enterococcus* BIOMARKER

BY

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Crop and Soil Sciences

2005

ABSTRACT

TECHNIQUE FOR DETECTING SOURCES OF HUMAN POLLUTION IN WATER USING AN *Enterococcus* BIOMARKER

By

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Microbial source tracking is a newly emerging field, which aims to identify sources of fecal pollution in a body of water. The first portion of this research aimed to develop a host-specific molecular marker for the identification of human fecal pollution targeting an *Enterococcus spp.* The second portion of this research aimed to examine natural waters for the marker and to develop a preliminary understanding of how the marker related to other common microbial indicators, including enteric viruses. Two different methods were examined for developing the molecular marker. The first method targeted the *ace* gene in *Enterococcus faecalis* and was based on its identification in individually isolated colonies. While this method did not prove to be host-specific for any of the human and animal species examined, it did show promise as a marker for the presence of *E. faecalis*. The second method targeted the *esp* gene in *Enterococcus faecium* and was based on evaluating the entire microbial population of a sample. This marker was shown to be human-specific in 97% (n=69) of the human fecal samples and in 0% (n=102) of the animal fecal samples analyzed. Nine rivers in the State of Michigan were examined for the *esp* marker and it was found in 22% of the samples. Culturable enteric viruses were found in 33% of the samples. The Grand and Rouge Rivers were positive for both indicators of human fecal pollution. This preliminary study demonstrates that the *esp* marker shows promise as being a reliable indicator of human fecal pollution.

ACKNOWLEDGEMENTS

I would like to thank the Michigan Sea Grant Project Number M/PD-10 for funding the Michigan river water quality monitoring portion of this research. I would also like to thank the Homer Nowlin Endowed Chair of Water Research for funding the remainder of this research.

Many thanks to my advisor Dr. Joan B. Rose, for her support and guidance throughout my Masters research. Also, thank you to my committee members, Dr. Thomas S. Whittam, Dr. Syed Anwar Hashsham, and Dr. Frank Dazzo, for their guidance during my research.

Lastly, I would like to thank the other graduate students and employees in Dr. Rose's lab for their help in many aspects of my research. Your time and effort is greatly appreciated.

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LITERATURE REVIEW

1.1 Waterborne Diseases and Risks Associated with Exposure to Human Sewage

Waterborne diseases range from gastroenteritis to respiratory ailments and are caused by enteric bacteria, viruses, and protozoans. These microorganisms are able to survive and re-infect upon transmission through saline and fresh waters (Table 1-1) (Lund 1982; Slifko *et al.* 2000; Russell and Jarvis 2001; Leclerc *et al.* 2002; Adams *et al.* 2003). The most severe symptoms occur in the young, elderly, and immunocompromised and globally, waterborne disease represents a large portion of the total disease burden on populations. World-wide, approximately four billion cases of diarrhea occur annually (Murray and Lopez 1996), leading to 2.2 million deaths, mostly in children under the age of five (World Health Organization 2000).

Fecal material, from animals and humans, and untreated or improperly treated wastewaters are the main sources of waterborne pathogens (Craun 1986). The pathogens are transmitted to human hosts via the fecal-oral cycle. The survivability and reproductive success of the pathogens outside of a host varies from pathogen to pathogen. Bacterial pathogens survive in both surface and ground waters for several weeks to months at low (4°C) temperatures, while exposure to elevated (25°C) temperatures result in reduced $\log_{10}$ numbers within a few hours to days (Guan and Holley 2003). Protozoan pathogens have developed a complex life-cycle with dormant, spore-like structures termed (oo)cysts, which are able to survive for longer periods (no significant loss of (oo)cyst integrity was observed after 30 days) as compared with bacteria at both low (4°C) and elevated (30°C) temperatures (Nasser *et al.* 2003). Viral pathogens,

Table 1-1. Key waterborne microorganisms and the symptoms and outbreaks they caused from 1999-2002 (FDA/CFSAN 1992; Lee *et al.* 2002; Blackburn *et al.* 2004; Yoder *et al.* 2004).

Microorganism (Disease)	**Symptoms**	**Drinking Water Outbreaks**	**Recreational Water Outbreaks**
Campylobacter jejuni (campylobacteriosis)	Watery or sticky diarrhea, fever, abdominal pain, nausea, headache, muscle pain	4	1
Shigella spp. (shigellosis)	Abdominal pain, cramps, diarrhea, fever, vomiting, mucus in stools	0	5*
Vibrio cholera (cholera)	Rice water diarrhea, abdominal cramps, nausea, vomiting, dehydration, shock	0	0
Pseudomonas aeruginosa (dermatitis)	Folliculitis (infection of hair follicles) or rash	0	30
Pathogenic *Escherichia coli*	Diarrhea, abdominal pain	6	8
Legionella pneumophila (Legionnaires disease)	Pneumonia	9*	2*
Cryptosporidium hominis (cryptosporidiosis)	Severe watery diarrhea, may also be respiratory with coughing and low-grade fever	2*	27*
Giardia intestinal (giardiasis)	Diarrhea	8	1
Naegleria fowleri (meningoencephalitis)	Varies from asymptomatic to mild gastroenteritis, dysentery	1	12
Norovirus (gastroenteritis)	Nausea, vomiting, diarrhea, abdominal pain	8	8

* Represents outbreaks caused by other species in the same genus.

particularly *Norovirus*, display a cold weather seasonality phenomenon. This seasonality trend infers that *Norovirus* is able to survive for longer periods in the environment in cold conditions compared with warm, summer conditions (Mounts *et al.* 2000). Because the protozoan and viral pathogens require a host in order to reproduce, they will not replicate

in the environment. Bacterial pathogens have the ability to replicate outside of a host; however, there are few field studies to support this hypothesis.

The Centers for Disease Control and Prevention (CDC) defines an outbreak as more than two people experiencing a similar illness after ingestion of drinking water or exposure to recreational/occupational waters, with the epidemiological evidence implicating water as the most probable source (Lee *et al.* 2002; Blackburn *et al.* 2004; Yoder *et al.* 2004). From January 2001 to December 2002, 31 outbreaks were associated with drinking water (Blackburn *et al.* 2004) and 65 with recreational water (Yoder *et al.* 2004) in the United States for a total of 96 outbreaks. In these two years, over 3,000 people become ill and 15 died after exposure to waterborne pathogens (Table 1-1).

The etiologic agents, including microbial and chemical agents, were identified in 81 of the outbreaks. The causes of the remaining 15 outbreaks remain unknown. This is the highest identification rate for the etiological agents for waterborne outbreak surveillance. However, several key outbreaks were not identified and this continues to be a major concern for those involved in preventing outbreaks and regulating the sources of potential contamination. Many factors are involved in the identification of the agent. The outbreak must be recognized by local health authorities and then reported to the CDC. In order to identify the agent causing the outbreak, laboratories at the local/state level need to have methods and equipment available for positive identification (Embrey *et al.* 2002; Hilborn *et al.* 2002). Samples, especially water, need to be collected as soon as the outbreak is identified to avoid the effects of pathogen dilution, transportation, and mortality, which hinders their detection. However, a lag time will always exist between the initial period of contamination and the identification that an outbreak is occurring due to the time

needed to go from the exposure to the illness (incubation period), to physicians reporting the pathogen/symptom, to health officials identifying that a cluster is occurring in pathogens/symptoms, and finally to the outbreak investigation. Timely identification of the pathogens enhances the probability that immediate and appropriate action can be taken to reduce the severity of the outbreak.

1.2 Water Quality Indicators

Although the microorganisms listed in Table 1-1 represent a large portion of the causative agents responsible for waterborne disease outbreaks, routine monitoring of drinking and recreational water quality does not screen for all of them. Instead, water samples are monitored for the presence of indicator microorganisms, used to infer the risk of acquiring an acute gastrointestinal illness upon consuming drinking water or the accidental ingestion of recreational waters (National Research Council of the National Academies (NRC) 2004). They indicate the presence of fecal pollution and the possible presence of enteric pathogens.

Historically, total coliforms were used to monitor water quality; fecal coliforms were later used to monitor fecal contamination, as they are more specific to feces than total coliforms (NRC 2004). However, in the 1970's and early 1980's, the United States Environmental Protection Agency (U.S. EPA) initiated a series of studies designed to determine if swimming in sewage-contaminated waters carried a health risk for bathers, and if quantitative relationships between water quality and health could be determined (Cabelli *et al.* 1979; Cabelli *et al.* 1982; Cabelli 1983). An additional goal was to identify the most appropriate indicator (U.S. EPA 1986). Results led the U.S. EPA to move from fecal coliforms to alternative indicators recommending enterococci as the indicator of

choice in marine environments, and both *Escherichia coli* and enterococci, with *E. coli* being the preferred indicator, in fresh water environments (Table 1-2).

Table 1-2. Surface water quality maximum allowable limits (CFU/100mL) for recreational use.

			Geometric Mean*	Single Sample Maximum
U.S. EPA[1]				
	Marine Water	enterococci	35	104
	Fresh Water	enterococci	33	61
		E. coli	126	235
Michigan Department of Environmental Quality[2]				
	Fresh Water	*E. coli*	130	300

[1] (U.S. EPA 1986)
[2] (MDEQ 2002)
* based on ≥ 5 samples equally spaced over a 30-day period

The U.S. EPA is currently conducting an extensive epidemiological investigation on the occurrence of enterococci at marine and fresh water beaches. The results of these studies will be critical in the re-evaluation of U.S. EPA's current recreational water quality standards. Based on these recommended guidelines, individual states set their own water quality guidelines or criteria. The State of Michigan has based their criteria on *E. coli* concentrations (Table 1-2). Additional microorganisms have also been recognized as being potential indicators of fecal pollution and the presence of enteric pathogens (Table 1-3).

Several recommendations have been made in regard to the levels of *E. coli* and enterococci in relationship to health risk (U.S. EPA 1986). However, prediction of health risks in recreational waters continues to be a source of debate among regulators and

scientists. Some studies maintain that these criteria are adequate for protecting bathers. Wade *et al.* (2003) examined the literature using a meta-analysis. After identifying 27

Table 1-3. Characteristics of the recommended and alternative water quality indicators.

Indicator	Definition	Target systems	Methods
Escherichia coli	Gram-negative, facultative anaerobe that is able to grow at elevated temperatures (45°C) by fermenting lactose with the production of carbon dioxide. Member of the fecal coliform group.	Fresh water systems used for recreation and drinking.	(American Public Health Association *et al.* 1999; U.S. EPA 2002a; U.S. EPA 2002d; U.S. EPA 2002e) Colilert®
Enterococci	Gram-positive microbes that are able to grow at low and elevated temperatures (10°C and 45°C), at elevated pH (9.5), and in 6.5% sodium chloride. Member of the fecal streptococci.	Marine and fresh water systems used for recreation.	(U.S. EPA 2002b; U.S. EPA 2002c) Enterolert™
Clostridium perfringens	Gram-positive, anaerobic spore-forming, rod-shaped bacterium.	Marine systems in Hawaii as an alternative to *E. coli* and enterococci.	(Bisson and Cabelli 1979)
Bacteriophage	Viruses that infect only bacteria.	Fresh surface and ground waters.	(U.S. EPA2001a; U.S. EPA 2001b)

studies that met their selection criteria by including data on human exposure to water, water quality measures, health outcomes, and study design, they concluded that, in marine waters, enterococci were an adequate indicator of gastrointestinal illness; and that *E. coli* was an adequate indicator in fresh waters. They also determined that the guidelines set by the U.S. EPA in 1986 are adequate based on epidemiological information available in both the published and non-published literature.

However, since the original recommendations were made in 1986, concern has been raised in using *E. coli* and enterococci as indicators in both marine and fresh water environments. Griffin *et al.* (2001) examined the different indicators being used in marine

waters, including *E. coli*, enterococci, *Clostridium perfringens*, and bacteriophages, and concluded that the existing recommendations needed to be reevaluated for coastal waters. The use of *E. coli* and possible enterococci are being challenged as data show that *E. coli* is able to replicate in warm, tropical environments (NRC 2004). There is also a debate on whether *E. coli* and enterococci are appropriate indicators of fecal pollution in fresh waters. Recent studies conducted in the Great Lakes concluded that beach sand (Whitman and Nevers 2003), gull feces (Fogarty *et al.* 2003; Haack *et al.* 2003), and algal mats (Whitman *et al.* 2003) are all potential sources of these indicator microorganisms; thereby providing a source of indicators that does not appropriately reflect the presence of human pathogens. Various *Enterococcus spp.* are also known to exist as epiphytes in the phylloplane of plants (Muller *et al.* 2001). How and to what degree these non-fecal sources of indicators affect water quality and therefore the human health risk are currently unknown.

1.3 Microbial Source Tracking

In 1999, the U.S. EPA proposed new rules (Total Maximum Daily Load (TMDL) program) to tackle the problem of non-point sources of pollution that enter waterways. This program outlines the framework for regulating the amount and types of pollution entering the water. First, the maximum amount of pollutant that a waterbody can receive and still meet water quality standards needs to be calculated. Second, the relative contribution of the pollutant from all sources needs to be determined (U.S. EPA 1999). Since *E. coli* and enterococci are frequently found in non-sewage sources, new techniques need to be developed to determine the source of the microorganisms in order to meet these new regulations.

Historically, the ratio between fecal coliforms and fecal streptococci was used to determine whether the fecal pollution in a waterway was from an animal or a human source. This ratio was used because it was known that animals secreted higher concentrations of fecal streptococci than fecal coliforms whereas the opposite was seen in human feces. A ratio > 4.0 would indicate human pollution and a ratio $\leq$0.7 would indicate animal pollution (Geldreich and Kenner 1969). However, this method has not proven to be accurate at sourcing the pollution. This method frequently fails due to differences in the survival rates of the two bacterial groups in water, and to differences in the bacterial concentrations excreted in the feces of various mammals. Pourcher *et al.* (1991) found that 39% of the samples from human origin (feces and wastewater) had ratios below 4 and the animals samples had ratios over 0.7, 86% of the time.

Recent advances in technology and microbiology have allowed researchers to revisit the concept of differentiating sources of fecal pollution. Thus, the scientific field of "Microbial Source Tracking" has emerged. This discipline involves methods, which utilize the phenotypic and genotypic characteristics of bacteria, viruses, and in some cases protozoa to assist in the identification of sources of fecal contamination; in addition the occurrence of some chemicals and the presence of certain microorganisms are also employed (Sinton *et al.* 1998; Scott *et al.* 2002; Simpson *et al.* 2002; Meays *et al.* 2004). The microbial source tracking methods can be divided into six categories: 1) phenotypic, 2) genotypic, 3) library-based, 4) non-library based or host-specific, 5) chemical, and 6) the detection of specific microorganisms.

Antibiotic resistance analysis, multiple antibiotic resistance, and carbon source utilization are the phenotypic methods commonly employed in microbial source tracking.

These methods characterize isolates from human and animal sources by either the isolates' resistance to a series of antibiotics or the carbon sources, which then can metabolize (Whitlock *et al.* 2002; Hagedorn *et al.* 2003; Kelsey *et al.* 2003). Several genotypic methods (ribotyping, RFLP, AFLP, rep-PCR) have been explored for microbial source tracking, but all of them are based on cutting and then profiling the target DNA (Parveen *et al.* 1999; Dombek *et al.* 2000; D'Agata *et al.* 2001).

The phenotypic and genotypic methods create profiles, which characterizes isolates from different fecal sources. The profiles from known fecal sources are placed together in a library, which is used to determine the source of isolates from unknown sources (Harwood *et al.* 2003; Myoda *et al.* 2003). The non-library or host-specific methods target a section of a microorganism's genome in a PCR-based assay (Bernhard and Field 2000b; Khatib *et al.* 2002). The main advantage of the non-library methods over the library methods is that the non-library methods are not subjected to the pitfalls of having to create a large dataset, which is useful over an unknown spatial and temporal period. However, the non-library methods are limited by the number of potential sources, which can currently be targeted (Field *et al.* 2003; Stewart *et al.* 2003).

The chemical methods mainly rely on the presence of caffeine (Rogers *et al.* 1986), fecal sterols (Leeming *et al.* 1996), and fluorescent whitening agents (Hayashi *et al.* 2002) to determine human sewage contamination in water, although the fecal sterols are also used to determine animal sources as well. It has also been demonstrated that the occurrence of some microorganisms can be used to determine the presence of fecal sources. *Bacteroides fragilis* bacteriophages (Tartera and Jofre 1987; Tartera *et al.* 1989) and human enteric viruses (Noble *et al.* 2003) have shown the presence of human

pollution, while animal enteric viruses (Maluquer de Motes *et al.* 2004) have been shown to determine animal pollution. The F-RNA coliphages are also used to determine human and animal sources of fecal pollution.

1.3.1 Phenotypic Library-Based Methods

1.3.1.1 Antibiotic Resistance Analysis

Antibiotic resistance analysis (ARA), multiple antibiotic resistance (MAR), and carbon source utilization are the main phenotypic methods used in microbial source tracking. These methods rely on the use of microorganisms that can be cultured. Generally, ARA is developed by isolating bacterial colonies, either *E. coli* (Parveen *et al.* 1997) or enterococci (Wiggins 1996), from known sources of fecal pollution and placing them onto media containing various antibiotics at four concentrations. Antibiotics vary from study to study; however, they are usually those used in animal feeds and clinical therapies (DuPont and Steele 1987). The results for each isolate are recorded into a library based on the greatest antibiotic concentrations that yields colony formation, for the purpose of creating an antibiotic resistance profile.

Discriminant analysis is often used to determine the classification accuracy of the library (Wiggins 1996). The average rate of correct classification (ARCC) and misclassification, which assesses the library's internal accuracy, must be determined before the library can be used to classify the source of unknown isolates. ARCC is obtained by adding the number of known source isolates correctly classified into each category and dividing by the total number of known source isolates and the rate of misclassification is obtained by adding the number of known source isolates incorrectly classified and dividing by the total (Harwood *et al.* 2000). In some instances, researchers

will perform a jackknife analysis to determine if the library contains adequate profiles. A jackknife is performed by removing a sub-set of known source isolates from the library and then testing the library with these isolates to determine how well they are classified (Harwood *et al.* 2000; Wiggins *et al.* 2003). The size of a library may partially determine the correct classification rate as small libraries have been shown to have a higher ARCC than a larger library (Wiggins *et al.* 2003; Johnson *et al.* 2004). In addition, the removal of duplicate fingerprints in a library has resulted in a further reduction of the ARCC by 21.7% (Johnson *et al.* 2004). However, a larger library is more representative of the microbial diversity present in the watershed (Wiggins *et al.* 2003).

For ARA analysis, the range of ARCC has been shown to be from 51% to 95%, which indicates the presence of variation in the design and evaluation of the libraries being used to determine sources of fecal pollution (Wiggins 1996; Harwood *et al.* 2000; Whitlock *et al.* 2002; Harwood *et al.* 2003; Wiggins *et al.* 2003). Higher ARCC are often obtained when groups of related animals are pooled together. For example, Wiggins (1996) obtained an ARCC of 72% when including isolates from beef and dairy cattle, chicken, turkey, human (wastewater), and streams. However, when they combined the beef and dairy cattle and the chicken and turkey, they obtained an ARCC of 82% (cattle, poultry, human, and streams). The average rate of misclassification has been shown to range from 7.2% to 30.7% (Harwood *et al.* 2000; Whitlock *et al.* 2002; Harwood *et al.* 2003). During a blind round-robin study, Harwood *et al.* (2003) found that the false-positive rate for ARA ranged from 39.4% to 54.6%. A false-positive occurs when a method yields a positive for a fecal source when the fecal source is not present in the sample.

After creating a library with 1,435 isolates from known sources of human and animal (cattle, poultry, and streams), Wiggins (1996) examined two local waterways in Virginia to determine the largest source of fecal pollution. Of 105 isolates collected from Cooks Creek and 88 from Muddy Creek, 96% and 95% (two-way classification human and animal), respectively, were classified as being of animal origin. Two-way classifications are performed via discriminant analysis on two sources of fecal contamination. Whitlock *et al.* (2002) created a similar library in Florida using isolates from cattle, canines, humans, and wild animals to determine that a creek, a puddle, and soil were indeed being impacted by a failing septic system (52.8% to 91.7% of isolates were two-way classified as human when comparing human versus animals).

1.3.1.2 Multiple Antibiotic Resistance

Multiple antibiotic resistance distinguishes resistant and sensitive isolates from fecal sources and is one of the tools in microbial source tracking (Parveen *et al.* 1997; Kelsey *et al.* 2003). The MAR index is determined by calculating the number of antibiotics that an isolate is resistant to and then dividing by the total number of antibiotics tested (Kaspar *et al.* 1990). A high MAR index indicates that the isolate has a high degree of resistance to the antibiotics tested. Parveen *et al.* (1997) used MAR to examine point (wastewater treatment effluent) and non-point (marsh runoff) sources of fecal pollution and determined the average MAR indices for the sources to be 0.25 and 0.13, respectively. An isolate with an indice of 1.0 would indicate that it is resistant to all of the antibiotics that were tested (100%) and an index of 0.1 would indicate resistance to 10% of the antibiotics. This is consistent with the finding that isolates from point sources have a higher resistance to single and multiple antibiotics. When the point and non-point

isolates were compared with isolates from humans and animals, it was shown that the human isolates contained resistance patterns that were more similar to those of point source isolates.

1.3.1.3 Carbon Source Utilization

Carbon source utilization can be used to determine the source of fecal pollution by examining an isolate's biochemical reactions (Pourcher *et al.* 1991; Hagedorn *et al.* 2003; Wallis and Taylor 2003). Hagadorn *et al.* (2003) examined the use of the Biolog GP2 MicroPlate™ for identifying fecal sources of *Enterococcus spp.* A library created from 365 known isolates of human and non-human (poultry, cattle, equine, canine, goose, deer, and muskrat) sources correctly classified the isolates with an ARCC of 92.7% (two-way classification). Samples from three bodies of water were then assayed to determine if the library would correctly identify obvious sources of contamination. At each of the three sites, the obvious source was correctly identified by a two-way classification (human versus non-human).

The PhenePlate technique has also been used with *Enterococcus spp.* to demonstrate the usefulness of carbon source utilization. Using Simpson's Diversity Index, which is used to measure bacterial diversity (ranging from 0.0 to 1.0 representing low to high diversity, respectively), it has been shown that *Enterococcus spp.* from wastewater contains a higher diversity of available biochemical pathways (mean diversity of 0.95) than seen in the different animals (seabirds, cattle, sheep, donkeys, and dogs) examined (mean diversity of 0.32-0.72) (Wallis and Taylor 2003). Therefore in recreational waters with a high diversity index, one would expect wastewater to be the dominant factor

impacting the water and waters with a low diversity index would indicate an animal source.

In the round-robin study which examined the efficacy in the different source tracking methodologies, carbon source utilization was found to have ARCC's above 80% with a misclassification rate of approximately 5% (Harwood *et al.* 2003). However, the methods false-positive rate was 51.5% to 66.7%. Therefore, the carbon source utilization faired equally with the ARA methods examined.

1.3.2 Genotypic Methods

1.3.2.1 Library-Based Methods

1.3.2.1.1 Ribotyping

Ribotyping is restriction fragment length polymorphism (RFLP) of rRNA genes and is used in differentiating between strains of a species as well as in tracking the source of microorganisms (Gordillo *et al.* 1993; Parveen *et al.* 1999; Carson *et al.* 2001; Svec *et al.* 2001; Carson *et al.* 2003; Kuntz *et al.* 2003; Scott *et al.* 2003). The methods used in ribotyping vary; however, the steps include bacterial isolation, digestion with restriction enzymes, Southern blotting, and then hybridization with probes specific for 16S and 23S rRNA. As with the phenotypic methods mentioned above, ribotyping requires the development of a library containing known isolates to which unknown isolates are compared.

Parveen *et al.* (1999) were the first to use ribotyping in source tracking. They were able to demonstrate the utility of using ribotyping by having a two-way classification of 84% for human and animal fecal sources. The library used by Parveen *et al.* (1999) consisted of only 59 *E. coli* isolates. Carson *et al.* (2001) examined a total of 287 *E. coli*

isolates from eight different sources of fecal pollution and yielded an ARCC of 97.1% for a two-way classification of human and non-human sources. When the eight-way classification was performed, the ARCC was 73.56% demonstrating that ribotyping has a greater discriminating power compared with the phenotypic methods. In the blind source study, ribotyping exhibited correct classification of unknown isolates to a small library between 81% and 100% of the time, with incorrect classification occurring between 19% and 57% of the time (Myoda *et al.* 2003).

While ribotyping has been shown to be more accurate than ARA, there are still problems with it. Hartel *et al.* (2003) has demonstrated that the ribotype profiles used will vary depending on the diet of the particular host animal. In deer, they observed more unique ribotypes in feces from wild deer compared with penned deer. Temporal variability has also been examined. Jenkins *et al.* (2003) examined ribotypes from yearling steers and determined that the majority were transient and unique to the specific sampling event. This implies that all of the isolates used in building a library need to be collected over a short temporal period and that a library developed in year one could not be used the following year.

A geographic variability in the ribotype profiles also exists. Hartel *et al.* (2002) showed that there is a very small percentage (0%-19%) of shared ribotypes between geographically distant sources. However, unique ribotypes do exist for each of the host animals (cattle, swine, chicken, and horses) examined. Based on their results, they recommended that the library of known isolates needs to be created from sources within 175km or the watershed being examined.

1.3.2.1.2 PFGE and AFLP

Pulse field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), like ribotyping, have been used in differentiating bacterial strains and also for determining sources of fecal pollution (Gordillo *et al.* 1993; D'Agata *et al.* 2001; Parveen *et al.* 2001; McLellan *et al.* 2003; Lu *et al.* 2004). While PFGE has proven to be a useful method in epidemiological studies (D'Agata *et al.* 2001; Borchardt *et al.* 2003), its usefulness in source tracking has yet to be determined. Parveen *et al.* (2001) found that PFGE was not able to differentiate between sources of fecal pollution and contributed this to the specificity of the method to detect small sequence differences, which may not be associated with a specific bacterial characteristic, such as host source. This conclusion was also reached when PFGE was used to examine genetic variability in irrigation water and sediments (Lu *et al.* 2004). In the blind source study, PFGE correctly classified the unknown isolates 81% of the time with misclassification occurring in 21% of the unknown isolates (Myoda *et al.* 2003).

AFLP has been used to show the existence of host specificity of vancomycin-resistant *E. faecium* in hospital patients and in a select group of agricultural animals (Willems *et al.* 2000). In this same study, AFLP was able to distinguish outbreaks, which occurred at two separate hospitals. Guan *et al.* (2002) showed that AFLP can be used to predict bacterial contamination by using discriminant analysis, instead of cluster analysis (Guan *et al.* 2002). Depending on the primers used in the analysis, ARCC (three-way) ranged from 52.78% to 80.00%. Recently, AFLP has yielded correct classification rates ranging from 90.6% to 97.97% when examining human, bovine, and pig sources of *E. coli* (Leung *et al.* 2004). In addition to high source classification, Leung *et al.* (2004) was also

able to assign isolates to their correct phenotype (non-pathogenic, VTEC, or ETEC) at a range of 90.9% to 100%.

1.3.2.1.3 Rep-PCR

Repetitive element anchored PCR (rep-PCR) targets repetitive extragenic palindromic PCR (REP), enterobacterial repetitive intergenic consensus (ERIC), or BOX elements, and has been used comparing bacterial genomes (McLellan 2004). Dombek *et al.* (2000) were the first to take this method and apply it to source tracking. Their studies examined two different primers, BOX A1R and REP 1R-REP 2I, for PCR amplification and found that the BOX A1R primers, while being equally capable of classifying human and sheep isolates as the REP primers, BOX was superior at classifying other animal sources (chickens, cows, ducks, geese, and pigs).

The ARCC has been found to be 79.3% when classifying three-ways using gull, cattle, and sewage (McLellan *et al.* 2003) and 96.65% when classifying two-ways using human and non-human (Carson *et al.* 2003). This difference in classification rates may be due to differences in the primers being used, McLellan *et al.* (2003) used REP primers and Carson *et al.* (2003) used BOX A1R primers. However, McLellan *et al.* (2003) also examined ERIC primers on the same isolates and determined that while the REP and ERIC primers generated comparable, but not identical dendrogram groups that these three methods yield comparable results. However, ERIC-PCR was found to be poor at assigning *E. coli* isolates based on the phenotypic groups of non-pathogenic, VTEC, and ETEC (Leung *et al.* 2004). In the blind source study, rep-PCR was performed using the BOX A1R primers. Unknown isolates were correctly classified between 86% and 90% of the time with misclassification occurring 38% to 52% (Myoda *et al.* 2003).

Recently, a new method has been added to the microbial source tracking toolbox, a horizontal, fluorophore-enhanced, repetitive extragenic palindromic-PCR DNA fingerprinting technique (HFERP). This method uses an internal ROX-labeled molecular weight marker in the gel lanes to align, correct, and normalize the fluorescently labeled, rep-PCR DNA fingerprint bands. HFERP reduces within-gel clustering of fingerprint groups, which is normally seen in rep-PCR as well as reducing between-gel variations due to band migration (Johnson *et al.* 2004). Johnson *et al.* (2004) examining only the unique bands found in rep-PCR and HFERP analysis were able to determine that the correct classification rates for human and non-human were 60.9% and 45.8%, respectively.

1.3.2.2 Library Independent Methods

1.3.2.2.1 Host-Associated Molecular Markers

The use of host-associated molecular markers (HAMM) circumvents all of the issues associated with using a library-based method. There are two approaches to developing HAMM in the literature for use in microbial source tracking. The first approach developed by Bernhard *et al.* (2000a; 2000b) uses a combination of length heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism (T-RFLP) analysis to identify unique gene fragments within the desired host. Once the fragments were identified and shown to be host-specific, primers were developed to target them using standard PCR. This procedure has been used to develop two sets of primers, one for humans and one for ruminants (cattle). The detection limit for this human marker was 4.2 x 10^5 bacterial cells per liter and was between 8.4 x 10^4 and 8.4 x 10^6 bacterial cells per liter for cattle.

The other approach being used in developing HAMM is to use sequences in public databases (NCBI or RDP-II) and create primers, which demonstrate host-specificity. This method has been used by Khatib *et al.* (2002; 2003) to develop two sets of primers, one that targets the LTIIa toxin gene in cattle (Khatib *et al.* 2002) and one that targets the STII toxin gene in swine (Khatib *et al.* 2003). For the cattle marker, 100% PCR positives were obtained when membranes containing an *E. coli* concentration of $\geq 10^5$ colonies per filter were analyzed (Khatib *et al.* 2002). For the swine marker, 100% PCR positives were obtained when membranes containing an *E. coli* concentration of ≥ 100 colonies per filter were analyzed (Khatib *et al.* 2003).

The library-independent genotypic methods described above were evaluated as part of the blind source study mentioned several times previously. There was a wide-spread inability to extract adequate amounts of DNA from some of the samples received, which especially hindered the use of T-RLFP for a *Bacteroidetes* marker (Field et. al.2003). An additional hindrance in the study was the lack of markers, which could correctly identify all of the sources in the samples analyzed. However, the ability of these methods used to correctly classify the appropriate source of fecal material was between 75% and 100% (Field *et al.* 2003).

1.4 Objectives of this Study

There is an ongoing need to further the microbial source tracking field, given the inaccuracies associated with the current methods. Thus, research into new markers is warranted. The specific goals of this research included:

I. The development of a host-specific molecular marker within *Enterococcus spp.* for use in microbial source tracking.

II. The development of a method using archived bacterial strains and animal fecal/lagoon samples to assay environmental samples for the host-specific molecular marker.

III. The application of the newly developed methods along with conventional water quality indicators in environmental water samples.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF A HUMAN-SPECIFIC *Enterococcus* MARKER

2.1 Introduction

Microbial source tracking continues to be a developing field of study where several approaches and techniques are being explored to answer the same question, "Can the microorganisms of fecal origin found in a body of water be traced back to their original host sources?" The approaches examined have been based on both phenotypic and genotypic characteristics of fecal coliforms, *E. coli*, and enterococci with each having its own advantages and disadvantages (Scott *et al.* 2002). However, it is hypothesized that the genotypic characteristics are better suited for use in determining the source origin of microbial contaminants (Simpson *et al.* 2002). Of the genotypic techniques explored thus far, it is hypothesized that the PCR-based host-associated molecular markers are more accurate and precise in determining source origins (Griffith *et al.* 2003).

With an overall goal of developing and validating a human-specific host molecular marker for use in microbial source tracking, this section examines the potential of two surface proteins within *Enterococcus*. The first protein targeted was Ace (adhesin of collagen from enterococci), which is a member of the collagen-binding MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and is encoded by the *ace* gene (Rich *et al.* 1999). Ace was first discovered in *Enterococcus faecalis* and primers developed by Nallapareddy *et al.* (2000) showed specificity to clinical isolates of *E. faecalis*. A preliminary assessment in clinical *Enterococcus faecium* yielded no

evidence of an *ace* homolog. Duh *et al.* (2001) explored a range of *Enterococcus spp.* for the presence of *ace* and found it in *E. faecalis* only. In exploring *ace* as a source tracking marker, enterococci isolates were collected from a variety of host sources and then examined individually for the presence of the *ace* markers developed.

The second protein targeted was Esp, the enterococcal surface protein (Shankar *et al.* 1999). This target was initially discovered in clinical isolates of *E. faecalis* only, but a homolog had been identified in isolates of *E. faecium* (Willems *et al.* 2001). The function of Esp (encoded by *esp*) is thought to increase virulence, which is supported by evidence that shows it is located within unique pathogenicity islands within *E. faecalis* (Shankar *et al.* 2001) and *E. faecium* (Leavis *et al.* 2004). While Hammerum and Jensen (2002) have identified *esp* in *E. faecalis* in swine and poultry in addition to humans, this gene has not been explored for a possible use in microbial source tracking. An initial screening of *E. faecium* isolates from clinical and sewage sources following the same methods used for the *ace* marker yielded no presence of the *esp* marker. Therefore, a new method needed to be explored, which scrutinized the whole enterococci population within a single sample.

2.2 Materials and Methods

Two different approaches were undertaken in the development and evaluation of a host-associated molecular marker for human sources of fecal pollution. Both individual isolates and total populations grown on a membrane on MEI media were used to screen for the markers (Figure 2-1).

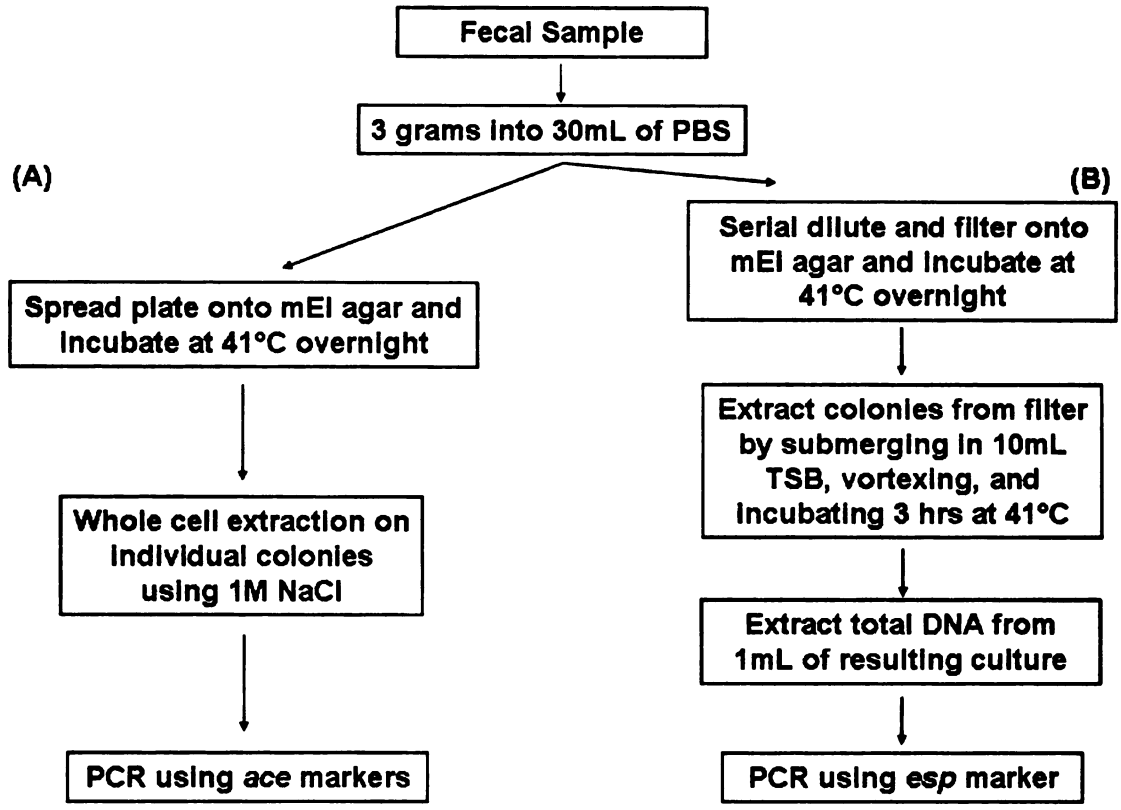

Figure 2-1. Sample processing outline for the fecal (solid) samples. (A) summarizes the method used to isolate and extract individual colonies for *ace* marker analysis. (B) outlines the method used to examine the microbial population of a sample via membrane filtration for the presence of the *esp* marker.

2.2.1 Sample Collection

Composite fecal samples were collected from animal wastewater lagoons at poultry (Bushnell, FL), dairy cattle (Hague, FL), and swine (Gainesville, FL) farms. Individual animal fecal samples were collected for poultry, Canada geese, dairy and beef cattle, and swine from Michigan State University's agricultural farms and wetlands. In addition, animal scat samples were collected for sea gulls (Grand Haven, MI and Florida Keys), pelicans (Florida Keys), and canines (canine park in Largo, FL). Human fecal samples were collected from domestic septic tanks from the Florida Keys, Gainesville, FL and from sites in Colorado. Domestic raw sewage, secondary sewage effluent, and filtered

wastewater were collected from wastewater treatment plants in Arizona, Florida, and Michigan. Dr. Valerie J. Harwood from the University of South Florida kindly provided enterococci isolates from clinical specimens. All samples were collected using aseptic techniques and were transported to the lab on ice before being processed.

2.2.2 *ace* Target

The initial gene target for use as a human-specific marker was the *ace* gene, which is found only in *E. faecalis* (Duh et al 2001).

2.2.2.1 Sample Processing

The fecal samples were analyzed according to Figure 2-1A. Three grams of sample were weighed and placed into a sterile 50mL centrifuge tube. Thirty milliliters of phosphate buffer saline (0.13M NaCl, 0.0051M Na_2HPO_4, 0.0015M KH_2PO_4; pH 7.4) (PBS) was added to the centrifuge tube, which was then vortex to suspend the sample. Either 100μL or 1mL of the resulting solution was then spread plated onto mEI agar; mEnterococcus (mE) agar (Difco) supplemented with indoxyl β-D-glucoside (Sigma-Aldrich, Co.) and incubated for 24 hours at 41°C. Animal lagoon samples were also spread plated (100μL or 1mL) onto mEI agar. Colonies that were surrounded by a blue halo were picked for speciation using the api 20 Strep (bioMerieux, Inc.) biochemical test strips. All isolates were placed into long-term storage at -80°C in TSB.

Human fecal samples from septic tanks and wastewater treatment plants were processed using membrane filtration. Serial dilutions were performed using PBS. The appropriate dilutions were filtered through a 0.45μm pore 47mm nitrocellulose-mixed ester membrane filter (GE Osmonics). Filters were then placed onto mEI agar and incubated at 41 ± 0.5°C for 24 hours (U.S. EPA 2002). Colonies that were surrounded by

a blue halo were picked for speciation using the api 20 Strep (bioMerieux, Inc.) biochemical test strips. All isolates were placed into long-term storage at -80 °C.

2.2.2.2 Whole Cell Extraction

Isolates were removed from long-term storage and placed into tryptic soy broth (TSB) (Difco) and grown overnight at 41°C. One milliliter of the resulting culture was placed into a microcentrifuge tube and centrifuged at maximum speed for four minutes. The supernatant was removed and 1 mL of a 1 M NaCl solution was placed into the microcentrifuge tube and then vortexed to suspend the pellet. The sample was centrifuged at maximum speed for four minutes and then was washed a total of three times with the 1 M NaCl solution. Finally, the pellet was suspended in 50 µL of DNase/RNase free water and placed into a -20 °C freezer until analyzed by polymerase chain reaction (PCR).

2.2.2.3 Polymerase Chain Reaction

The PCR primers used for detecting the *ace* gene, which is known to be in *Enterococcus faecalis*, were previously developed by Nallapareddy *et al.* (2000). From the primers examined by Nallapareddy *et al.* (2000), a forward primer, AceF2 (aceA forward 5'-GAG CAA AAG TTC AAT CGT TGA C-3') and a reverse primer, AceR3 (aceA reverse 5'-GTC TGT CTT TTC ACT TGT TTC T-3') were chosen.

The PCR reactions were performed in a 20 µL reaction mixture containing 1X PCR Buffer (Qiagen, Inc.), 200 µM of each of the four deoxyribonucleotides (USB, Co.), 0.3 µM of each primer, 0.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 1 µL of template DNA (whole cell extractions). The PCR amplification was performed with an initial step at 95 °C for 15 minutes (to activate Taq), followed by 35 cycles of 94°C for 1

minute, 55 °C for 1 minute, 72°C for 1 minute with a final extension at 72°C for 5 minutes. The PCR products were then separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (Cambrex Bio Science, Inc.) and viewed under ultraviolet (UV) light.

Two additional forward primers were developed in house to assist in distinguishing human and non-human sources of feces. The DNA fragments from two human and three poultry *E. faecalis* isolates, which all contained *ace*, were extracted and purified from the agarose gel using the QIAquick Gel Extraction Kit and Protocol (Qiagen, Inc.). One shot chemical transformation was performed on the fragments using the TOPO TA Cloning Kit (Invitrogen, Co.). The cloned DNA was then purified using the Wizard Plus SV Minipreps DNA Purification System (Promega, Co.) and electrophoresed to ensure that the plasmid was inserted correctly. All clones were then sequenced.

The sequences obtained were then aligned using Jellyfish Biowire Software. Unique differences were present between the human and poultry sequences. Two new forward primers were developed using the same software package. AceA-1, 5'-TGG AAT GAC CGA GAA CGA TAG T-3', was designed to identify animal sources. AceA-2, 5'-CGG AAT GAC CGA GAA CGA TGG C-3', was designed to identify human sources. The aceA-reverse primer was used in all reactions.

The PCR reactions were performed in a 20 µL reaction mixture containing 1X PCR Buffer (Qiagen, Inc.), 200 µM of each of the four deoxyribonucleotides, 0.3 µM of each primer, 0.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 1 µL of template DNA (whole cell extractions). The PCR amplification was performed with an initial step at 95°C for 15 minutes (to activate Taq), followed by 35 cycles of 94°C for 1 minute, 62°C

for 1 minute, 72°C for 1 minute with a final extension at 72°C for 5 minutes. The PCR products were then separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (Cambrex Bio Science, Inc.) and viewed under UV light.

2.2.3 *esp* Target

The second gene target examined for use as a human-specific marker was the *esp* gene, whose primers were developed to only target the *E. faecium* sequence.

2.2.3.1 Bacterial Strains

In addition to the isolates provided by Dr. Harwood, other members of Enterobacteriaceae were evaluated to assess the potential for cross-reactivity of the PCR primers developed in this portion of this study. The species tested were *E. faecium* (ATCC# 19434), *E. faecalis* (ATCC# 19443), *Enterococcus casseliflavus* (ATCC# 700327), *Enterococcus avium* (ATCC# 14025), *Enterococcus gallinarum* (ATCC# 49573), *Enterococcus durans* (ATCC# 6056), *Streptococcus bovis* (ATCC# 15351), *Escherichia coli* (ATCC# 15597 and 13706), and *Klebsiella pneumoniae* (ATCC# 43816). *E. faecium* strain C68, which contains the *esp* gene and was used as a positive control in all PCR reactions, was kindly provided by Dr. Louis B. Rice of the Louis Stokes Cleveland Veterans Affairs Medical Center in Cleveland, Ohio.

2.2.3.2 Sample Processing

The fecal samples were analyzed according to Figure 2-1B. Three grams of each sample was weighed and placed into a sterile 50mL centrifuge tube. Thirty milliliters of PBS was added to the centrifuge tube, which was then vortex to suspend the sample. The suspended fecal samples (Figure 2-1B) along with the animal lagoon and the human septic tank and wastewater samples (Figure 2-2A) were filtered through a 0.45μm pore

47mm nitrocellulose-mixed ester membrane filter (GE Osmonics). Filters were then placed onto mEI agar and incubated at 41 ± 0.5 °C for 24 hours (U.S. EPA 2002). Membrane filtration was used to concentrate the microorganisms and not as a means of isolating individual colonies. Serial dilutions were used to estimate bacterial densities for purposes of enumeration (Figure 2-2B).

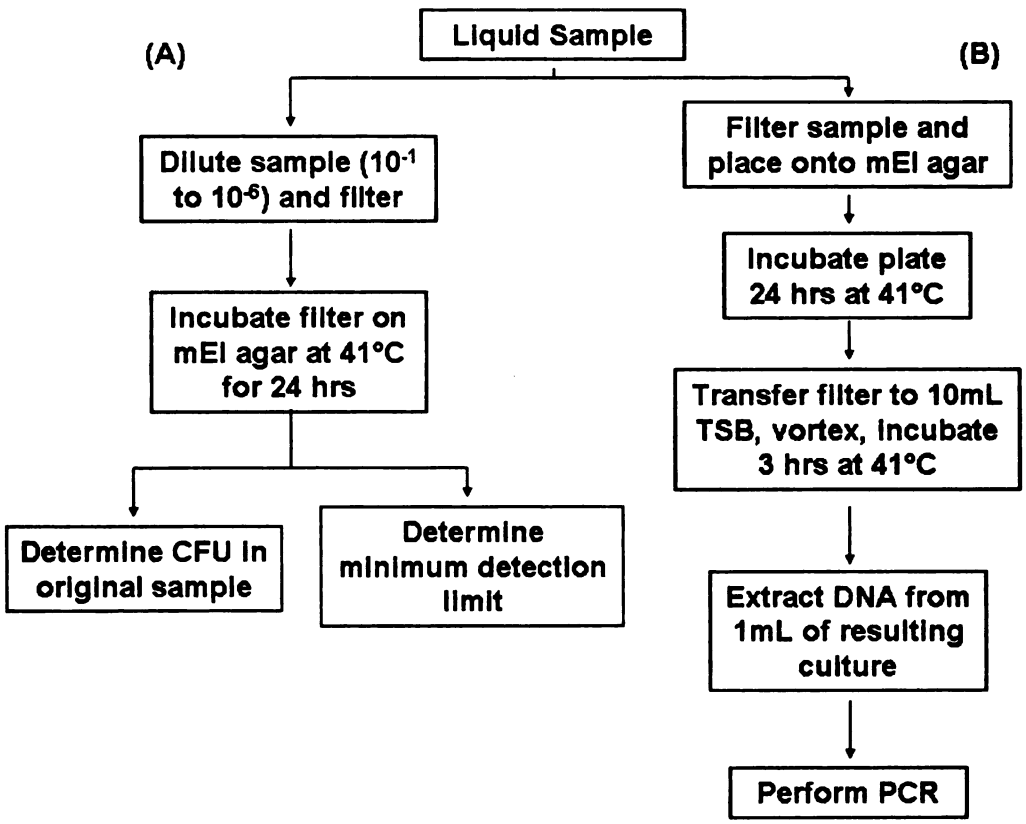

Figure 2-2. Schematic diagram that outlines how liquid (animal lagoon, wastewater, and environmental water) samples were analyzed for the presence of enterococci (A) and the *esp* marker (B).

2.2.3.3 Membrane Elution

After overnight incubation, membranes were lifted off of the agar and placed into a 15 mL centrifuge tube (Figure 2-2A). Ten milliliters of TSB was added to the centrifuge

tube, which was then vortexed to ensure that the membrane was submerged with broth. The tubes were then placed into a 41°C incubator for 3 hours. The centrifuge tubes were then removed from the incubator and vortexed again. The resulting bacterial culture containing both target and non-target microorganisms allowed for the analysis of the microbial population of the sample, which included both culturable and unculturable *Enterococcus spp.*. One milliliter of the resulting culture was then placed into a microcentrifuge tube to be used as the sample that underwent DNA extraction.

2.2.3.4 DNA Extraction

The microcentrifuge tube from the membrane elution was used as the sample for total DNA extraction (Figure 2-2A). The tube was centrifuged at maximum speed for five minutes. The supernatant was removed and 180 µL of lysis buffer (20 mg/ml lysozyme; 20 mM Tris•HCl, pH 8.0; 2 mM EDTA; 1.2% Triton®) was added. The pellet was resuspended and then placed into a 35°C water bath for 30 minutes. Then 20 µL of Proteinase K (Qiagen, Inc.) and 200 µL of Lysis Solution (Qiagen, Inc.) was added to the tube and vortexed. The tube was then placed into a 45°C water bath for 30 minutes. The temperature of the water bath was increased to 95°C for 15 minutes. The microcentrifuge tube was then removed from the bath and kept at room temperature. DNA extraction was performed using QIAamp DNA Mini Kit according to the manufacture's instructions (Qiagen, Inc.).

2.2.3.5 Polymerase Chain Reaction

The PCR primers used for detecting the *esp* gene, which is in *E. faecium* and *E. faecalis*, from the DNA extractions were developed in order to detect the *E. faecium esp* gene and not the *E. faecalis esp* gene. Primers were developed by aligning two *E. faecium*

sequences (GenBank accession numbers AF444000 and AF443999) with one *E. faecalis* sequence (GenBank accession number AF034779). Unique differences were identified between these genes, and PCR primers were developed to specifically amplify only the *E. faecium* variant (Jellyfish Biowire Software). The forward primer designed in this study, which is specific for the *E. faecium esp* gene is: (5'-TAT GAA AGC AAC AGC ACA AGT T-3'). A conserved reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3') developed previously by Hammerum and Jensen (2002) was used for all reactions.

The PCR reactions were performed in a 20 μL reaction mixture containing 1X PCR Buffer (Qiagen, Inc.), 200 μM of each of the four deoxyribonucleotides (USB, Co.), 0.3 μM of each primer, 0.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.), and 1 μL of template DNA. The PCR amplification was performed with an initial step at 95°C for 15 minutes (to activate Taq), followed by 35 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute with a final extension at 72°C for 5 minutes. The PCR products were then separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (Cambrex Bio Science, Inc.) and viewed under UV light. The PCR product is 680 base pairs in length.

The PCR product from the *E. faecium* C68 was purified using a QIAquick PCR Purification Kit (Qiagen, Inc.). The purified PCR product was cloned using the TOPO TA Cloning ® Kit (Invitrogen, Co.). Sequencing of the product was performed at the Genomics Technology Support Facility at Michigan State University using an ABI Prism ® 3100 Genetic Analyzer to verify the sequence of the positive control and specificity of the primers.

2.2.3.6 Minimum Detection Limit

Five sewage, septic tank, and marine environmental samples from the Florida Keys suspected of containing human sewage contamination were collected. The samples were serially diluted in PBS, and 1 mL of each dilution was membrane filtered thru a 0.45µm pore 47mm nitrocellulose-mixed ester membrane filter (GE Osmonics) and placed onto mEI media as described above (Figure 2-2B). Each filter was enumerated for total enterococci and was prepared for analysis by PCR as described previously. Total viable enterococci were then compared to PCR results in order to estimate the colony densities that must be present to ensure detection of the human associated marker.

2.2.3.7 Persistence in Environmental Samples

In order to associate the presence of the *esp* gene and its detection with direct colony counts, autoclaved fresh water and simulated marine (Instant Ocean) water samples were spiked with primary sewage influent containing approximately 10^4 enterococci. Each type of water sample (n=3) was then divided equally (25 ml) into 10 separate 50 ml polypropylene tubes and were incubated in a water bath at 30°C. At intervals of 0, 3, 5, 7, 10, and 15 days, individual sample tubes were processed and analyzed for total enterococci and the *esp* gene by plate counts and PCR, respectively, as described previously.

2.2.4 Statistical analysis.

All statistical analyses (Chi-squared, t-tests) were performed using Pro-Stat statistical software (Polysoftware International, Inc.).

2.3 Results

2.3.1 Speciation of Source Isolates

A total of 337 enterococci isolates that were surrounded by a blue halo when cultivated on mEI agar were speciated using api 20 Strep. Four species, *E. faecalis, E. faecium, E. durans*, and *E. gallinarum* were identified from seven different fecal sources as outlined in Table 2-1. Overall, the human sources contained similar amounts of *E. faecalis* (50%) and *E. faecium* (43%). However, the clinical sources were dominated by *E. faecalis* (76%) and the sewage sources contained a proportionately higher concentration of *E. faecium* (56%) compared with *E. faecalis* (35%).

Table 2-1. Speciation (api 20 Strep, BioMeriux) of *Enterococcus* isolates collected from human and animal sources of fecal contamination.

Source	Number (%) of Isolates Speciated Per Source			
	E. faecalis	***E. faecium***	***E. durans***	***E. gallinarum***
Clinical	35 (76%)	10 (22%)	1 (2%)	0 (0%)
Sewage	28 (35%)	44 (56%)	7 (9%)	0 (0%)
Poultry	57 (97%)	2 (3%)	0 (0%)	0 (0%)
Swine	20 (57%)	14 (40%)	1 (3%)	0 (0%)
Dairy Cattle	14 (15%)	34 (36%)	46 (48%)	1 (1%)
Beef Cattle	2 (29%)	0 (0%)	5 (71%)	0 (0%)
Canine	0 (0%)	0 (0%)	16 (100%)	0 (0%)
Total Human	63 (50%)	54 (43%)	8 (7%)	0 (0%)
Total Animal	93 (44%)	50 (23.5%)	68 (32%)	1 (0.5%)

Overall, the animal sources examined contained a greater number of isolates from *E. faecalis* (44%) > *E. durans* (32%) > *E. faecium* (23.5%) (Table 2-1). *E. gallinarum* was only isolated once from dairy cattle feces. The poultry samples examined yield the greatest concentration (97%) of *E. faecalis* from the seven sources analyzed. While swine

contained a more equal ratio between *E. faecalis* and *E. faecium* isolates. The dairy cattle had higher proportions of *E. faecium* (35.8%) and *E. durans* (48.4%) than any of the other animal sources. While beef cattle contained a high proportion of *E. durans* (71.4%), the canines examined contained exclusively *E. durans*.

2.3.2 *ace* Target

Table 2-2 shows the results of a total of 145 *Enterococcus* isolates from six sources screened using the AceA primers previously developed by Nallapareddy *et al.* (2000). For the animals, only poultry, swine, and dairy cattle were further characterized for the presence of the *ace* gene. The percentage of positive results varied between 21% (n=29) for the dairy cattle and 67% (n=6) for the swine. Overall, 17 out of 47 or 36% of the animal source isolates examined were positive for the *ace* gene. The results from Nallapareddy's primers showed no specificity to source type. Therefore, the PCR band from two human and three poultry isolates was cloned and sequenced (Appendix I). From these sequences two new forward primers were designed (AceA-1 and AceA-2) and all samples positive with the AceA primers were then characterized with the new primers.

Table 2-2. Results of the AceA, AceA-1, and AceA-2 primers, which target the *ace* gene, for all the *Enterococcus* isolates examined.

Source	Number of Isolates Positive		
	AceA	AceA-1	AceA-2
Clinical	27 (n=41)	12 (n=27)	10 (n=27)
Sewage	9 (n=29)	2 (n=9)	6 (n=9)
Septic	28 (n=28)	0 (n=28)	21 (n=28)
Poultry	7 (n=12)	5 (n=7)	2 (n=7)
Swine	4 (n=6)	0 (n=4)	2 (n=4)
Dairy Cattle	6 (n=29)	0 (n=6)	1 (n=6)
Total Human	64 (n=98)	14 (n=64)	37 (n=64)
Total Animal	17 (n=47)	5 (n=17)	5 (n=17)

Of the seven poultry samples that were positive with the AceA primers, five (71%) were positive with AceA-1 and two (29%) with AceA-2 (Table 2-2). The swine isolates were all negative using the AceA-1 primer, but two (50%) were positive with the AceA-2 primer. Again, all of the dairy cattle isolates were negative with the AceA-1 primer and one (17%) isolate was positive with AceA-2. The above results demonstrate that not all of the isolates, which were positive with AceA were also positive with AceA-1 and AceA-2 describing a reduction in the sensitivity of the two newly developed primers.

Clinical, sewage, and septic tanks were examined as sources of human fecal contamination. Table 2-2 shows that a total of 98 samples were characterized for the *ace* gene and 65% were positive with AceA. The 28 isolates from septic tanks were all positive. However, only nine (31%) sewage isolates were positive. The AceA primers were originally developed for use in detecting ace in clinical samples (Nallapareddy *et al.* 2000). In this study, 66% (27 out of 41) of the clinical isolates were positive.

When the primers developed in this study were examined in the human sources, 22% were positive with AceA-1 and 58% were positive with AceA-2. This difference is due to zero AceA-1 positives from the septic tank isolates while 21 were positive with AceA-2. In the clinical isolates, 12 of 27 (44%) were positive with AceA-1 and 10 out of 27 (37%) were positive with AceA-2. For the sewage isolates, two out of nine (22%) were positive with AceA-1 and six out of nine (67%) were positive with AceA-2.

Table 2-3 shows the results of the AceA, AceA-1, and AceA-2 primers for source isolates that were identified by api 20 Strep as *E. faecalis*. Overall, 78% of the human source isolates were positive with AceA, 14 of 35 for AceA-1, and 15 of 35 for AceA-2. Twenty-six of the clinical isolates (n=30) were positive for AceA. Comparing this result

with the clinical results showed in Table2-2 reveals that one isolate that was identified as *E. faecium* (Table 2-4) was positive using the AceA primers. However, this *E. faecium* isolate was negative with AceA-1 and AceA-2.

Table 2-3. Results of the AceA, AceA-1, and AceA-2 primers, which target the *ace* gene, for *Enterococcus faecalis* isolates.

Source	Number of Isolates Positive		
	AceA	AceA-1	AceA-2
Clinical	26 (n=30)	12 (n=26)	9 (n=26)
Sewage	9 (n=15)	2 (n=9)	6 (n=9)
Poultry	7 (n=12)	5 (n=7)	2 (n=7)
Swine	4 (n=5)	0 (n=4)	2 (n=4)
Dairy Cattle	6 (n=8)	0 (n=6)	1 (n=6)
Total Human	35 (n=45)	14 (n=35)	15 (n=35)
Total Animal	17 (n=25)	5 (n=17)	5 (n=17)

Table 2-4. Results of AceA primers in *E. faecalis*, *E. faecium*, and *E. durans* from clinical, sewage, poultry, swine, and dairy cattle samples examined.

PCR Result (Isolates)	Number (%) of Species		
	E. faecalis	*E. faecium*	*E. durans*
Positive	52 (74%)	1 (3%)	0 (0%)
Negative	18 (26%)	29 (97%)	17 (100%)

2.3.3 *esp* Target

The detection limit of the naturally occurring *esp* gene in five separate sewage, septic, and ambient water samples was determined, based on the coupled membrane filtration and PCR method developed. As shown in Table 2-5, an average of 58 $\pm$ 24 enterococci colony forming units (CFU) per membrane filter was necessary to ensure the detection of the *esp* gene and to identify the presence of the marker. The marine water samples being

influenced by failing septic tanks contained lower (< 10^4 CFU) overall concentrations of culturable enterococci and therefore was only evaluated at those lower concentrations.

Table 2-5. The detection limit for naturally occurring *esp* in sewage influent, septic tank effluent and marine waters being impacted with improperly working septic tanks.

Sample Type (Number of Samples)	Number[a] of enterococci screened[b] (Number of Positive Samples)				
	> 10^4 - < 10^5	> 10^3 - < 10^4	100 - 1000	10 – 100 (μ = 58 ± 24)	1 – 10 (μ = 5 ± 3)
Primary Influent (5)	+ (5)	+ (5)	+ (5)	+ (5)	-
Septic Tank (5)	+ (5)	+ (5)	+ (5)	+ (5)	-
Water Samples[c] (5)	NA[c]	+ (5)	+ (5)	+ (5)	-

[a] Higher values extrapolated from plates in the countable range (30-300 CFU).
[b] CFU/100 mL.
[c] Water samples contained ~ 1.5 x 10^3 CFU/100 mL and were collected from a marine environment (Florida Keys) with a defined sewage input. NA indicates that the counts were not in this range for any of these samples.

Primers specific for the *E. faecium esp* gene developed in this study as a marker of human source fecal pollution were used to screen composite fecal samples from humans, livestock, and birds to test for potential cross-reactivity in various sources of fecal contamination. A total of 171 samples containing 10^1 – 10^7 enterococci CFUs per membrane filter were screened. The gene was detected only in samples from primary sewage influent (n=40), secondary sewage effluent (n=10), filtered wastewater (n=5), and septic tanks (n=14). The marker was not detected in any samples from livestock or birds (n=102) (Table 2-6). Overall, this gene was detected in 97% of the sewage and septic tank samples. The two septic tank samples, which were negative for the marker, contained low levels of culturable enterococci.

Six reference strains of enterococci along with three reference strains from other Enterobacteriaceae were screened to test for further cross-reactivity. The primers did not amplify any PCR products in any of these reference strains (data not shown). In addition, 16 *E. faecium* isolates from clinical samples were evaluated for the presence of the *esp* gene using the developed primers. These isolates were also negative (data not shown). In addition, the *E. faecium* C68 strain positive by these primers was sequenced for verification (Appendix II).

Table 2-6. Detection of the *esp* marker in composite DNA samples extracted from enterococci isolated from domestic sewage, livestock, and birds.

Source	Sample Type	# of CFUs Screened	# of Samples	*esp* Results	
				Positive	Negative
Sewage	Primary Influent	10^6 - 10^7	40	40	0
Sewage	Secondary Effluent	10^3 - 10^4	10	10	0
Sewage	Filter Effluent	10^1 – 10^2	5	5	0
Human	Septic Tank	10^2 - 10^5	14	12	2[a]
Poultry	Lagoon/Feces	10^5 - 10^6	6	0	6
Swine	Lagoon/Feces	10^5 - 10^6	9	0	9
Dairy Cattle	Lagoon/Feces	10^5 - 10^6	28	0	28
Beef Cattle	Feces	10^5 - 10^6	4	0	4
Canada Geese	Feces	10^2 - 10^3	12	0	12
Seagull	Feces	10^4 - 10^5	28	0	28
Pelican	Feces	10^5 - 10^6	7	0	7
Wild Birds	Feces	10^4 - 10^5	8	0	8
Total Human			69	67[b]	2
Total Animal			102	0[b]	102

[a] Total *Enterococcus* levels were < 1 x 10^2 CFU and showed atypical colony morphology.
[b] Significantly different statistically as shown by a chi square analysis $p < 0.001$.

Survival studies, which used naturally-occurring enterococci from raw sewage inoculated into fresh water and simulated sea water, were conducted and the results are shown in Table 2-7. These studies were performed under elevated temperatures (30°C) to

enhance the die-off rates. The number of culturable enterococci dropped rapidly within three days; falling to 19 CFU per mL by Day 7 in fresh water and were no longer detectable by Day 15. In simulated sea water, the level of culturable enterococci declined to 132 CFU by Day 7, to 71 CFU by Day 10 and were no longer detectable by Day 15. The *esp* marker was only detectable up to Day 5, when levels of enterococci were between 19 and 122 CFU/mL in fresh water and up to Day 10 at levels above 70 CFU/mL in simulated sea water.

Table 2-7. Persistence of the naturally occurring *esp* gene from raw sewage in culturable enterococci in fresh water and simulated sea water survival studies conducted at 37°C.

Water Type	Total Culturable Enterococci (CFU/mL, $\mu \pm sd$)					
	Day 0	Day 3	Day 5	Day 7	Day 10	Day 15
Fresh Water (n=3)	1.80 (± 0.53) x 10^{4a*}	2.51 (± 0.64) x 10^{2a}	1.22 (± 0.30) x 10^{2a}	1.9 (± 0.30) x 10^{1a}	9.0 (± 0.4) x 10^{0a}	< 6.7 x 10^{-3**}
PCR Results (+/-)	+	+	+	-	-	-
Simulated Sea Water (n=3)	1.34 (± 0.42) x 10^{4a}	5.01 (± 0.81) x 10^{2b}	2.65 (± 0.46) x 10^{2b}	1.32 (± 0.41) x 10^{2b}	7.1 (± 0.3) x 10^{1b}	< 6.7 x 10^{-3**}
PCR Results (+/-)	+	+	+	+	+	-

* Values in the same column followed by the same letter are not statistically different at $p < 0.05$

** < indicates the detection limit of the samples

2.4 Discussion

In order for microbial source tracking (MST) methods to be most useful, they should be specific and applicable over a broad geographic region. Recent research has indicated that methods requiring a reference database may have limited utility for identification of specific sources of fecal pollution when isolates are collected from multiple watersheds

(Griffith *et al.* 2003; Scott *et al.* 2003; Johnson *et al.* 2004). Molecular methods currently exist that identify specific sources of fecal pollution without relying on a database. These methods can circumvent the inherent drawbacks of existing, library-dependent MST methodologies (Bernhard and Field 2000a, 2000b; Khatib *et al.* 2002; Khatib *et al.* 2003; Noble *et al.* 2003). These targeted methods also generally cost less and can produce results within 1-2 days, which is significantly better than methods such as ribotyping, which can take 7-14 days.

Most MST methods attempt to characterize indicator microorganisms (either genotypically or phenotypically) on the basis of the host from which they were isolated. However, the ubiquitous nature of most of these indicators makes this task particularly challenging. The *Enterococcus* genus contains multiple species and several researchers have reported on the variable distribution of species of enterococci in different animal hosts (Pourcher *et al.* 1991; Wheeler *et al.* 2002).

In this study, speciation of *Enterococcus* was carried out using cultivation and biochemical methods. These methods resulted in the identification of only four species; *E. faecalis, E. faecium, E. durans*, and *E. gallinarum* from a total of seven human and non-human sources. The use of biochemical assays to speciate *Enterococcus* has been addressed previously (Harwood *et al.* 2004; Velasco *et al.* 2004), and the use of molecular based assays for identification have been suggested as being superior (Ozawa *et al.* 2000; Manero *et al.* 2002). More specific biochemical tests for speciation of *Enterococcus* and further development of species or source-specific molecular markers would be extremely useful for identifying sources of fecal pollution impacting a water body.

The *ace* gene of *E. faecalis* is composed of a single A and repeated B domains. The primers developed and used in this study target the A domain, which is 46% similar to the *Staphylococcus aureus* Cna (collagen adhesion) A domain (Rich *et al.* 1999). Since the Ace A domain has been shown to be responsible for an isolate's ability to bind to collagen type I (Rich *et al.* 1999), it was the target of the primers developed initially by Nallaparredy *et al.* (2000) to characterize the gene and applied in this study for its potential use as a source tracking marker. However, as this study examined the occurrence of this gene in other sources beyond not only the clinical arena, but also examined its occurrence in multiple *Enterococcus spp.* it has been concluded that it is found in a variety of sources both human and non-human. Its occurrence in other *Enterococcus spp.* suggests that it may be on a transposable element as a positive was detected in a clinical *E. faecium* isolate. However, this conclusion has not been confirmed in the literature. Duh *et al.* (2001) examined 103 *E. faecium* and 62 *E. faecalis* isolates all from clinical specimens and detected *ace* only in the *E. faecalis* isolates. However, the primers used in this study and by Duh *et al.* (2001) targeted different base pair locations based on the sequence from *E. faecalis* strain V583. In addition, it is possible that this clinical isolate was incorrectly speciated. *E. faecalis* and *E. faecium* have very similar biochemical characteristics differing most predominately in their ability to ferment pyruvate (*E. faecalis* is positive and *E. faecium* is negative) (Wood and Holzapfel 1995).

Based on the cloning results from seven isolates, two new forward primers were developed to enhance the host identification of *E. faecalis* isolates. These two new primers were shown to be too restrictive in their ability to identify the *ace* gene, which is shown by a reduction in positives achieved with these primers after being positive with

the less restrictive primers developed by Nallaparreddy *et al.* (2000). In addition, these primers were not shown to be more specific for a human vs. a non-human source.

Much like the approach of Khatib *et al.* in their identifications of a bovine and a porcine-specific biomarkers in *E. coli* (2002; 2003), the intent of this research was to target potential enterococcal virulence factors that have been associated only with human clinical disease (Nallapareddy *et al.* 2000; Nallapareddy *et al.* 2003; Rice *et al.* 2003). Multiple targets were evaluated and fecal samples from domestic sewage, septic tanks, livestock, and birds were screened. The research has demonstrated the *esp* method presented here is more useful for tracking sources of human fecal pollution than the *ace* methods.

The *esp* gene target is believed to be located on the bacterial chromosome (personal communication, Dr. Louis B. Rice); therefore, the target is assumed to be stable and would be less likely to be transferred (via horizontal transfer) to non-target microorganisms in the environment. However, recent laboratory-based research indicated that conjugative transfer of this gene between species is a possibility (Oancea *et al.* 2004) as it has been found within a pathogenicity island (Leavis *et al.* 2004).

This test method circumvented the need to isolate and characterize individual colonies of *Enterococcus* by examining the entire population of enterococci in a water or fecal sample and specifically targeting a single HAMM, and allowed for the screening of millions of bacteria from multiple hosts. In agreement with previous literature (Shankar *et al.* 1999; Willems *et al.* 2001; Eaton and Gasson 2002; Hammerum and Jensen 2002), the primers specific for the *E. faecium esp* gene produced a product only in populations of microorganisms cultured from fecal samples of human origin.

The marker was consistently detected when levels of human derived enterococci in a water or diluted fecal sample totaled approximately 50 to 100 colony-forming units (CFU). This is useful, as the current recommendation for permissible levels of enterococci as a fecal indicator in marine waters is less than 104 CFU/100ml for a single sampling event (U.S. EPA 1986). Current federal fresh water recommendations of less than 61 CFU/100mL for a single sample event (U.S. EPA 1986) also falls within the detection limit of 58 ± 24 CFU/membrane filter determined from naturally occurring sources of *esp*. The current water quality guidelines are based on total numbers of enterococci, regardless of composition. While these guidelines have been shown to be reliable as general indicators of water quality and health risks (Cabelli *et al.* 1979; Cabelli *et al.* 1982; Wade *et al.* 2003), microbial source tracking methods should seek to better characterize the constituent species within this group in order to develop specific tests for specific sources of fecal pollution.

Survival studies showed that the marker was detectable in both fresh water and simulated seawater for as long as total numbers of culturable enterococci were above 70 CFU, thus indicating that differential survival of enterococci populations in primary sewage influent did not significantly affect the utility of this test. The study was conducted at a relatively high environmental temperature (30°C). Therefore, the experiment was a worst-case scenario of temperature and matrix (i.e. predation by sewage microorganisms) and was designed only to indicate the stability of the marker relative to culturable enterococci. In addition, the marker was no longer detected in filter concentrated cells once enterococci were no longer able to be cultured from the sample.

A literature review by Wade *et al.* supports culturable enterococci as indicators of human health risk in recreational waters (2003) and culturable enterococci were also shown to be superior markers for risk of disease due to contamination of groundwater used for drinking when septic tanks were the source of contamination (Borchardt *et al.* 2003). Finally, culturable enterococci have been shown to be extremely useful indicators of the efficacy of wastewater treatment for purposes of reclamation (Rose *et al.* 2001).

Following along from the above mentioned studies, I propose that the presence of culturable enterococci and the detection of the *E. faecium esp* gene in a water sample indicates the presence of human fecal contamination and potential human health risk, while the absence of the marker in association with high counts of enterococci could then also indicate an alternative source of contamination. Both results therefore will aid in the further investigation of the contamination and use of the water body. The method developed herein allows for the identification of viable microorganisms, as well as quantification and is compatible with current methods utilizing U.S. EPA approved membrane filtration methods.

The ideal source-tracking tool is one that employs multiple targets for each individual source group/animal. Overall, the results of the current study suggest that better genetic characterization of the communities of enterococci present in different animal hosts warrants further investigation. Future studies may reveal valuable information, which could subsequently be used to design more specific tests for the identification of multiple sources of fecal pollution. While the primers designed in this study for the *ace* gene have thus far not demonstrated to be specific to a single source, it does show specificity to *E. faecalis*. This specificity to *E. faecalis* is important from a public health aspect in that *E*

faecalis is a major cause of nosacomial infections (Koch *et al.* 2004). The use of PCR primers specific for the *esp* gene in *Enterococcus faecium* as an index of human fecal pollution may be a useful addition to the ever expanding microbial source tracking toolbox. As with all source tracking methods, as they are utilized in new regions of the country and with new potential sources of fecal pollution it is recommended that a set of field collected Quality Assurance/Quality Control samples (known sources of fecal contamination) be evaluated. The enterococci are becoming more useful indicators of water pollution and of public health risk and the source of this risk should continue to be investigated by research directed at further characterization of these microorganisms.

CHAPTER 3

COMPARISION OF THE *E. faecium esp* MARKER WITH MICROBIAL AND VIRAL INDICATORS IN WATER

3.1 Introduction

The State of Michigan is a peninsula surrounded by three of the five Great Lakes. This coupled with 49,141 miles of rivers and streams and over 11,000 ponds and in-land lakes makes the state an attractive area for enjoying recreational water activities (MDEQ 2002). Therefore, these waters need to be monitored and protected from a public health perspective. Studies are being done within the Great Lakes region to monitor waters for fecal indicators (Byappanahalli *et al.* 2003; Kinzelman *et al.* 2003; Whitman and Nevers 2003). However, studies within Michigan have been limited in scope to the beach areas along the coast (Cabelli *et al.* 1982; Haack *et al.* 2003; Wheeler Alm *et al.* 2003). In addition while these studies enumerate the fecal bacterial concentrations, they do not address the issues of bacterial sources and human health risks.

Microbial source tracking approaches need to be undertaken in order to better understand where the microbial population is originating as this relates directly to human health risks. The source tracking tools available are wide and varied. However, an enterococci host-specific molecular marker in humans has been developed and could serve as a first approach in understanding sources within Michigan waters. In addition, monitoring for human pathogens themselves assists in grasping the risk of being exposed to human pathogens during recreational activities. While it is expensive to monitor for all pathogens, viruses serve as a good choice because of their host specificity and low infectious doses (Payment *et al.* 2000).

The purpose of this field study was to survey a range of recreating waters to determine if the *esp* marker in enterococci was detectable under field conditions and to examine how the presence/absence of the *esp* marker related to other microbial and viral indicators commonly used in monitoring recreational water quality.

3.2 Materials and Methods

3.2.1 Sample Collection

Nine rivers (AuSable, Clinton, Grand, Kalamazoo, Raisin, Rouge, Saginaw, Shiawassee, and Thunder Bay) that drain into the Great Lakes were chosen for analysis of bacterial indicators and enteric viruses (see page , Figure 3-1). One site from each river was sampled once during July 2003 under normal base-flow conditions. The location of the sampling sites was based on previous data collected by the Michigan Department of Environmental Quality (MDEQ), if available; otherwise, sampling occurred near the river's outflow into the receiving Great Lake (Table 3-2). One-liter grab samples were collected for processing the bacterial indicators. The enteric viruses were collected via filtration through Virusorb 1 MDS filters (Cuno Inc.) according to the U.S. EPA Manual of Methods for Virology (2001a). At each site a minimum of 40 gallons (151.2 liters) of surface water was filtered for enteric virus analysis. All samples were transported on ice to the Water Quality and Health Laboratories at Michigan State University and processed within six hours.

3.2.2 Indicator Analysis

Grab samples were analyzed for the presence of fecal coliforms, *Escherichia coli*, enterococci and coliphages. Bacterial analysis was performed by aseptic membrane filtration of water samples through a 0.45μm pore 47mm nitrocellulose-mixed ester

membrane filter (GE Osmonics). Membranes for fecal coliform analysis were placed onto mFC agar (Difco) sealed in a water-proof whirl-pak bag and submerged into a 44.5 ± 0.2°C waterbath for 24 ± 2 hours according to Method 9222D (American Public Health Association *et al.* 1999). Colonies, which yielded a blue morphology color, were counted as fecal coliforms. For *E. coli* analysis, membranes from the fecal coliform analysis were aseptically transferred to EC with MUG (Difco) supplemented with 1.5% agar (Difco) plates and were incubated at 44.5 ± 0.2°C for 24 ± 2 hours according to Method 9222G (American Public Health Association *et al.* 1999). Colonies that fluorescenced upon exposure to ultraviolet light at 365 nm were counted as *E. coli*. Membranes for enterococci analysis were placed onto mEI (Difco) agar supplemented with indoxyl-β-D-glucoside (Sigma Inc.) and were incubated at 41 ± 0.5°C for 24 hours according to Method 1600 (U.S. EPA 2002). Colonies that were surrounded by a blue halo were counted as enterococci.

Coliphage analysis was done using a modified double-agar overlay method. Two host bacteria, *E. coli* F^+amp and *E. coli* C3000 (ATCC 15597) were used for coliphage analysis. Samples for coliphage analysis were from the 1MDS filter elutions, except for the Shiawassee River, which used a one-liter grab sample for its analysis. Either 1 or 2 mls of sample were mixed with 0.5 mls of a 4-hour log phase bacterial host in 1% tryptic soy agar (Difco) overlays and then poured using aseptic techniques onto 1.5% agar tryptic soy plates (Difco) and incubated at 37 ± 1°C for 20 ± 4 hours according to Method 1602 (U.S. EPA 2001b). Clear zones (plaques) on the bacterial lawn were counted as coliphage plaque forming units (PFU).

3.2.3 Enteric Virus Analysis

Samples were analyzed for enteric viruses according to the U.S. EPA's Manual of Methods for Virology (2001a). The Virusorb 1MDS filters (Cuno, Inc.) were eluted with 1 L of 1.5% beef extract (BBL) with 0.05M glycine (Sigma, Inc.) (pH 9.2, 25°C). The elution was reconcentrated using organic flocculation by the addition of 1M HCl until the pH reached 3.5. However at pH 7.0, 50 mLs of the elution was removed and used in the coliphage analysis (except for Shiawassee River). The remaining elution solution was further concentrated by centrifugation and the floc containing the viruses was resuspended in 0.15 M sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$) and an antibiotic-antimycotic solution (Cellgro) was added before being filter sterilized. The concentrated samples were then placed onto MA-104, BS-C-1, and RD cell lines. The cells (samples and negative controls) were observed for cytopathic effect (CPE) as an indication of the presence of viable, infectious enteric viruses for 14 days. All positive results were confirmed with a second passage.

Integrated cell culture reverse transcription polymerase chain reaction (ICC RT-PCR) was performed on samples that were positive via cell culture to further characterize the infectious viruses present. Viral RNA was extracted and purified from the cell culture supernatant using the QIAamp Viral RNA Mini Kit according to the manufacture's instructions (Qiagen, Inc.). QIAGEN OneStep RT-PCR Kit was used according to the manufacture's instructions for the RT-PCR reactions (Qiagen, Inc.). For the detection of enteroviruses, primers (Table 3-1) for the RT and Nested PCR were previously developed by Puig *et al.* (1994) based on human clinical specimens. For the detection of rotavirus,

the primers (Table 3-1) for the RT and Nested PCR were previously developed by LeGuyader *et al.* (1994).

The RT-PCR reactions were run using a 50 μL reaction mixture containing 1X QIAGEN OneStep RT-PCR Buffer (Qiagen, Inc.), 400 μM of each of the four deoxyribonucleotides (USB, Co.), 0.6 μM of each primer, 2.0 μL QIAGEN One-Step RT-PCR Enzyme Mix, and 10 μL of viral RNA extraction. For enterovirus detection, the RT-PCR amplifications were performed with an initial step at 50°C for 30 minutes (RT) followed by 95°C for 15 minutes (to activate Taq polymerase), followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 5 minutes. For rotavirus detection, the same amplification was performed with an annealing temperature of 50°C.

The nested-PCR reactions were run using a 50 μL reaction mixture containing 10X PCR buffer (Qiagen, Inc.), 1.5 mM $MgCl_2$ (Qiagen, Inc.), 200 μM of each of the four deoxyribonucleotides (USB, Co.), 0.3 μM of each primer, 2.5 U of HotStarTaq DNA polymerase (Qiagen, Inc.) and 2.5 μL of the RT-PCR product. For enterovirus detection, the nested-PCR amplifications were performed with an initial step at 95°C for 15 minutes (to activate Taq polymerase), followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72°C for 5 minutes. For rotavirus detection, the same amplification was performed with an annealing temperature of 50°C. PCR products were then separated on a 1.5% agarose gel stained with GelStar nucleic acid stain (Cambrex Bio Science, Inc.) and viewed under a UV light.

Table 3-1. Enterovirus and rotavirus primers used for the RT and Nested PCR reactions.

Virus	PCR	Primer	Sequence
Enterovirus[a]	RT	Ent1	5'-CGG TAC CTT TGT ACG CCT GT-3'
	RT	Ent2	5'-ATT GTC ACC ATA AGC AGC CA-3'
	Nested	neEnt1	5'-TCC GGC CCC TGA ATG CGG CTA-3'
	Nested	neEnt2	5'-GAA ACA CGG ACA CCC AAA GTA-3'
Rotavirus[b]	RT	R1	5'-GGC TTT AAA AGA GAG AAT TTC CGT CTG G-3'
	RT	R2	5'-GAT CCT GTT GGC CAT CC-3'
	Nested	R3	5'-GTA TGG TAT TGA ATA TAC CAC-3'
	Nested	Rp	5'-TCC ATT GAT CCT GTT ATT GG-3'

[a] Puig *et al.* (1994)
[b] Le Guyader *et al.* (1994)

3.2.4 Microbial Source Tracking

Sites that were positive for culturable enterococci using membrane filtration were further evaluated to determine whether the fecal pollution present was from humans. This evaluation was based on the presence of the target gene, *esp*, which codes for the enterococcal surface protein found in *Enterococcus faecium*. Membrane filters from the enumeration process on mEI agar, which contained greater than 58 CFUs were aseptically lifted, suspended in tryptic soy broth (Difco), vortexed vigorously and incubated at 41°C for two hours. DNA extraction was performed on 1 ml of the resulting culture of bacteria using the QIAamp DNA Mini Kit (Qiagen, Inc.) as described in Chapter 2. PCR was then performed as described in Chapter 2 for the detection of the human *esp* marker. All negative samples were analyzed for PCR inhibitors by spiking the sample DNA with DNA extracted from the positive control, *E. faecium* strain C68.

3.3 Results

3.3.1 Presence of Indicators

Nine rivers in Michigan were sampled for the presence of microbial fecal indicators (Figure 3-1; Table 3-2). The Rouge River had the greatest fecal coliform concentration of

155,000 CFU/100mL compared with the AuSable River, which had the lowest fecal coliform concentration of 83 CFU/100mL (Table 3-2). If an 800 CFU/100mL fecal coliform level is used, then 56% (5 of 9) of the samples exceeded the water quality guidelines. Six of the rivers (67%) exceeded U.S. EPA's single sample maximum of 235 CFU/100mL for swimmable water based on *E. coli* concentrations (U.S. EPA 1986). If Michigan's single sample limit of 300 CFU/100mL (MDEQ 2002) was used, five rivers (56%) exceeded allowable limits. The largest number of exceedances of seven rivers (78%) occurred when U.S. EPA's single sample maximum of 61 CFU/100mL based on enterococci (U.S. EPA 1986) concentrations were used. The AuSable and Thunder Bay Rivers, which are considered pristine by the State of Michigan, were the only two rivers analyzed in this study that did not exceed any of the bacterial water quality standards currently in use.

The presence of two types of coliphage, bacteriophages that infect *E. coli*, was examined in each of the rivers (Table 3-2). In each river, both somatic (C3000 bacterial host) and F^+-specific phage, which attached to the F pili of *E. coli*, (F^+amp bacterial host) were detected. While the numbers of phage present in the rivers varied from less than 64 plaque forming units (PFU)/100L to greater than 63,000 PFU/100L, there was a general increase in the presence of the coliphages from the rivers not listed as Areas of Concern (AOCs) on EPA's Great Lakes web site (AuSable, Grand, Shiawassee, and Thunder Bay Rivers) to the rivers listed as AOCs (Clinton, Kalamazoo, Raisin, Rouge, and Saginaw Rivers).

3.3.2 Presence of Enteric Viruses

Three rivers (Grand, Kalamazoo and Rouge Rivers) were positive for CPE indicating the presence of viable enteric viruses, which was confirmed with a second cell line passage (Table 3-2). The Grand and Kalamazoo Rivers contained viable viruses, which generated CPE on MA-104 cells and the Rouge River presented CPE on MA-104 and BS-C-1 cells. The concentrations of viable enteric viruses ranged from 1 to 3 most probable number (MPN)/100L. Table 3-2 presents the MPN results of the three positive rivers as well as the detection limits for the samples, which were negative for CPE after 14 days.

Table 3-2. Location of sampling sites with the results from the indicator microorganisms, enteric viruses, and the microbial source tracking gene (*esp*).

River[a] (city)	Fecal coliforms	*Escherichia coli*	Enterococci	Coliphage		Enteric viruses[b]	*esp*
	CFU/100 mL			PFU/100 L		MPN/100 L[c]	
				C3000	F^+amp		
AuSable* (Oscoda)	83	27	18	580	< 64	< 0.64	-
Clinton* (Mt. Clemens)	1200	700	250	265	397	< 0.53	-
Grand (Jackson)	1950	900	780	3075	1872	3.12	+
Kalamazoo (Galesburg)	4050	375	216	1443	1924	3.5	-
Raisin (Clinton)	310	235	290	245	489	< 0.98	-
Rouge* (Dearborn)	155000	8500	250	21,179	63,536	1.55	+
Saginaw* (Bay City)	895	400	780	1605	988	< 0.62	-
Shiawassee (Owosso)	515	165	370	20,000[d]	< 10,000[d]	< 0.62	-
Thunder Bay* (Alpena)	325	110	11	1175	365	< 0.41	-

[a] Rivers with an asterisk indicates that the sampling sites were located near the river's outfall into the surrounding Great Lake.

[b] Values with an < indicates that the microorganism was not detected based upon the limits of detection.
[c] Most probable number (MPN)/100 L.
[d] Results are based on a one-liter grab sample and not the Virusorb 1MDS filter elution

Cell culture supernatant containing viable enteric viruses was further evaluated for the presence of enterovirus or rotavirus via RT and Nested PCR. The Rouge River was positive for enteroviruses and negative for rotavirus. The Grand and Kalamazoo Rivers were negative for both enterovirus and rotavirus (data not shown). Thus, it is likely that other enteric viruses were associated with the cell culture CPE.

3.3.3 Microbial Source Tracking

The Grand and Rouge Rivers, which contained elevated concentrations of *E. coli* and viable enteric viruses, tested positive for human fecal pollution based upon the *esp* enterococci marker (Table 3-2). The remaining river samples tested negative for the human fecal pollution marker and did not contain PCR inhibitors. The AuSable and Thunder Bay Rivers were negative for the enterococci marker due to low concentrations of enterococci in the samples, which were below the detection limit of 58 CFU for the *esp* marker. Figure 3-1 shows the sites and results of the sampling in Michigan.

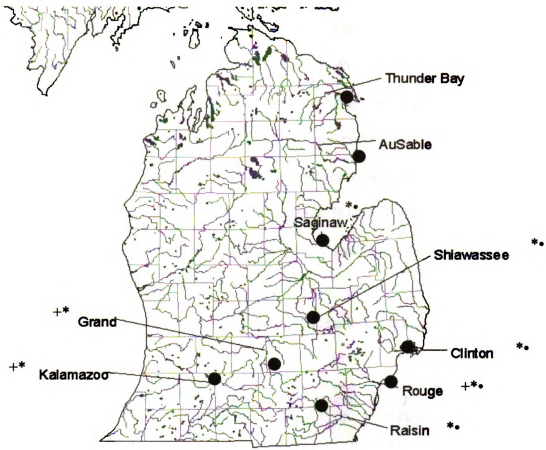

Figure 3-1. Map of the rivers sampled in the State of Michigan.
● sampling locations
+ rivers were positive for enteric viruses via cell culture
* rivers had *E. coli* levels which exceeded U.S. EPA guidelines (235 CFU/100mL)
▪ rivers had enterococci levels which exceeded U.S. EPA guidelines (61 CFU/100mL)
Grand and Rouge rivers positive for *esp* marker

3.4 Discussion

Recent publications examining the occurrence of indicator bacteria in the water and sediments at Great Lake beaches have implied an increase in potential public health impacts (Murray *et al.* 2001; Byappanahalli *et al.* 2003; Kinzelman *et al.* 2003; Wheeler Alm *et al.* 2003; Whitman and Nevers 2003). Relative pollution rankings for each river were assigned based upon the bacterial and somatic coliphage data collected in this preliminary study (Table 3-3) (Griffin *et al.* 1999). These rankings show the Rouge River (rank 9) as being the most polluted and the AuSable River (rank 1) as being the least polluted. The Shiawassee (rank 5) and the Raisin (rank 3) Rivers, while listed as Areas of Concern (AOC), had relatively low concentrations of fecal contamination present in this single sample assessment.

Table 3-3. Relative pollution ranking of rivers and results of viral analysis and source tracking marker.

Rivers	Ranking[a]	AOC[b]	Cell Culture	Enteroviruses (RT-PCR)	*esp* Marker	Wastewater Facilities[c]
Rouge	9	Yes	+	+	+	13
Grand	8	No	+	-	+	64
Saginaw	7	Yes	-	-	-	4
Kalamazoo	6	Yes	+	-	-	22
Shiawassee	5	No	-	-	-	14
Clinton	4	Yes	-	-	-	4
Raisin	3	Yes	-	-	-	24
Thunder Bay	2	No	-	-	-	2
AuSable	1	No	-	-	-	4

[a] Ranking is based upon bacterial and somatic coliphage indicator data (1 is cleanest to 9 is the most contaminated).
[b] Areas of Concern (AOC).
[c] Data courtesy of the Michigan Department of Environmental Quality and includes all wastewater treatment facilities along the rivers (Figure 2).

A variety of pollution sources, including wastewater treatment plants, septic tanks, combined sewer overflows (CSOs), and agricultural and wild animals, may be impacting a river. The microbial indicators used in determining water quality do not define the source of the contamination. The relationship of the pollution ranking and the presence of a human source (*esp* and/or enteric virus positives) were compared to the number of sewage treatment facilities located along the rivers. Figure 3-2 shows the location of wastewater treatment facilities (based on data provided by MDEQ). The Grand River (rank 8) had the highest number of sewage treatment facilities and was positive for viruses and the *esp* human marker (Table 3-3). The Saginaw River (rank 7), which ranked high for fecal contamination, had fewer treatment plants located on it and was negative for the enterococci marker and human enteric viruses. In contrast, the Kalamazoo River (rank 6) did have a higher number of treatment plants and was positive for enteric viruses. The Shiawassee and Raisin Rivers showed relatively less pollution compared with the other rivers, even with more sewage treatment facilities discharging to them. Further assessment of discharge volumes, the types of wastewater disinfection, CSOs, rainfall and distances between discharge and sampling sites is needed in future comprehensive monitoring studies.

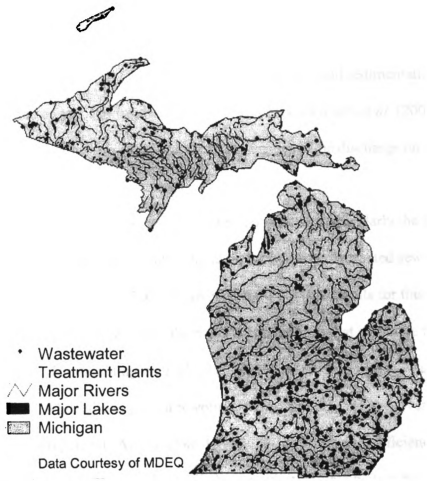

Figure 3-2. **Wastewater treatment locations in the State of Michigan based on data available from the Michigan Department of Environmental Quality.**

The presence of enteric viruses in three of these rivers suggests that wastewaters with inadequate sewage treatment or faulty septic systems are impacting these waterways. Payment *et al.* (2000) examined the occurrence of bacterial indictors and pathogens in the Saint Lawrence River in Canada. Human enteric viruses were found in 39% of the 381 samples analyzed. These results are mimicked in this preliminary study in that 33% of the

Michigan samples were also positive. In addition to examining the source water used by drinking water plants along the Saint Lawrence River, Payment *et al.* (2001) examined the current treatment process used by the Montreal Urban Community wastewater treatment plant before discharging into the river. The current treatment being used is a physico-chemical treatment based on flocculation and sedimentation. Based on 104 untreated and 104 treated wastewater samples, Payment *et al.* (2001) showed a 0% removal of human enteric viruses. The impact of the discharge on recreational users needs to be further evaluated.

The widespread occurrence of bacteriophages, particularly the F^+-specific bacteriophages, in this study indicates that improperly treated sewage is impacting these waters (Noble *et al.* 2003). However, the coliphage results for this study are not likely to be an accurate measure of the mean concentrations that would be found normally in these waters. The sampling method used, collection of bacteriophages using Virusorb 1MDS filters, while allowing a large volume to be sampled, has a recovery efficiency of less than two percent (Alonso *et al.* 1994). This low recovery efficiency greatly underestimates the levels of coliphage present in these waterways. The use of the eluent from the 1MDS filter allows only for a quick assessment of bacteriophage presence. This preliminary study indicates that it would be advantageous to examine the waters directly for bacteriophages in the future.

Enterococci is currently being used by the U.S. EPA for its' National Epidemiological and Environmental Assessment of Recreational Water Study being conducted at Great Lake beaches as well as in marine waters and may be preferred as a human health risk marker based on the outcome of this large-scale study. In this preliminary study, the

enterococci levels did not correlate well with the *E. coli* levels with a correlation value of –0.038.Yet, two of the top three most contaminated sites, as measured by *E. coli* levels, were positive for the human enterococci marker. In addition, these same two sites were also positive for enteric viruses. Therefore, the MST methods bring a more comprehensive understanding of the risk involved when recreating in areas impacted by these waters.

Kinzelman *et al.* (2003) examined indicator levels in the water column and found that by using enterococci as the indicator for beach closures instead of *E. coli*, there would have been 56 more unsafe-recreational-water-quality advisories for the cities of Milwaukee and Racine along Lake Michigan. This study found that while *E. coli* was generally present in higher concentrations than enterococci, the stricter enterococci guideline would have precipitated more closures for recreational activities. These results are reflected in this study with enterococci levels exceeding the guidelines in 78% of the samples and *E. coli* exceeding State standards 56% of the time.

This preliminary survey suggests that human sewage is impacting waters of the State of Michigan and the surrounding Great Lakes. The findings in these single samples indicate a need to further examine the water quality and public health impacts associated with the designated uses of Michigan waters. This study demonstrates that the U.S. EPA enterococci guidelines result in more warnings of a human health risk than do the Michigan *E. coli* criteria. Therefore, it is recommended that the states in the Great Lakes region begin to monitor recreational waters for enterococci. High levels of coliphage, the presence of the enterococci human marker, and the detection of viral pathogens suggest that a more comprehensive microbial/pathogen monitoring program is needed to evaluate

the impact of sewage discharges to Michigan waterways on public health. This should include source tracking, alternative indicators and pathogen monitoring. These types of studies will assist in prioritizing impaired waters and watershed management strategies to maximize water quality and public health protection.

CHAPTER 4

FINAL DISCUSSION

4.1 Introduction

The field of microbial source tracking is new and evolving and will continue to do so in the near future as the methods are adapted from research laboratories to those monitoring recreational waters. Those applying these methods want to know the variety of sources that are impacting their waters, thus causing them to exceed the U.S. EPA's criteria. However, the methods are currently limited in their ability to answer these questions accurately and because of their expense and/or time requirements (library-dependent) (Scott *et al.* 2002) or their sensitivity (amount of marker present) or ability to detect only one-specific source of contamination (library-independent) (Field *et al.* 2003). In addition, the range of methods available makes it cumbersome for managers to implement the necessary method(s) that will answer their specific questions. The U.S. EPA is addressing this very issue by drafting a guidance document, which is designed to assist managers in developing sample schemes tailored to their specific situation (personal communication Jorge Santo-Domingo, U.S. EPA).

4.2 Future Application of Host-Specific Molecular Markers

The major goal of this research was to develop a host-specific molecular marker that could be added to the microbial source tracking toolbox. Because the presence of human fecal pollution in water indicates a higher potential for the presence of human pathogens than the presence of animal feces, such as seagulls, the research focused around developing a human-specific marker. In addition, the ability to develop a marker in a

bacterial group already being used in routine monitoring allows for the method to be easily implemented in routine testing laboratories.

While two different markers were examined, only the *esp* marker demonstrated host-specificity. Further research and development needs to be undertaken with the *ace* gene in order for it to be used as a source tracking marker. This research targeted only a small region of the ace protein. Further primer development and research may yet lead to a host-specific molecular marker using the *ace* gene. In addition, the *ace* gene could still be a valuable marker as it is specific to *Enterococcus faecalis*.

While this research has shown that the *esp* marker is specific to human fecal pollution, several areas need to be further addressed in order for this marker to reach its full potential in source tracking. The validation of the *esp* marker examined fecal sources from several geographic locations including Florida, Michigan, Colorado, and Arizona:, however, a more comprehensive study of potential geographic variability needs to be undertaken. Until this is done, additional tests on known sources should be performed by monitoring programs that are using this marker for identifying human fecal impacts.

Our initial research in the *esp* marker showed that it was not present in our library of clinical isolates. In addition, two of the septic samples examined were negative for the marker. Therefore, the prevalence of the *esp* gene in the human population needs to be determined. Thus, a negative result cannot be interpreted as meaning there is no human impact. Research also needs to be undertaken to examine how the *esp* gene behaves in the environment. For example, do strains with the marker regrow in the water column or sand?

4.3 Further Monitoring Surveys

The monitoring survey undertaken in Michigan was very preliminary. A more comprehensive study including multiple sites along each river and sample collections would allow for a more complete understanding of the water quality in these rivers. It would be especially valuable in the three rivers that were positive for either the *esp* marker or the enteric viruses. A comprehensive study would increase the understanding of how the marker is transported in a natural system and further relate it to the occurrence of human pathogens back as viruses.

APPENDIX I

Human clinical isolate H13 *ace* gene sequence

> GACAAAATTCAATCGTTGACAAAGTAGAATTAGATCACACTACTTTATAT
CAAGGGGAGATGACCTCCATTAAAGTATCTTTTAGTGACAAAGAAAATCAGA
AAATAAAACCCGGAGATACAATTACTTTAACCTTACCAGACGCACTAGTCGG
AATGACCGAGAACGATGGCTCACCACGAAAAATCAATTTAAATGGTTTAGGA
GAAGTTTTCATTTATAAAGATCATGTTGTAGCAACATTTAACGAAAAAGTTGA
ATCTTTACATAATGTGAATGGGCATTTTTCTTTCGGGATTAAAACGCTTATCA
CCAATAGTTCGCAACCGAATGTGATAGAAACGGATTTCGGAACAGCAACGG
CGACTCAACGTTTGACGATTGAAGGAGTGACTAACACAGAGACTGGTCAAA
TTGAGCGAGACTATCCTTTTTTTATAAAGTAGGCGATTTGGCTGGAGAGTCA
AATCAAGTACGTTGGTTTTTAAATGTGAACCTCAATAAATCCGATGTCACAGA
AGATATTTCAATTGCGGATCGACAAGGAAGTGGTCAACAATTAAATAAAGAG
AGTTTTACATTTGATATTGTGAATGACAAAGAAACTAAATATATTTCACTTGC
CGAGTTTGAACAACAAGGTTATGGCAAAATTGACTTTGTGACAGATAACGAC
TTTAATTTACGTTTTTATCGGGATAAAGCACGCTTTACTTCCTTTATCGTCCGT
TACACTTCGACAATCACGGAAGCGGGGCAACATCAAGCGACATTTGATAATA
GTTATGACATCAATTATCAACTAAACAATCAAGACGCAACGAATGAAAAAAA
TACATCACAGGTTAAAAATGTTTTTGTAGACGGTGAGGCAAGCGGCAATCA
AAATGTGGAAATGCCAACAGAAGAAAGTCTAGACATTCCTTTAGAGACAATA
GAAGAATGGGACCAAAGACACCTACTTCGGAACAGGCAACAGGAACAAGTG
AAA

Human clinical isolate H15 *ace* gene sequence

> GAGCAAAATTCAATCGTTGCCAAAGTAGAATTAGATCACACTACTTTATAT
CAAGGGGAGATGACCTCCATTAAAGTATCTTTTAGTGACAAAGAAAATCAGA
AAATAAAACCCGGAGATACAATTACTTTAACCTTACCAGACGCACTAGTCGG
AATGACCGAGAACGATGGCTCACCACGAAAAATCAATTTAAATGGTTTAGGA
GAAGCTTTCATTTATAAAGATCATGTTGTAGCAACATTTAACGAAAAAGTTG
AATCTTTACATAATGTGAATGGGCATTTTTCTTTCGGGATCAAAACGCTTATC
ACCAATAGTTCGCAACCGAATGTGATAGAAACGGATTTCGGAACAGCAACGG
CGACTCAACGTTTGACGATTGAAGGAGTGACTAACACAGAGACTGGTCAAA
TTGAGCGAGACTATCCTTTTTTTATAAAGTAGGCGATTTGGCTGGAGAGTCA
AATCAAGTACGTTGGTTTTTAAATGTGAACCTCAATAAATCCGATGTCACAGA
AGATATTTCAATTGCGGATCGACAAGGAAGTGGTCAACAATTAAATAAAGAG
AGTTTTACATTTGATATTGTGAATGACAAAGAAACTAAATATATTTCACTTGC
CGAGTTTGAACAACAAGGTTATGGCAAAATTGACTTTGTGACAGATAACGAC
TTTAATTTACGTTTTTATCGGGATAAAGCACGCTTTACTTCCTTTATCGTCCGT
TACACTTCGACAATCACGGAAGCGGGGCAACATCAAGCGACATTTGATAATA
GTTATGACATCAATTATCAACTAAACAATCAAGACGCAACGAATGAAAAAA
TACATCACAGGTTAAAAATGTTTTTTGTAGACGGTGAGGCAAGCGGCAATCA

AAATGTGGAAATGCCAA

Poultry isolate C15 *ace* gene sequence

> GAGCAAAATTCAATCGTTACCAAAGTAGAATTAGATCACACTACTTTATAT
CAAGGAGAGATGACCTCAATTAAAGTATCTTTTAGTGACAAAGAAAATCAGA
AAATAAAACCTGGAGATACTATTACTTTAACTTTACCAGACGCACTAGTTGG
AATGACCGAGAACGATAGTTCACCACGAAAAATCAATTTAAATGGTTTAGGG
GAAGTTTTTATCTATAAAGATCATGTTGTAGCAACATTTAACGAAAAAGTTGA
ATCTTTACATAATGTGAATGGGCATTTTTCTTTCGGGATTAAAACGCTTATCA
CCAATAGTTCTCAACCGAATGTGATAGAAACGGATTTCGGAACAGCAACGG
CGACTCAACGTTTGACGATTGAAGGAGTGACTAACACAGAGACTGGCCAAA
TTGAGCGAGACTATCCTTTTTTTATAAAGTAGGCGATTTGGCTGGAGAGTCA
AATCAAGTACGTTGGTTTTTAAATGTGAACCTCAATAAATCCGATGTCACAGA
AGATATTTCAATTGCGGATCGACAAGGAAGTGGTCAACAATTAAATAAAGAG
AGTTTTACATTTGATATTGTGAATGACAAAGAAACTAAATATATTTCACTTGC
CGAGTTTGAGCAACAAGGTTATGGCAAAATTGACTTCGTAACAGATAATGAC
TTTAACTTACGTTTTTATCGGGATAAAGCACGCTTTACTTCCTTTATCGTCCGT
TACACTTCGACAATCACAGAAGCAGGCCAACATCAAGCGACATTTGAAAATA
GTTATGACATCAATTATCAACTAAACAATCAAGACGCAACGAATGAAAAAAA
TACATCACAGGTTAAATATGTTTTTGTAGAAGGCGAGGCAAGCGGCAATCA
AAATGTGGAAATGCCAACAGAAGAAAGTCTAGACATTCCTTTAGAGACAATA
GATGAATGGGA

Poultry isolate C17 *ace* gene sequence

> GAGCAAAATTCAATCTTGACAAAGTAGAATTAGATCACACTACTTTATAT
CAAGGAGAGATGACCTCAATTAAAGTATCTTTTAGTGACAAAGAAAATCAGA
AAATAAAACCTGGAGATACTATTACTTTAACTTTACCAGACGCACTAGTTGG
AATGACCGAGAACGATAGTTCACCACGAAAAATCAATTTAAATGGTTTAGGG
GAAGTTTTTATCTATAAAGATCATGTTGTAGCAACATTTAACGAAAAAGTTGA
ATCTTTACATAATGTGAATGGGCATTTTTCTTTCGGGATTAAAACGCTTATCA
CCAATAGTTCTCAACCGAATGTGATAGAAACGGATTTCGGAACAGCAACGG
CGACTCAACGTTTGACGATTGAAGGAGTGACTAACACAGAGACTGGCCAAA
TTGAGCGAGACTATCCTTTTTTTATAAAGTAGGCGATTTGGCTGGAGAGTCA
AATCAAGTACGTTGGTTTTTAAATGTGAACCTCAATAAATCCGATGTCACAGA
AGATATTTCAATTGCGGATCGACAAGGAAGTGGTCAACAACTAAATAAAGAG
AGTTTTACATTTGATATTGTGAATGACAAAGAAACTAAATATATTTCACTTGC
CGAGTTTGAGCAACAAGGTTATGGCAAAATTGACTTCGTAACAGATAATGAC
TTTAACTTACGTTTTTATCGGGATAAAGCACGCTTTACTTCCTTTATCGTCCGT
TACACTTCGACAATCACAGAAGCAGGCCAACATCAAGCAACATTTGAAAATA
GTTATGCCATCAATTATCAACTAAACAATCAAGACGCAACGAATGAAAAAAA
TACATCACAGGTTAAAAATGTTTTTGTAGGAGGCGAGGCAAGCGGCAATCA
AAATGTGGGAATGCCAACAGAAGAAAGTCTAGACATTCCTTTAGAGACAATA
GATGAGTGGG

Poultry isolate C18 *ace* gene sequence

> GAGCAAAAGTTCAATCTTACCAAAGTAGAATTAGATCACACTACTTTATAT
CAAGGAGAGATGACCTCAATTAAAGTATCTTTTAGTGACAAAGAAAATCAGA
AAATAAAACCTGGAGATACTATTACTTTAACTTTACCAGACGCACTAGTTGG
AATGACCGAGAACGATAGTTCACCACGAAAAATCAATTTAAATGGTTTAGGG
GAAGTTTTTATCTATAAAGATCATGTTGTAGCAACATTTAACGAAAAAGTTGA
ATCTTTACATAATGTGAATGGGCATTTTTCTTTCGGGATTAAAACGCTTATCA
CCAATAGTTCTCAACCGAATGTGATAGAAACGGATTTCGGAACAGCAACGG
CGACTCAACGTTTGACGATTGAAGGAGTGACTAACACAGAGACTGGCCAAA
TTGAGCGAGACTATCCTTTTTTTATAAAGTAGGCGATTTGGCTGGAGAGTCA
AATCAAGTACGTTGGTTTTTAAATGTGAACCTCAATAAATCCGATGTCACAGA
AGATATTTCAATTGCGGATCGACAAGGAAGTGGTCAACAATTAAATAAAGAG
AGTTTTACATTTGATATTGTGAATGACAAAGAAACTAAATATATTTCACTTGC
CGAGTTTGAGCAACAAGGTTATGGCAAAATTGACTTCGTAACAGATAATGAC
TTTAACTTACGTTTTTATCGGGATAAAGCACGCTTTACTTCCTTTATCGTCCGT
TACACTTCGACAATCACAGAAGCAGGCCAACATCAAGCAACATTTGAAAATA
GTTATGTCATCAATTATCAACTAAACAATCAAGACGCAACGAATGAAAAAAA
TACATCACAGGTTAAAAATGTTTTTGTAGAAGGCGAGGCAAGCGGCAATCA
AAATGTGGAAATGCCACCAGAAGTAAGTCTAGACATTCCTTTAGAGACAATA
GATGAATGGGACCAAAGACACCTACTTCGG

APPENDIX II

Enterococcus faecium strain C68 *esp* gene sequence

> CGTCGAAAGTTCGATTTCCGATCTTAACAGTTACAATACCTTTCGTACTAT
CAGTAACGTTAGAAGTATCAGGCTCTGTTTTCCATGAATACTCTGCATCTTCA
GGTAAGTTTTTATTATTCCGAATTGATTCTTTAGCATCTGGTTTAAATCCAACT
ACCACGGTTTGTTTATCAACCGCTTTTGGTGATTCCTTAATAACGGTTGAACC
TTCTTCTGGTTTATCAAAACCTGGAGAAACGATTTGGATTTTTAATTCATCTTT
CGCGATTAATTTGCTTGAATCTACACCCGTAAATTCAAATTCTACAGTATCTG
AAATTGGAGCCCCATCTTTTTCATTTGGAGCGATAGTTTTTTCTGCTAATACA
GTACCTTCTTTATCAACAAGTTGGGCTTTGTGACCTGTTCCCGCTAACTCGTG
GATGAATACTTTCCCCTTAACTGTTGTGTCAACATCATATACTTCTTCTAATTC
AGGTTTCCCGATATATGGATTCGTGTCTCCGCTCTCTTCTTTATCGTCAGGGTT
TCCATCACCATCAATATCTTGCAAATAATAATAACCTGTTCCATAAGTGTTCT
GGATTAATTTTTTATTCGTATCC

REFERENCES

Adams, B. L., T. C. Bates and J. D. Oliver (2003). Survival of *Helicobacter pylori* in a natural freshwater environment. Appl. Environ. Microbiol. 69(12): 7462-7466.

Alonso, M. C., J. M. Sanchez, M. A. Morinigo and J. J. Borrego (1994). A direct membrane filter method for enumerating somatic coliphages in drinking water. Microbiologia 10(3): 285-296.

American Public Health Association, American Water Works Association and Water Environment Federation, Eds. (1999). Standard methods for the examination of water and wastewater.

Bernhard, A. E. and K. G. Field (2000a). Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. 66(4): 1587-1594.

Bernhard, A. E. and K. G. Field (2000b). A PCR Assay To Discriminate Human and Ruminant Feces on the Basis of Host Differences in Bacteroides-Prevotella Genes Encoding 16S rRNA. Appl. Environ. Microbiol. 66(10): 4571-4574.

Bisson, J. W. and V. J. Cabelli (1979). Membrane filter enumeration method for *Clostridium perfringens*. Appl. Environ. Microbiol. 37(1): 55-66.

Blackburn, B. G., G. F. Craun, J. S. Yoder, V. Hill, R. Calderon, N. Chen, S. H. Lee, D. A. Levy and M. J. Beach (2004). Surveillance for waterborne-disease outbreaks associated with drinking water-United States, 2001-2002. Morb. Mortal. Wkly. Rep. 53(SS-8): 23-44.

Borchardt, M. A., M. E. Stemper and J. H. Standridge (2003a). Aeromonas isolates from human diarrheic stool and groundwater compared by pulsed-field gel electrophoresis. Emerging Infect. Dis. 9(2): 224-228.

Borchardt, M. A., P. D. Bertz, S. K. Spencer and D. A. Battigelli (2003b). Incidence of Enteric Viruses in Groundwater from Household Wells in Wisconsin. Appl. Environ. Microbiol. 69(2): 1172-1180.

Byappanahalli, M., M. Fowler, D. Shively and R. Whitman (2003). Ubiquity and persistence of *Escherichia coli* in a midwestern coastal stream. Appl. Environ. Microbiol. 69(8): 4549-4555.

Cabelli, V. J. (1983). Health effects for marine recreation waters. United States Environmental Protection Agency 600: 1-80-031.

Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe and P. W. Haberman (1979). Relationship of microbial indicators to health effects at marine bathing beaches. Am. J. Public Health 69(7): 690-696.

Cabelli, V. J., A. P. Dufour, M. A. Levin and L. J. McCabe (1982). Swimming-associated gastroenteritis and water quality. Am. J. Epidemiol. 115: 606-616.

Carson, C. A., B. L. Shear, M. R. Ellersieck and J. D. Schnell (2003). Comparison of ribotyping and repetitive extragenic palindromic-PCR for identification of fecal *Escherichia coli* from humans and animals. Appl. Environ. Microbiol. 69(3): 1836-1839.

Carson, C. A., B. L. Shear, M. R. Ellersieck and A. Asfaw (2001). Identification of fecal *Escherichia coli* from humans and animals by ribotyping. Appl. Environ. Microbiol. 67: 1503-1507.

Craun, G. F. (1986). Waterborne Diseases in the United States. Boca Raton, FL, CRC Press, Inc.

D'Agata, E. M. C., M. M. Gerrits, Y.-W. Tang, M. Samore and J. G. Kusters (2001). Comparison of pulsed-field gel electrophoresis and amplified fragment-length polymorphism for epidemiological investigations of common nosacomial pathogens. Infection Control & Hospital Epidemiology 22: 550-554.

Dombek, P. E., L. K. Johnson, S. T. Zimmerley and M. J. Sadowsky (2000). Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl. Environ. Microbiol. 66: 2572-2577.

Duh, R.-W., K. V. Singh, K. Malathum and B. E. Murray (2001). In vitro activity of 19 antimicrobial agents against enterococci from healthy subjects and hospitalized patients and use of an ace gene probe from *Enterococcus faecalis* for species identification. Microb. Drug Resist. 7(1): 39-46.

DuPont, H. L. and J. H. Steele (1987). Use of antimicrobial agents in animal feeds: implications for human health. Rev. Infect. Dis. 9: 447-460.

Eaton, T. J. and M. J. Gasson (2002). A variant enterococcal surface protein esp_{fm} in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiol. Lett. 216(2): 269-275.

Embrey, M. A., R. T. Parkin and J. M. Balbus, Eds. (2002). Handbook of CCL Microbes in Drinking Water.

FDA/CFSAN. 1992. Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. U.S. Food and Drug Administration/Center for Food Safety and Applied Nutrition.

Field, K. G., E. C. Chern, L. K. Dick, J. Fuhrman, J. F. Griffith, P. A. Holden, M. G. LaMontagne, B. H. Olson and M. T. Simonich (2003). A comparative study of culture-independent, library-independent genotypic methods of fecal source tracking. J. Water Health 1(4): 181-194.

Fogarty, L. R., S. K. Haack, M. J. Wolcott and R. L. Whitman (2003). Abundance and characteristics of the recreational water quality indicator bacteria Escherichia coli and enterococci in gull faeces. J. Appl. Microbiol. 94(5): 865-878.

Geldreich, E. E. and B. A. Kenner (1969). Concepts of fecal streptococci in stream pollution. J. Water Pollut. Control Fed. 41: R335-R352.

Gordillo, M. E., K. V. Singh and B. E. Murray (1993). Comparison of ribotyping and pulsed-field gel electrophoresis for subspieces differentiation of strains of *Enterococcus faecalis*. J. Clin. Microbiol. 31(6): 1570-1574.

Griffin, D. W., C. J. I. Gibson, E. K. Lipp, K. Riley, J. H. Paul and J. B. Rose (1999). Detection of viral pathogens by reverse-transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. Appl. Environ. Microbiol. 65(9): 4118-4125.

Griffin, D. W., E. K. Lipp, M. R. McLaughlin and J. B. Rose (2001). Marine recreation and public health microbiology: quest for the ideal indicator. BioScience 51(10): 817-825.

Griffith, J. F., S. B. Weisberg and C. D. McGee (2003). Evaluation of microbial source tracking methods using mixed fecal sources in aqueous test samples. J. Water Health 1(4): 141-151.

Guan, S., R. Xu, S. Chen, J. Odumeru and C. Gyles (2002). Development of a Procedure for Discriminating among *Escherichia coli* Isolates from Animal and Human Sources. Appl. Environ. Microbiol. 68(6): 2690-2698.

Guan, T. Y. and R. A. Holley (2003). Pathogen Survival in Swine Manure Environments and Transmission of Human Enteric Illness--A Review. J Environ Qual 32(2): 383-392.

Haack, S. K., L. R. Fogarty and C. Wright (2003). *Escherichia coli* and enterococci at beaches in the Grand Traverse Bay, Lake Michigan: Sources, characteristics, and environmental pathways. Environ. Sci. Technol. 37(15): 3275-3282.

Hagedorn, C., J. B. Crozier, K. A. Mentz, A. M. Booth, A. K. Graves, N. J. Nelson and R. B. Reneau (2003). Carbon source utilization profiles as a method to identify sources of faecal pollution in water. J. Appl. Microbiol. 94(5): 792-799.

Hammerum, A. M. and L. B. Jensen (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(11): 4396.

Hartel, P. G., J. D. Summer and W. I. Segars (2003). Deer diet affects ribotype diversity of *Escherichia coli* for bacterial source tracking. Water Res. 37: 3263-3268.

Hartel, P. G., J. D. Summer, J. L. Hill, J. V. Collins, J. A. Entry and W. I. Segars (2002). Geographic variability of *Escherichia coli* ribotypes from animals in Idaho and Georgia. J. Environ. Qual. 31(4): 1273-1278.

Harwood, V. J., J. E. Whitlock and V. Withington (2000). Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in prediction the source of fecal contamination in subtropical Florida waters. Appl. Environ. Microbiol. 66: 3698-3704.

Harwood, V. J., N. C. Delahoya, R. M. Ulrich, M. F. Kramer, J. E. Whitlock, J. R. Garey and D. V. Lim (2004). Molecular confirmation of *Enterococcus faecalis* and *E. faecium* from clinical, faecal and environmental sources. Lett. Appl. Microbiol. 38(6): 476-482.

Harwood, V. J., B. Wiggins, C. Hagedorn, R. D. Ellender, J. Gooch, J. Kern, M. Samadpour, A. C. H. Chapman, B. J. Robinson and B. C. Thompson (2003). Phenotypic library-based microbial source tracking methods: Efficacy in the California collaborative study. J. Water Health 1(4): 153-166.

Hayashi, Y., S. Managaki and H. Takada (2002). Fluorescent whitening agents in Tokyo Bay and adjacent rivers: Their application as anthropogenic molecular markers in coastal environments. Environ. Sci. Technol. 36: 3556-3563.

Hilborn, E. D., M. O. Royster and D. J. Drabkowski (2002). Survey of US public health laboratories: microbial pathogens on the CCL. Journal AWWA 94(6): 88-95.

Jenkins, M. B., P. G. Hartel, T. J. Olexa and J. A. Stuedemann (2003). Putative temporal variability of *Escherichia coli* ribotypes from yearling steers. J. Environ. Qual. 32(1): 305-309.

Johnson, L. K., M. B. Brown, E. A. Carruthers, J. A. Ferguson, P. E. Dombek and M. J. Sadowsky (2004). Sample size, library composition, and genotypic diversity among natural populations of *Escherichia coli* from different animals influence accurarcy of determining sources of fecal pollution. Appl. Environ. Microbiol. 70(8): 4478-4485.

Kaspar, C. W., J. L. Burgess, I. T. Knight and R. R. Colwell (1990). Antibiotic resistance indexing of *Escherichia coli* to identify sources of fecal contamination in water. Can. J. Microbiol. 36: 891-894.

Kelsey, R. H., G. I. Scott, D. E. Porter, B. Thompson and L. Webster (2003). Using multiple antibiotic resistance and land use characteristics to determine sources of fecal coliform bacterial pollution. Environ. Monit. Assess. 81: 337-348.

Khatib, L. A., Y. L. Tsai and B. H. Olson (2002). A biomarker for the identification of cattle fecal pollution in water using the LTIIa toxin gene from enterotoxigenic *Escherichia coli*. Appl. Microbiol. Biotechnol. 59: 97-104.

Khatib, L. A., Y. L. Tsai and B. H. Olson (2003). A biomarker for the identification of swine fecal pollution in water, using the STII toxin gene from enterotoxigenic *Escherichia coli*. Appl. Microbiol. Biotechnol. 63: 231-238.

Kinzelman, J., C. Ng, E. Jackson, S. Gradus and R. Bagley (2003). Enterococci as indicators of Lake Michigan recreational water quality: Comparison of two methodologies and their impacts on public health regulatory events. Appl. Environ. Microbiol. 69(1): 92-96.

Koch, S., M. Hufnagel, C. Theilacker and J. Huebner (2004). Enterococcal infections: host response, therapeutic, and prophylactic possiblities. Vaccine 22: 822-830.

Kuntz, R. L., P. G. Hartel, D. G. Godfrey, J. L. McDonald, K. W. Gates and W. I. Segars (2003). Targeted sampling protocol as prelude to bacterial source tracking with *Enterococcus faecalis*. J. Environ. Qual. 32(6): 2311-2318.

Le Guyader, F., E. Dubois, D. Menard and M. Pommepuy (1994). Detection of hepatitis A virus, rotavirus, and enterovirus in naturally contaminated shellfish and sediment by reverse transcription-seminested PCR. Appl. Environ. Microbiol. 60(10): 3665-3671.

Leavis, H., J. Top, N. Shankar, K. Borgen, M. Bonten, J. van Embden and R. J. L. Willems (2004). A vovel putative enterococcal pathogenicity island linked to the esp virulence gene of *Enterococcus faecium* and associated with epidemicity. J. Bacteriol. 186(3): 672-682.

Leclerc, H., L. Schwartzbrod and E. Dei-Cas (2002). Microbial agents associated with waterborne diseases. Crit. Rev. Microbiol. 28(4): 371-409.

Lee, S. H., D. A. Levy, G. F. Craun, M. J. Beach and R. L. Calderon (2002). Surveillance for Waterborne-Disease Outbreaks---United States, 1999-2000. Morb. Mortal. Wkly. Rep. 51(SS-8): 1-28.

Leeming, R., A. Ball, N. Ashbolt and P. Nichols (1996). Using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters. Water Res. 30(12): 2893-2900.

Leung, K. T., R. Mackereth, Y.-C. Tien and E. Topp (2004). A comparison of AFLP and ERIC-PCR analysis for discriminating Escherichia coli from cattle, pig and human sources. FEMS Microbiol. Ecol. 47: 111-119.

Lu, L., M. E. Hume, K. L. Sternes and S. D. Pillai (2004). Genetic diversity of *Escherichia coli* isolates in irrigation water and associated sediments: implications for source tracking. Water Res. 38(18): 3899-3908.

Lund, E. (1982). Waterborne virus diseases. Ecol. Dis. 1(1): 27-35.

Maluquer de Motes, C., P. Clemente-Casares, A. Hundesa, M. Martin and R. Girones (2004). Detection of bovine and porcine adenoviruses for tracing the source of fecal contamination. Appl. Environ. Microbiol. 70(3): 1448-1454.

Manero, A., X. Vilanova, M. Cerda-Cuellar and A. R. Blanch (2002). Characterization of sewage waters by biochemical fingerprinting of Enterococci. Water Res. 36(11): 2831-2835.

McLellan, S. L., A. D. Daniels and A. K. Salmore (2003). Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA fingerprinting. Appl. Environ. Microbiol. 69(5): 2587-2594.

McLellan, S. L. (2004). Genetic diversity of *Escherichia coli* isolated from urban rivers and beach water. Appl. Environ. Microbiol. 70(8): 4658-4665.

Meays, C. L., K. Broersma, R. Nordin and A. Mazumder (2004). Source tracking fecal bacteria in water: a critical review of current methods. J. Environ. Manage. 73(1): 71-79.

Michigan Department of Environmental Quality Surface Water Division. 2002. Water quality and pollution control in Michigan: 2002 Section 305(b) report. 323.1062 Microorganisms (Rule 62).

Mounts, A. W., T. Ando, M. Koopmans, J. S. Bresee, J. Noel and R. I. Glass (2000). Cold weather seasonality of gastroenteritis associated with Norwalk-like viruses. J. Infect. Dis. 181: S284-S287.

Muller, T., A. Ulrich, E.-M. Ott and M. Muller (2001). Identification of plant-associated enterococci. J. Appl. Microbiol. 91(2): 268-278.

Murray, C. and A. Lopez (1996). Global burden of disease and injury. Global health statistics. Cambridge, MA, Harvard School of Public Health on behalf of the World Health Organization and the World Bank. Series Vol. 2.

Murray, K. S., L. E. Fisher, J. Therrien, B. George and J. Gillespie (2001). Assessment and use of indicator bacteria to determine sources of pollution to an urban river. J. Great Lakes Res. 27(2): 220-229.

Myoda, S. P., A. Carson, J. J. Fuhrmann, B.-K. Hahm, P. G. Hartel, H. Yampara-Iquise, L. Johnson, R. L. Kuntz, C. H. Nakatsu, M. J. Sadowsky and M. Samadpour (2003). Comparison of genotypic-based microbial source tracking methods requiring a host origin database. J. Water Health 1(4): 167-180.

Nallapareddy, S. R., G. M. Weinstock and B. E. Murray (2003). Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol. Microbiol. 47(6): 1733-1747.

Nallapareddy, S. R., K. V. Singh, R.-W. Duh, G. M. Weinstock and B. E. Murray (2000). Diversity of ace, a Gene Encoding a Microbial Surface Component Recognizing Adhesive Matrix Molecules, from Different Strains of *Enterococcus faecalis* and Evidence for Production of Ace during Human Infections. Infect. Immun. 68(9): 5210-5217.

Nasser, A. M., N. Zaruk, L. Tenenbaum and Y. Netzan (2003). Comparative survival of *Cryptosporidium, coxsackievrius* A9 and *Escherichia coli* in stream, brackish and sea waters. Water Sci. Technol. 47(3): 91-96.

National Research Council of the National Academies, Ed. (2004). Indicators for Waterborne Pathogens. Washington D.C., National Academies Press.

Noble, R. T., S. M. Allen, A. D. Blackwood, W. Chu, S. C. Jiang, G. L. Lovelace, M. D. Sobsey, J. R. Stewart and D. A. Wait (2003). Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial source tracking comparison study. J. Water Health 1(4): 195-208.

Organization, W. H. 2000. The world health report: making a difference. Geneva, World Health Organization.

Ozawa, Y., P. Courvalin and M. Galimand (2000). Identification of enterococci at the species level by sequencing of the genes for D-alanine:D-alanine ligases. Syst. Appl. Microbiol. 23: 230-237.

Parveen, S., R. L. Murphree, L. Edmiston, C. W. Kaspar, K. M. Portier and M. L. Tamplin (1997). Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. Appl. Environ. Microbiol. 63: 2607-2612.

Parveen, S., K. M. Portier, K. Robinson, L. Edmiston and M. L. Tamplin (1999). Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. Appl. Environ. Microbiol. 65(7): 3142-3147.

Parveen, S., N. C. Hodge, R. E. Stall, S. R. Farrah and M. L. Tamplin (2001). Phenotypic and genotypic characterization of human and nonhuman *Escherichia coli*. Water Res. 35(2): 379-386.

Payment, P., A. Berte, M. Prevost, B. Menard and B. Barbeau (2000). Occurrence of pathogenic microorganisms in the Saint Lawrence River (Canada) and comparison of health risks for populations using it as their source of drinking water. Can. J. Microbiol. 46(6): 565-576.

Payment, P., R. Plante and P. Cejka (2001). Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. Can. J. Microbiol. 47(3): 188-193.

Pourcher, A.-M., L. A. Devriese, J. F. Hernandez and J. M. Delattre (1991). Enumberation by a miniaturized method of *Escherichia coli, Streptococcus bovis* and enterococci as indicators of the origin of faecal pollution of waters. J. Appl. Bacteriol. 70(6): 525-530.

Puig, M., J. Jofre, F. Lucena, A. Allard, G. Wadell and R. Girones (1994). Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. Appl. Environ. Microbiol. 60(8): 2963-2970.

Rice, L. B., L. Carias, S. Rudin, C. Vael, H. Goossens, C. Konstabel, I. Klare, S. R. Nallapareddy, W. Huang and B. E. Murray (2003). A Potential Virulence Gene, hyl_{Efm}, Predomiantes in *Enterococcus faecium* of Clinical Origin. J. Infect. Dis. 187(1): 508-512.

Rich, R. L., B. Kreikemeyer, R. T. Owens, S. LaBrenz, S. V. L. Narayana, G. M. Weinstock, B. E. Murray and M. Hook (1999). Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. J. Biol. Chem. 274(38): 26939-26945.

Rose, J. B., D. E. Huffman, K. Riley, S. A. Farrah, J. Lukasik and C. L. Hamann (2001). Reduction of enteric microorganisms at the Upper Occoquan Sewage Authority Water Reclamation Plant. Water Environ. Res. 73: 711-720.

Russell, J. B. and G. N. Jarvis (2001). Practical mechanisms for interrupting the oral-fecal lifecycle of *Escherichia coli*. J. Mol. Microbiol. Biotechnol. 3(2): 265-272.

Scott, T. M., S. Parveen, K. M. Portier, J. B. Rose, M. L. Tamplin, S. A. Farrah, A. Koo and J. Lukasik (2003). Geographical variation in ribotype profiles of Escherichia coli isolates from humans, swine, poultry, beef, and dairy cattle in Florida. Appl. Environ. Microbiol. 69(2): 1089-1092.

Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah and J. Lukasik (2002). Microbial Source Tracking: Current Methodology and Future Directions. Appl. Environ. Microbiol. 68(12): 5796-5803.

Shankar, N., C. V. Lockatell, A. S. Baghdayan, C. Drachenberg, M. S. Gilmore and D. E. Johnson (2001). Role of *Enterococcus faecalis* surface protein esp in the pathogenesis of ascending urinary tract infection. Infect. Immun. 69(7): 4366-4372.

Shankar, V., A. S. Baghdayan, M. M. Huycke, G. Lindahl and M. S. Gilmore (1999). Infection-derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. Infect. Immun. 67(1): 193-200.

Simpson, J. M., J. W. Santo Domingo and D. J. Reasoner (2002). Microbial source tracking: state of the science. Environ. Sci. Technol. 36(24): 5279-88.

Sinton, L. W., R. K. Finlay and D. J. Hannah (1998). Distinguishing human from animal faecal contamination in water: a review. N. Z. J. Mar. Freshwater Res. 32: 323-348.

Slifko, T. R., H. V. Smith and J. B. Rose (2000). Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30(12-13): 1379-1393.

Stewart, J. R., R. D. Ellender, J. A. Gooch, S. C. Jiang, S. P. Myoda and S. B. Weisberg (2003). Recommendations for microbial source tracking: Lessons from a methods comparison study. J. Water Health 1(4): 225-231.

Svec, P., I. Sedlacek, R. Pantucek, L. A. Devriese and J. Doskar (2001). Evaluation of ribotyping for characterization and identification of *Enterococcus haemoperoxidus* and *Enterococcus moraviensis* strains. FEMS Microbiol. Lett. 203(1): 23-27.

Tartera, C. and J. Jofre (1987). Bacteriophages active against *Bacteroides fragilis* in sewage-polluted waters. Appl. Environ. Microbiol. 53(7): 1632-1637.

Tartera, C., F. Lucena and J. Jofre (1989). Human origin of *Bacteroides fragilis* bacteriophages present in the environment. Appl. Environ. Microbiol. 55(10): 2696-2701.

United States Environmental Protection Agency. 1986. Ambient Water Quality Criteria for Bacteria-1986. EPA440/5-84-002.

United States Environmental Protection Agency. 2001a. Manual of Methods for Virology. EPA 600/4-84/013 (N14).

United States Environmental Protection Agency. 2002a. Method 1103.1: *Escherichia coli* (E. coli) in water by membrane filtration using membrane-thermotolerant *Escherichia coli* agar (mTEC). EPA 821-R-02-020.

United States Environmental Protection Agency. 2002b. Method 1106.1: enterococci in water by membrane filtration using membrane-Enterococcus-esculin iron agar (mE-EIA). EPA 821-R-02-021.

United States Environmental Protection Agency. 2002c. Method 1600: enterococci in water by membrane filtration using membrane-Enterococcus indoxyl-β-D-glucoside agar (mEI). EPA 821-R-02-022.

United States Environmental Protection Agency. 2001b. Method 1601: male-specific (F^+) and somatic coliphage in water by two-step enrichment procedure. EPA 821-R-01-030.

United States Environmental Protection Agency. 2001c. Method 1602: male-specific (F^+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA 821-R-01-029.

United States Environmental Protection Agency. 2002d. Method 1603: *Escherichia coli* (E. coli) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC). EPA 821-R-02-023.

United States Environmental Protection Agency. 2002e. Method 1604: Total coliforms and *Escherichia coli* in water by membrane filtration using a simultaneous detection technique (MI medium). EPA 821-R-02-024.

United States Environmental Protection Agency. 1999. Total maximum daily load (TMDL) program. EPA 841-F-99-003A.

Velasco, D., S. Perez, F. Pena, M. A. Dominguez, M. Cartelle, F. Molina, R. Moure, R. Villanueva and G. Bou (2004). Lack of correlation between phenotypic techniques and PCR-based genotypic methods for identification of *Enterococcus spp*. Diagn. Microbiol. Infect. Dis. 49: 151-156.

Wade, T. J., N. Pai, J. N. S. Eisenberg and J. J. Colford (2003). Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. 111(8): 1102-1109.

Wallis, J. L. and H. D. Taylor (2003). Phenotypic population characteristics of the enterococci in wastewater and animal faeces: implications for the new European directive on the quality of bathing waters. Water Sci. Technol. 47(3): 27-32.

Wheeler, A. L., P. G. Hartel, D. G. Godfrey, J. L. Hill and W. I. Segars (2002). Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. J. Environ. Qual. 31(4): 1286-1293.

Wheeler Alm, E., J. Burke and A. Spain (2003). Fecal indicator bacteria are abundant in wet sand at freshwater beaches. Water Res. 37(16): 3978-3982.

Whitlock, J. E., D. T. Jones and V. J. Harwood (2002). Identification of the sources of fecal coliforms in an urban watershed using antibiotic resistance anlaysis. Water Res. 36: 4273-4282.

Whitman, R. L. and M. B. Nevers (2003). Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan beach. Appl. Environ. Microbiol. 69(9): 5555-5562.

Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers and M. N. Byappanahalli (2003). Occurrence of *Escherichia coli* and enterococci in Cladophora (Chlorophyta) in nearshore water and beach sand of Lake Michigan. Appl. Environ. Microbiol. 69(8): 4714-4719.

Wiggins, B. A. (1996). Discriminant analysis of antibiotic resistance patterns in fecal streptococci, a method to differentiate human and animal sources of fecal pollution in natural waters. Appl. Environ. Microbiol. 62: 3997-4002.

Wiggins, B. A., P. W. Cash, W. S. Creamer, S. E. Dart, P. P. Garcia, T. M. Gerecke, J. Han, B. L. Henry, K. B. Hoover, E. L. Johnson, K. C. Jones, J. G. McCarthy, J. A. McDonough, S. A. Mercer, M. J. Noto, H. Park, M. S. Phillips, S. M. Purner, B. M. Smith, E. N. Stevens and A. K. Varner (2003). Use of Antibiotic Resistance Analysis for Representativeness Testing of Multiwatershed Libraries. Appl. Environ. Microbiol. 69(6): 3399-3405.

Willems, R. J. L., J. Top, N. van den Braak, A. van Belkum, H. Endtz, D. Mevius, E. Stobberingh, A. van den Bogaard and J. D. A. van Embden (2000). Host specificity of vancomycin-resistant *Enterococcus faecium*. J. Infect. Dis. 182(3): 816-823.

Willems, R. J. L., W. L. Homan, J. Top, M. van Santen-Verheuvel, D. Tribe, X. Manzioros, C. Gaillard, C. M. J. E. Vandenbroucke-Grauls, E. M. Mascini, E. van Kregten, J. D. A. van Embden and M. J. M. Bonten (2001). Variant esp gene as a marker of a distinct genetic lineage of vancomycin-reistant *Enterococcus faecium* spreading in hospitals. Lancet 357: 853-855.

Yoder, J. S., B. G. Blackburn, G. F. Craun, V. Hill, D. A. Levy, N. Chen, S. H. Lee, R. Calderon and M. J. Beach (2004). Surveillance for waterborne-disease outbreaks associated with recreational water-United States, 2001-2002. Morb. Mortal. Wkly. Rep. 53(SS-8): 1-20.