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Cow Enterococcus ID™

Detection of the *Enterococcus hirae* Cattle Gene Biomarker for Cattle Fecal Contamination by Polymerase Chain Reaction (PCR) DNA Analytical Technology

Submitter: Raintree Lake Property Owners Association

Submitter #'s: Hidden Cove and Sunset Cove

Source Molecular #'s: SM 11723 and SM 11724

Samples Received: August 17, 2006

Date Reported: August 23, 2006

SM #	Client #	Enterococci (CFU/100mL) ⁷	Enterococci Analyzed	DNA Analytical Results
SM 11723 SM 11724	Hidden Sunset	2,500 1,000	2,500 1,000	Cattle Gene Biomarker Detected Negative

Laboratory Comments

The submitted water samples were filtered for *Enterococcus spp.* and the *Enterococci* were enumerated on petri plates. Afterwards, the *Enterococci* were eluted and centrifuged directly from the filter for DNA analysis.

All reagents, chemicals and apparatuses were verified and inspected beforehand to ensure that no false negatives or positives could be generated. In that regard, positive and negative controls were run to attest the integrity of the analysis. All inspections and controls tested negative for possible extraneous contaminants, including PCR inhibitors.

Sample Sunset Cove (Our Ref: SM 11724) tested negative for the *Enterococcus hirae* cattle gene biomarker. It is important to note that a negative result does not mean that the sample does not definitely have cattle contamination. In order to strengthen the result, a negative sample should be analyzed further for cattle fecal contamination with other DNA analytical tests such as the Cow Bacteroidetes ID™ and Cow Fecal Virus ID™ services.

Sample Hidden Cove (Our Ref: SM 11723) tested positive for the *Enterococcus hirae* cattle gene biomarker suggesting that cattle fecal contamination is present in this water sample. The client is nonetheless encouraged to conduct other DNA analytical tests such as the services mentioned above to further confirm the result.

DNA Analytical Method Explanation

100 ml of water was filtered through 0.45 micron membrane filters. The filters were placed on mEnterococcus media supplemented with indoxyl substrate and the plates were incubated for 24 hours at 41°C according to the protocol outlined in EPA Method 1600.⁷ Colonies exhibiting a blue halo were enumerated as *Enterococci*.

DNA extraction was prepared using the Qiagen DNA extraction kit, as per manufacturers instructions. Five micro-liter aliquots of purified DNA extraction were used directly as template for subsequent PCR reactions. Amplification of PCR primers were carried out using HotStarTaq polymerase (Qiagen, Inc.) and master mix, which contained a final concentration of 1.5 mM MgCl₂, 150 mM dNTP, and 0.3 mM of each primer.

An Eppendorf Gradient Thermocycler was used with the following cycling parameters: 95°C for 15 minutes (to lyse cells and activate polymerase), followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and a final extension at 72°C for 5 minutes. PCR products were electrophoresed on 2% agarose gels, stained with GelStar nucleic acid stain (Cambrex, Inc.) and visualized under UV light.

DNA Analytical Theory Explanation

Enterococci are a subgroup of Fecal *Streptococci* and are characterized by their ability to grow in 6.5% sodium chloride, at low and elevated temperatures (10°C and 45°C), and at elevated pH (9.5). These microorganisms have been used as indicators of fecal pollution for many years and have been especially valuable in the marine environment and recreational waters as indicators of potential health risks and swimming-related gastroenteritis.^{1,2,3}

Enterococci are benign bacteria when they reside in their normal habitat such as the gastrointestinal tracts of human or animals. Outside of their normal habitat, *Enterococci* are pathogenic causing urinary tract and wound infections, and life-threatening diseases such as bacteraemia, endocarditis, and meningitis. *Enterococci* easily colonize open wounds and skin ulcers.

Compounding their pathogenesis, *Enterococci* are also some of the most antibiotic resistant bacteria.^{4,5} Studies have shown that certain strains of *Enterococci* are resistant to expensive and potent antibiotics such as vancomycin. This is particularly worrisome for the medical community since these antibiotics are given as a last resort to fight severe bacterial infections.

Several intrinsic features of the *Enterococcus* genus allow it to survive for extended periods of time, leading to its extended survivability and diffusion. For example, *Enterococci* have been shown to survive for 30 minutes at 60°C and persist in the presence of detergents. As such, the inherent ruggedness of *Enterococcus* confers it a strong tolerance to many classes of antibiotics.

The Cow Enterococcus ID™ service is designed around the principle that certain DNA sequences contained within strains of the *Enterococcus* genus are specific to cattle. These *Enterococci* sequences can be used as indicators of cattle fecal contamination.⁶ Strains of *Enterococcus hirae* and *Enterococcus mundtii* have been shown to be from cattle and other ruminant sources.⁶ The Cow Enterococcus ID™ service targets the cattle gene biomarker in *Enterococcus hirae*.

One of the advantages of the Cow Enterococcus ID™ service is that the entire population of *Enterococci* of the selected portion of the water sample is screened. As such, this method avoids the randomness effect of selecting isolates off a petri dish.

Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected.

Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available for detection by gel electrophoresis.

The gel electrophoresis apparatus uses an electrical field to distinguish different DNA fragments according to their molecular weights. Lighter DNA fragments will move farther along the gel than their heavier counterparts. At the end of the procedure different bands of accumulated DNA fragments will aggregate at different parts of the gel. It is this accumulation of DNA fragments that creates a band on the gel. Researchers use these bands to distinguish certain genomes such as the cattle gene biomarker from *Enterococcus hirae*.

These banding patterns confirm or negate the presence of the *Enterococci* cattle gene biomarker. As such, the banding patterns provide a reliable indicator of cattle fecal contamination. To strengthen the validity of the results, the Cow Enterococcus ID™ service should be combined with other DNA analytical services such as the Cow Bacteroidetes ID™ and Cow Fecal Virus ID™ services.

¹ Scott, Troy M., Rose, Joan B., Jenkins, Tracie M., Farrah, Samuel R., Lukasik, Jerzy **Microbial Source Tracking: Current Methodology and Future Directions.** Appl. Environ. Microbiol. (2002) 68: 5796-5803.

² Scott, T.M., T.M. Jenkins, J. Lukasik, and J.B. Rose. 2005. **Potential Use of a Host Associated Molecular Marker in *Enterococcus faecium* as an Index of Human Fecal Pollution.** Environ. Sci. Technol. 39: 283-287.

³ Bahirathan ML, Puente L, Seyfried P. 1998. **Use of yellow-pigmented enterococci as a specific indicator of human and nonhuman sources of faecal pollution.** Can J Microbiol 44:1066-1071.

⁴ Quednau, M., Ahrne, S., Molin, G. **Genomic Relationships between *Enterococcus faecium* Strains from Different Sources and with Different Antibiotic Resistance Profiles Evaluated by Restriction Endonuclease Analysis of Total Chromosomal DNA Using EcoRI and PvuII.** Appl. Environ. Microbiol. 1999 65: 1777-1780.

⁵ 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: 4396.

⁶ Soule, Marilyn, Kuhn, Edward, Loge, Frank, Gay, John, Call, Douglas R. **Using DNA Microarrays To Identify Library-Independent Markers for Bacterial Source Tracking** Appl. Environ. Microbiol. 2006 72: 1843-1851.

⁷ EPA Method 1600: Membrane Filter Test Method for Enterococci In Water (1997).

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