FIELD-TESTING *ENTEROCOCCUS FAECALIS* FOR BACTERIAL SOURCE TRACKING

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Abstract. A waste stabilization pond was suspected of causing fecal contamination to Holt Branch, a tributary of Juniper Creek, near Byron, Georgia. We used ribotyping, a genotypic (DNA-based) bacterial source tracking (BST) method, to match *Enterococcus faecalis* isolates from Holt Branch and the waste stabilization pond. Fecal enterococci in the effluent from the waste stabilization pond averaged 13,863 colony-forming units (CFU) per 100 mL, and numbers of fecal enterococci from Holt Branch averaged 3,290 CFU per 100 mL. The 17 *Ent. faecalis* isolates from the Holt Branch yielded 4 ribotypes, and the 41 isolates obtained from the waste stabilization pond yielded 9 ribotypes. All four ribotypes from Holt Branch matched the ribotypes from the waste stabilization pond at a 100% similarity index, which suggested that the waste stabilization pond was the source of fecal contamination to Holt Branch. This is the first use of *Ent. faecalis* for BST.

INTRODUCTION

A waste stabilization pond located in Byron, Georgia, was suspected as a source of fecal contamination of Holt Branch, a tributary of Juniper Creek. The Georgia Environmental Protection Agency requested that this suspected contamination be confirmed because Juniper Creek supplied water to a pond used by the Boy Scouts of America for swimming. Because the flow of Holt Branch was solely dependent on the effluent from the waste stabilization pond, the situation provided conditions ideal to field-test *Ent. faecalis,* a fecal indicator bacterium, for BST.

BACKGROUND AND RELATED WORK

In most BST methods, environmental isolates of a fecal bacterium are matched to isolates of the same bacterium obtained from various warm-blooded animals either phenotypically (based on characteristics expressed by the bacterium, like antibiotic resistance; e.g., Wiggins et al., 1999) or genotypically (e.g., Parveen et al., 1999). By matching the two, the host origin of the environmental isolates can be determined.

One BST method is ribotyping (Farber, 1996). This method is based on identifying highly conserved sections of DNA (not mutated readily) that encode for ribosomal RNA. Ribotyping yields banding patterns (ribotypes) that can be compared to each other for similarity. As a method for BST, it has excellent reproducibility, good discriminatory power, excellent ease of interpretation, and good ease of performance. However, manual ribotyping is tedious and time consuming. It is possible to automate the process with a RiboPrinter (DuPont Qualicon, Wilmington, DE). This instrument increases the accuracy and precision of ribotyping while making the method quicker and simpler.

One of the most important objectives of BST is to distinguish between human and other sources of fecal contamination. Water managers are interested in this because human feces are commonly presumed to be the reservoir of many human pathogens. Also, compared to other sources of fecal contamination (e.g., wildlife), human sources are relatively easy to fix and remedy. One bacterium that may be linked to humans and not to many other warm-blooded animals is *Ent. faecalis* (Pourcher et al., 1991; Wheeler et al., 2002).

EXPERIMENTAL DESIGN

If the *Ent. faecalis* ribotypes from Holt Branch matched *Ent. faecalis* ribotypes from the waste stabilization pond, then the hypothesis that the waste stabilization pond was a source of human fecal contamination to Holt Branch is supported.

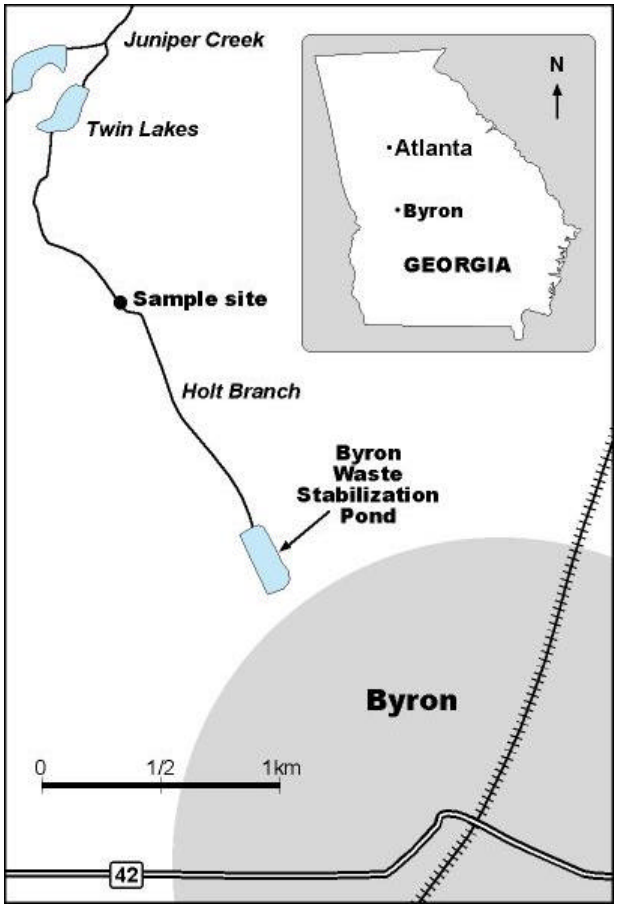

Fig. 1. Location of the sampling site and the waste stabilization pond near Bryon, GA.

METHODS

The Byron waste stabilization pond is approximately 3 ha in size and receives 1.6 ML of wastewater per day with a typical retention time of 42 days (M. Phipps, personal communication, 2002). On 5 Mar 2002, three replicate water samples were obtained from the effluent pipe of the pond and a sampling location 1 km downstream on Holt Branch (Fig. 1). Samples were collected in sterile, 500-mL bottles and were placed on ice in a cooler for transport to Athens, Georgia. The samples were analyzed within 6 hours.

The method to identify fecal contamination in the effluent and water samples was the Enterolert system (IDEXX, Westbrook, ME). This system estimates the most-probable-number (MPN) of fecal enterococci, which are recognized by the American Public Health Association as reliable indicators of fecal contamination (Clesceri et al., 1998). The Enterolert system uses defined substrate technology with the nutrient indicator substrate, 4-methylumbelliferyl-β-D-glucoside, which fluoresces when metabolized by the enterococci. A 100-mL sample of water or effluent was poured from each sample bottle into a separate sterile polystyrene bottle. In addition, a 100-fold dilution of each sample was obtained by adding 1 mL of water or effluent to a sterile polystyrene bottle containing 99 mL of sterile water. A package of powdered Enterolert reagent was added to each polystyrene bottle and, after the reagent was dissolved, the contents were added to a Quanti-tray, a sterile disposable panel containing 97 wells. Each Quanti-tray was sealed and was placed in triplicate 1-quart Ziploc bags. The Quanti-trays were incubated submerged in a water bath at 41±0.2 °C. After 24 hours, fluorescing (positive) wells were counted under 365-nm UV light and the number of positive wells converted to an MPN using manufacturer-supplied tables.

To speciate the fecal enterococci, all positive wells in each Quanti-Tray were marked with an acetate marker. The back surface of the Quanti-tray was disinfected with 70% ethanol, and each positive well was slit open with a sterile scalpel. A 10-µL portion was removed with a sterile plastic loop and was streaked onto a 5-cm plate of Enterococcosel agar (Becton Dickinson and Company, Sparks, MD). A total of 116 wells from the waste stabilization pond and 146 wells from Holt Branch were streaked. Plates were incubated in Ziploc bags at 37 °C. After 48 hours, all plates had black colonies (indicating esculin hydrolysis). A single well-isolated colony was picked from each Enterococcosel plate with a sterile plastic stab and was suspended in 125 µL of saline-phosphate buffer contained in a well of a 96-well microtiter plate. Three wells of the 96-well plate were reserved for American Type Culture Collection (ATCC, Manassas, VA) controls, *Ent. faecalis* ATCC #19433, *Ent. faecium* ATCC #19434, and *Ent. gallinarum* ATCC #49573, and three wells were reserved for randomly placed uninoculated controls. Each isolate was inoculated with a replicator (Sigma, St. Louis, MO) into separate microtiter plates containing Brain Heart Infusion (BHI) broth (Difco Laboratories, Sparks, MD) with 6.5% NaCl, arginine hydrolysis medium with and without arginine, and modified pyruvate, arabinose, and raffinose carbon utilization media as described by Wheeler et al. (2002). Plates were incubated at 37 °C and reactions were recorded after 24 and 48 h. A catalase test with

Table 1. Enterococcal isolates obtained from the Holt Branch of Juniper Creek and a waste stabilization pond near Byron, GA

Source	Total Isolates	*Ent. faecalis*	*Ent. faecium*	*Ent. gallinarum*	Other Enterococci	Non Enterococci
	number	----------number (percent)----------				number
Waste stabilization pond	116	41 (35)	41 (35)	9 (8)	24 (21)	1
Holt Branch	146	17 (12)	57 (40)	45 (31)	24 (17)	3
Total	262	58 (22)	98 (38)	54 (21)	48 (19)	4

8.82 *M* H_20_2 was performed in the remaining saline-phosphate cell suspension to ensure each isolate was catalase negative. For BST, each isolate identified as *Ent. faecalis* was processed on a RiboPrinter. Briefly, each *Ent. faecalis* isolate was streaked onto BHI agar (Difco) and incubated at 37 °C for 24 hours.

Each sample was obtained by touching the end of a sterile, colony pick to a solid lawn of growth, and the sample was mixed with 40 µL of sample buffer. This step was repeated once before 30 µL of the buffer was transferred to a sample carrier. After the samples were inactivated in a heat treatment station, 5 µL each of two lysing agents were added to each sample and the sample carrier was placed into the RiboPrinter system. The DNA of each sample was digested with the restriction enzyme *Pvu*II. The DNA fragments were size-separated by electrophoresis on a precast agarose gel and were transferred to a nylon membrane. In order for the DNA fragments to chemiluminesce, the membrane was exposed to a series of chemical and enzymatic treatments. The banding pattern image created was captured by a CCD camera and stored as a TXT file. The files were imported into GelCompar II (Version 3.0, Applied Maths, Kortrijk, Belgium) for analysis. Variations among the gels were assessed with the *Ent. faecalis* ATCC #19433 strain. Optimization (shift between any two patterns) was set at 1.56% and tolerance (maximum distance between two band positions on different patterns) was set at 1.00%. Both optimization and tolerance settings were default settings for the software program. Similarity indices were determined using Dice's coincidence index and the distance among clusters calculated using the unweighted pair-group method using arithmetic averages (UPGMA). Banding patterns had to be 100% similar, a perfect match, to be considered the same ribotype.

CONCLUSIONS

The numbers of fecal enterococci in the effluent from the Bryon waste stabilization pond averaged 13,863 CFU per 100 mL; the numbers of fecal enterococci in the water from Holt Branch averaged 3,290 per 100 mL. Of 116 fecal enterococcal isolates obtained from the Bryon waste stabilization pond, 41 (35%) were identified as *Ent. faecalis*; of 146 isolates from Holt Branch, 17 (12%) were identified as *Ent. faecalis* (Table 1). The numbers and percentages of *Ent. faecium*, other enterococci, and non-enterococci in the two sources were similar, with the exception of higher numbers of *Ent. gallinarum* in the Holt Branch (45 isolates for 31%).

The 41 *Ent. faecalis* isolates from the waste stabilization pond yielded 9 ribotypes, whereas the 17 isolates from the Holt Branch yielded 4 ribotypes. The ribotypes from the Holt Branch all matched the ribotypes from the waste stabilization pond at a 100% similarity index (Fig. 2).

DISCUSSION

This is the first field test of *Ent. faecalis* for BST. Ribotyping was not strictly necessary because the waste stabilization pond was the sole source of water to Holt Branch. Nevertheless, this provided ideal conditions to test *Ent. faecalis* for BST. The ribotypes of the 17 isolates from Holt Branch perfectly matched the ribotypes of from the waste stabilization pond. Although a potential source of *Ent. faecalis* is birds (Wheeler et al., 2002), given the limited loading rate of birds and that subspecies of a specific bacterium are associated with specific animal species (Amor et al., 2000), it is unlikely that birds were the host origin for the

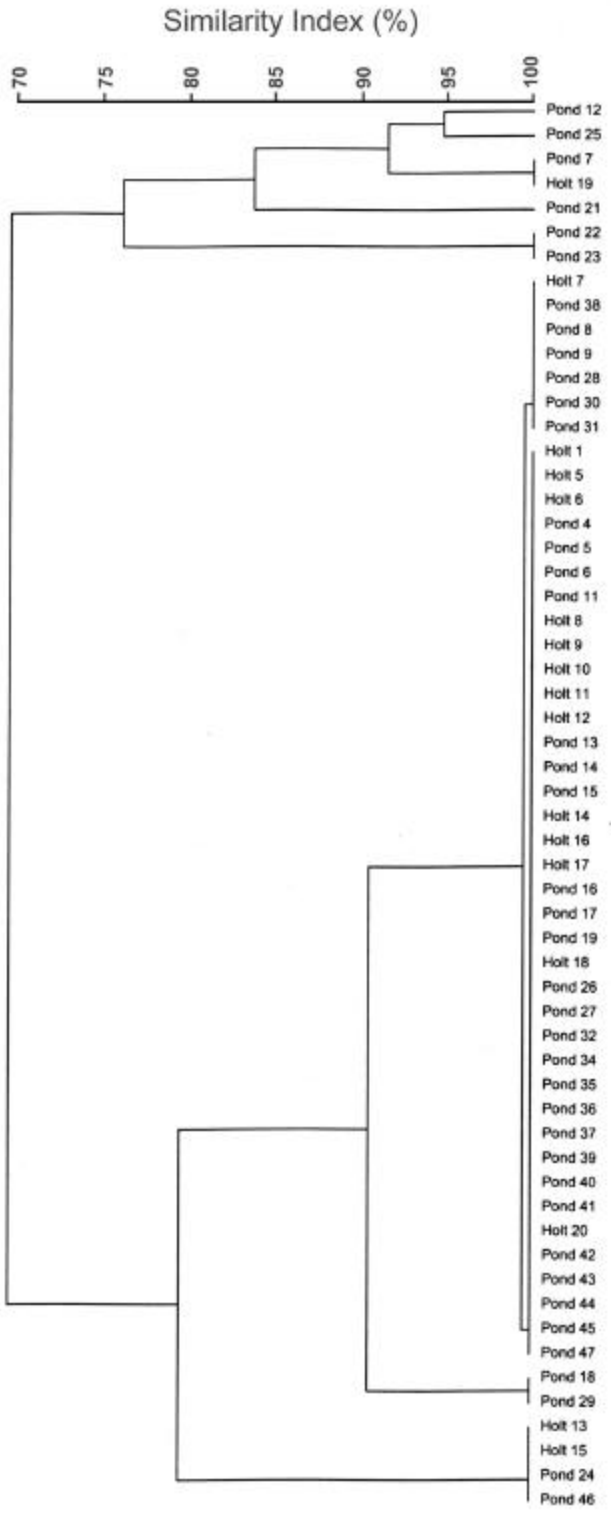

Fig. 2. Dendrogram of the ribotypes of 41 *Ent. faecalis* isolates from the waste stabilization point and 17 *Ent. faecalis* isolates from Holt Branch. The similarity index is given on the top scale.

matched fecal enterococci in the stream. Therefore, the *Ent. faecalis* isolates in the Holt Branch came from the waste stabilization pond. This is the first use of the RiboPrinter with *Ent. faecalis* for BST. Although the RiboPrinter was expensive to operate ($37 per isolate), this was offset by its increased precision and accuracy.

The standard for fecal enterococci in recreational freshwater is 33 per 100 mL (Clesceri et al., 1998). Thus, Holt Branch (3,290 fecal enterococci per 100 mL) was grossly contaminated. Because Georgia has 167 waste stabilization ponds (M. Phipps, personal communication, 2002), this suggests that the other streams that receive effluent from these ponds be checked to avoid similar contamination problems.

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