

# Occurrence of the *Enterococcus faecium* esp Genetic Marker Among Enterococci Isolated from Massachusetts Municipal Raw Sewage

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## INTRODUCTION

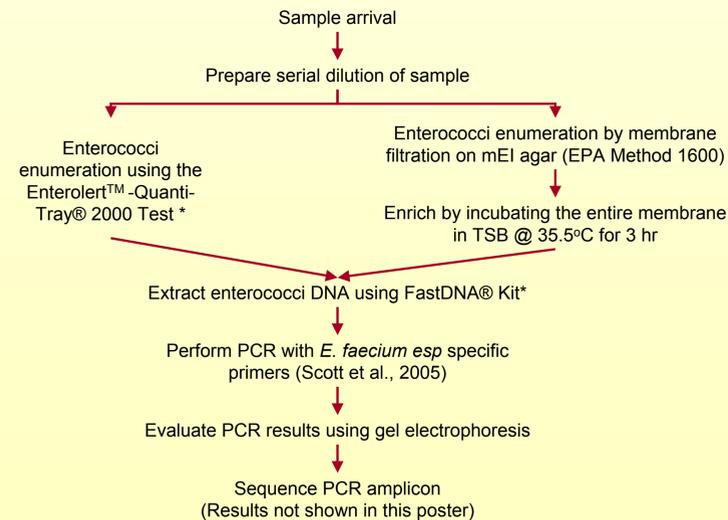
Library-independent methods for tracking microbial/fecal sources in watersheds are currently being evaluated in numerous laboratories. The PCR assay targeting the host-specific *Enterococcus faecium* exocellular surface protein (*esp*) marker is one such method that has been found by several laboratories, including ours, to be highly specific and sensitive for detecting sewage in non-blind trials. The *esp* gene was first detected in enterococci isolated from hospital patients and has subsequently been used by numerous researchers to detect putative human fecal contamination in hospital environments. However, researchers have found that the *esp* marker is not carried by every person. As a result, the *esp* marker may not be detected in sewage samples generated by smaller populations (e.g., in septic tanks serving a small household). In order to accurately interpret and use the results produced by the *esp* marker PCR assay, it is critically important to determine the sensitivity and specificity of the primer set in blind trials and the percentage of the enterococci population in raw sewage from a test area that carry the *esp* marker.

## OBJECTIVES

The goals of this study were to:

- Determine the *esp* gene PCR assay sensitivity and specificity for sewage in blind trials.
- Determine the detection limit of the *esp* marker in raw sewage samples from several Massachusetts municipal wastewater treatment plants of varying daily flow.
- Evaluate the feasibility of *esp* gene DNA recovery from Enterolert™-Quanti-Tray®-2000\* cultures.
- Determine the occurrence of the *esp* gene in enterococci isolates obtained using membrane filtration (EPA Method 1600) from 24-hr raw sewage composite samples taken from a 52 million-gallons/day Massachusetts municipal treatment plant during dry weather and thereby assess the potential *esp* marker false negative rate for raw sewage from a mid-size plant.

## METHODOLOGY



## REFERENCES

- 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.
- Whitman, R. L., K. Przybyla-Kelly, D. A. Shively, and M. N. Byappanahalli. 2007. Incidence of the enterococcal surface protein (*esp*) gene in human and animal fecal sources. *Environ. Sci. Technol.* 41:6090-6095.

## EXPERIMENTAL DATA

**Table 1.** Validation of *esp* marker PCR assay with known fecal sources and comparison with results from other investigators<sup>1</sup>

Fecal Source	This Study	Scott et al. <sup>2</sup>	Whitman et al. <sup>3</sup>
Cat	0/17	---	0/34
Chicken (composite)	0/4	0/6	---
Cow	0/15	0/32 (feces/lagoon)	---
Deer	---	---	0/4
Dog	3/19	---	9/43
Goat	0/7	---	---
Goose (composite)	0/1	0/12	0/18
Horse	0/8	---	---
Human	0/16 (feces)	8/10 (septic tanks)	6/20 (septic tanks) 0/15 (toilets)
Mouse	---	---	0/22
Pig	0/2 (feces)	0/9 (feces/lagoon)	---
Pigeon (composite)	0/3	---	---
Raccoon	---	---	0/23
Seagull (composite)	0/26	0/28	2/34
Sheep	1/9	---	---
Songbird	---	---	0/55
Wastewater	8/10	55/55	27/29
Wild birds	---	0/8	---

<sup>1</sup> Positive for the marker / total number of samples tested

<sup>2</sup> Adapted from Scott et al., 2005

<sup>3</sup> Adapted from Whitman et al., 2007

**Table 2.** Single- and double-blind challenges of the *esp* marker PCR assay.

Sample ID <sup>1</sup>	<i>esp</i> Marker	Sample Content
Blind 01	-	Diluted individual human feces
Blind 02	-	Diluted individual cat feces
Blind 03	-	Diluted individual dog feces
Blind 04	-	Diluted individual human feces
Blind 05	-	Diluted individual human feces
Blind 06	-	Diluted individual dog feces
Blind 07	-	Diluted individual seagull feces
Blind 08	-	Diluted individual goose feces
Blind 09	-	Diluted individual human feces
Blind 10	-	Field blank (sterile buffered water)
Sample 01	+	Diluted (1:50) raw sewage
Sample 02	-	Diluted individual dog feces
Sample 03	+	Diluted (1:50) raw sewage
Sample 04	-	Diluted goose feces
Sample 05	+	Diluted individual dog feces
Sample 06	-	Diluted goose feces
Sample 07	-	Field blank (sterile buffered water)
Sample 08	-	Field blank (sterile buffered water)
Sample 09	-	Field blank (sterile buffered water)
Sample 10	-	Field blank (sterile buffered water)
Sample 11	-	Field blank (sterile buffered water)
Sample 12	-	Field blank (sterile buffered water)
Sample 13	-	Diluted (1:50) raw sewage
Sample 14	-	Diluted (1:50) raw sewage
Sample 15	+	Diluted (1:50) raw sewage
Sample 16	-	Field blank (sterile buffered water)

<sup>1</sup> Blind samples consisted of sterile buffered water submitted as a blank or spiked with diluted sewage or diluted individual human/animal feces; single-blind samples were labeled as "Blind" samples when submitted to our laboratory while double-blind samples were submitted labeled as routine field samples with Sample Field ID.

**Table 3.** Detection limit of the *E. faecium* *esp* sewage marker in Massachusetts municipal raw sewage samples.

Wastewater Treatment Plant	Average Daily Flow (mgd)	<i>E. coli</i> (10 <sup>6</sup> CFU /100 mL)	Fecal Coliform (10 <sup>6</sup> CFU /100 mL)	Enterococci (10 <sup>6</sup> CFU /100 mL)	Enterococci CFU & <i>esp</i> Marker Results for Sewage Volume Assayed by EPA Method 1600 & PCR <sup>1</sup>					
					1 mL	0.1 mL	0.01 mL	0.001 mL	0.0001 mL	0.00001 mL
Grafton	2.4	7.0	6.9	2.4	TNTC	TNTC	TNTC	24	1	0
Newburyport <sup>2</sup>	3.4	4.7	5.3	1.3	TNTC	TNTC	139	5	4	0
Lowell	32	4.8	6.9	1.3	TNTC	TNTC	126	17	2	0
Greater Lawrence Sanitary District	52	1.5	Not Tested	1.1	TNTC	TNTC	62	11	1	0
Upper Blackstone	56	6.2	6.3	2.8	TNTC	TNTC	28	3	0	0
MWRA Deer Island (Charles River) <sup>2</sup>	1270	4.4	4.4	0.56	TNTC	TNTC	56	11	2	0

Wastewater Treatment Plant	Average Daily Flow (mgd)	<i>E. coli</i> (10 <sup>6</sup> CFU /100mL)	Fecal Coliform (10 <sup>6</sup> CFU /100mL)	Enterococci (10 <sup>6</sup> MPN /100mL)	Enterococci MPN & <i>esp</i> Marker Results for Sewage Volume Assayed by Enterolert™ Quanti-Tray® Test & PCR <sup>1</sup>					
					1 mL	0.1 mL	0.01 mL	0.001 mL	0.0001 mL	0.00001 mL
Greater Lawrence Sanitary District	52	1.2	Not Tested	0.37	> 2419	373	39	4	0	0

<sup>1</sup> "+", *esp* marker detected; "-", *esp* marker not detected; "NT", not tested (no bacterial DNA available for extraction).

<sup>2</sup> Sample collected following a rainstorm.

**Table 4.** Percentage of *esp* marker positive enterococci isolated from 24-hr composite raw sewage samples from the Greater Lawrence Sanitary District Wastewater Treatment Plant.

Sampling Date	2/5/2007	3/22/2007	11/29/2007	1/29/2008
Enterococci (CFU/100mL)	1,100,000	600,000	940,000	1,200,000
Total # of Isolates	41	63	93	77
# of Isolates <i>esp</i> positive	9	7	29	47
% of Isolates <i>esp</i> positive	22	11	31	61

\* Use of trade or firm names in this poster is for identification purposes only and does not constitute endorsement by MassDEP.

## SAMPLES TESTED

- Scat samples**
  - Cat (individuals)
  - Chicken (composites)
  - Cow (individuals)
  - Dog (individuals)
  - Goat (individuals)
  - Goose (composite)
  - Horse (individuals)
  - Human (individual fecal swabs)
  - Pig (individuals)
  - Pigeon (composites)
  - Seagull (composites)
  - Sheep (individuals)
- Massachusetts Wastewater Samples**
  - Grafton Wastewater Treatment Plant
  - Newburyport Wastewater Treatment Plant
  - Lowell Wastewater Treatment Plant
  - Greater Lawrence Sanitary District Treatment Plant
  - Upper Blackstone Wastewater Treatment Plant
  - Massachusetts Water Resources Authority (MWRA) Deer Island Sewage Treatment Plant
  - Wastewater Collection (Sewer) Pipe in Watertown, MA
- Proficiency Test (PT) Samples**
  - Single blind
  - Double blind

## RESULTS & DISCUSSION

- For samples of known fecal sources (Table 1):
  - The *esp* marker was detected in all sewage samples tested, except for one sample collected from a sewer pipe receiving raw sewage from one block of houses in Watertown (MA) and for a raw sewage sample collected from the intake of a very small municipal wastewater treatment plant.
  - The *esp* marker was not detected in individual human fecal samples; similar results were reported by Whitman et al. (2007) – these investigators did not detect the *esp* marker in samples collected from toilets.
  - The *esp* marker was not detected in non-human fecal samples, except for one sheep sample (out of 9 individual sheep) and 3 out of 19 fecal samples from individual dogs; Whitman et al. (2007) also detected the *esp* marker in some samples of dog feces.
- In 10 single-blind and 16 double-blind samples submitted to our laboratory consisting of 50-fold diluted raw municipal sewage, diluted individual human fecal samples, or diluted individual animal fecal samples, the *esp* marker was detected in (see Table 2):
  - Three of five municipal raw sewage samples;
  - None of four individual human fecal samples; and
  - Only 1 of 17 non-human/non-sewage samples (i.e., only in a sample containing diluted dog feces).
- For these 26 blind samples:
  - The sensitivity of the *esp* marker PCR assay for sewage was 60% (i.e., 40% false negative rate). Sensitivity (%) is defined as  $\frac{a}{a+c} \times 100$ , where a = # of true positives and c = # of false negatives.
  - The specificity of the *esp* marker PCR assay for sewage was 95% (i.e., 5% false positive rate). Specificity (%) is defined as  $\frac{b}{b+d} \times 100$ , where b = # of false positives and d = # of true negatives.
- As shown in Table 3, we detected the *esp* marker in serial dilutions up to 10<sup>-3</sup> of raw sewage samples collected during dry weather from several Massachusetts municipal wastewater treatment plants with average daily flows of 2.4 to 52 mgd.
- We also demonstrated that *esp* marker DNA can be recovered directly (without the need for enrichment) from Enterolert™-Quanti-Tray®-2000 cultures after 24 hours of incubation (i.e., comparable results to recovery from EPA Method 1600 membranes with enrichment) (see Table 3).
- In four 24-hour composite raw sewage samples from the Greater Lawrence Sanitary District Wastewater Treatment Plant, we found the presence of the *esp* gene in as little as 11% and as high as 61% of enterococci isolates (see Table 4), possibly indicating high variability in the percentage of enterococci carrying the *esp* gene among contributing sources on different days.
- There are potential false positives and especially false negatives when applying the *esp* marker PCR assay for sewage detection, and therefore, this method should be used in concert with other source tracking methods to more accurately identify sewage sources in watersheds.

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