

SOURCE MOLECULAR CORPORATION

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Cow Enterococcus "Quantification" ID™

Detection and Quantification of the *Enterococcus hirae* Cattle Gene Biomarker for Cattle Fecal Contamination by Real-Time Quantitative Polymerase Chain Reaction (qPCR) DNA Analytical Technology

Submitter: ABC Beach Park

Submitter #'s: 875, 876, 877 and 878

Source Molecular #'s: SM 0825, SM 0826, SM 0827 and SM 0828

Samples Received: January 3rd, 2011

Date Reported: January 10th, 2011

SAMPLE

SM #	Client #	Enterococci (CFU/100 mL)***	Total <i>E. faecium</i> Quantified*	Total <i>E. hirae</i> Cattle Biomarker Quantified*	DNA Analytical Results
SM 0825	875	400	2.95×10^7	BDL**	Negative **
SM 0826	876	1,500	1.15×10^8	3.55×10^4	Cattle Gene Biomarker Detected
SM 0827	877	1,400	2.25×10^8	BDL**	Negative **
SM 0828	878	5,400	4.65×10^8	BDL**	Negative **

* After 24 hours of incubation. Total is copy no./ml of extract.

** Detection limit is < 500 copy no./ml of DNA extract.

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

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Laboratory Comments
Submitter: ABC Beach Park
Report Date: August 17, 2006

The submitted water samples were filtered and incubated for 24 hours. **Please note that the *Enterococci* numbers given in the table in the previous page are after cultivation.** Afterwards, the filters were eluted in a buffer. The buffer was centrifuged and DNA was extracted from the resultant pellet. qPCR (i.e.: real-time quantitative PCR) targeting total *E. faecium* and the *E. hirae* cattle gene biomarker was performed on the DNA extract.

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.

All the samples in this report except sample 876 (SM 0826) tested negative (i.e. below the detection limit) 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 IDTM and Cow Fecal Virus IDTM services. On the other hand, one can infer the presence of mammalian sources of fecal pollution since generic forms of *Enterococcus faecium* were found present in the negative samples.

Preliminary Interpretation of Positive Result

Sample 876 (SM 0826) tested positive for the *Enterococcus hirae* cattle gene biomarker suggesting that cattle fecal contamination is present in this water sample. Using real-time quantitative PCR DNA analytical technology (qPCR), the *E. hirae* with the cattle gene marker was quantified and compared to the total *E. faecium* population. The *E. hirae* with the cattle gene marker gave ratio of 0.031% of the total *E. faecium* population. It is important to take into account the context of the sample when interpreting the percentage provided.

Our preliminary interpretation suggests that cattle fecal sources of contamination are a minor component of the positive sample. Using our internal ratios, the cattle fecal pollution would seem to be less than 1% of the overall fecal pollution of the sample.

The client is encouraged nonetheless to submit additional samples from this site both during wet and dry events to get a better understanding of the cattle fecal pollution contribution. Furthermore, a baseline of cow dung samples from the surrounding area of study would help gain a better understanding of the percentage of the cattle marker present within the geographic region. A more precise interpretation would be available to the client with the submittal of such baseline samples. The client is also encouraged to conduct other DNA analytical tests such as the services mentioned above to further confirm the positive result.

DNA Analytical Method Explanation

100 ml of water was filtered through 0.45 micron membrane filters and placed on mEI agar. The samples were incubated for 24 hours. Each filter was removed, placed in a buffer and vortexed vigorously. Once the buffer was spun to pellet the bacteria, the supernatant was removed and the pellet was resuspended in a small volume of water. DNA extraction was prepared using the Qiagen DNA extraction kit, as per manufacturer's instructions.

2.0 micro-liter aliquots of purified DNA extraction were used directly as template for subsequent qPCR reactions. All assays were run on an Applied Biosystems StepOne thermal cycler under the following thermal cycling conditions: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 10 seconds and 60 °C for 1 minute. Default data collection parameters were employed. The Taqman master mix supplied by Applied Biosystems was used with the forward and reverse primers added to a final concentration of 900nM and the probe added to a final concentration of 0.250uM with a 20ul final total reaction volume.

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* “Quantification” 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* “Quantification” ID™ service targets the cattle gene biomarker in *Enterococcus hirae*.

One of the advantages of the Cow *Enterococcus* “Quantification” 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 analysis.

Real-time quantitative PCR (qPCR) adds a variant to the PCR step by inserting of a fluorescent probe within the primer set. This fluorescent probe serves as a molecular beacon for the quantification step. During each PCR cycle, real-time quantification PCR monitors the fluorescence emitted during the reaction. This is done in “real-time” during the first PCR cycles as a way to quantify the targeted gene.

The Cow Enterococcus “Quantification” ID™ service uses real-time quantification PCR to simultaneously confirm and quantify total *Enterococcus faecium*, which is used as an indicator of total *Enterococcus* loading, and the cattle gene biomarker in *E. hirae*. This PCR technology avoids the cumbersome process of distinguishing DNA bands on a gel electrophoresis apparatus. The results are presented on a computer screen and printout thus avoiding ambiguities in interpretation.

Once each targeted gene is quantified, a relative percentage can be calculated. As such, it has been hypothesized that relative levels of cattle fecal pollution can be interpreted by the proportion of the cattle gene biomarker found in *E. hirae* relative to the total population of *E. faecium* in the water sample. Nonetheless this data should serve only as a preliminary indicator of relative cattle fecal pollution in the water sample. Furthermore, the context of the samples should be taken into account when interpreting the relative percentage provided. To strengthen the validity of the results, the Cow Enterococcus “Quantification” ID™ service should also 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, Jay **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.

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