COMPARISON OF THE TEMPORAL VARIABILITY OF ENTEROCOCCAL CLUSTERS IN IMPACTED STREAMS USING A MULTIPLEX POLYMERASE CHAIN REACTION PROCEDURE

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Abstract. Understanding how fecal indicator bacteria and/or fecal indicator genotypes vary over time is important to determine the sources of fecal contamination. *Enterococcus* is one of the two indicators recommended by the EPA to monitor freshwaters for fecal contamination. Along with *Escherichia coli,* it has been used by a number of researchers to infer sources of fecal contamination, an area identified as microbial source tracking (MST). Our objective in this study was to identify changes in the seasonal distribution of enterococcal populations in streams directly impacted by cattle farming. The sites under study are located in Madison County, Ga., in farms where cows have unrestricted access to first order streams. Enterococci were counted and isolated monthly from water samples using membrane filtration. The isolates were identified using a multiplex PCR procedure. From a total of nine species identified in stream samples, only the most frequently observed species (*E. faecalis, E. casseliflavus, E. flavescens, E. faecium and E. hirae)* were used to develop groupings of enterococcal populations via cluster analysis. This analysis revealed that *E. casseliflavus* and *E. faecalis* dominated the enterococcal community during spring and fall, respectively. The cluster dominated by *E. faecium* seemed to increase during winter. This study indicates that enterococcal communities exhibit seasonal variability; and suggests that cluster analysis is a robust approach to identify this variability. In conclusion, to determine the true impact of certain farming operations on stream water quality using enterococcal species as indicators, it is important to consider the temporal variability of key enterococcal communities.

INTRODUCTION

For microbial source tracking (MST) methodology to be effective, the source indicator bacteria selected should be part of the resident population of the source species. In addition, it should be part of a clonal population that is stable through time as suggested by Gordon (2001) for *Escherichia coli*. Jenkins et al. (2003) found a rather low percentage of *Escherichia coli* ribotypes to be part of the resident population in yearling steers sampled four times over a 129-day period. Specifically, only 8.3% of 240 ribotypes were determined to be resident in the host species. In addition, no ribotype was found at all four sampling times or in all of the steers sampled from a total of 20 resident ribotypes. These results suggest that there is a high probability that the majority of ribotypes obtained from a single host species at any given time belong to transient populations. This variability should reflect on the variability of the indicators recovered from environmental samples. Establishment of host origin libraries could require continuous updating in order for a particular MST methodology to be able to track the host species (Jenkins et al. 2003) over an extended period of time in complex environmental matrices such as stream water and sediments. Moreover, once the indicator organisms are present in the environment, the lack of information on their temporal persistence may highly confound data interpretation.

In this study, we sampled stream water monthly for over a year at two separate farms where cattle have direct access to the streams. We also included stream locations upstream from the farms. The objectives of the study were to: 1) determine the temporal variability of species of *Enterococcus* sp. in stream water samples; and 2) evaluate the use of a community analysis approach vs. an individual species approach to identify seasonal enterococcal species persistence in stream water.

STUDY AREA

The South Fork Broad River (SFBR) is a 635.01 square kilometer (156,915 acres) tributary of the Broad River Watershed located in the Savannah River Basin. The Savannah River Basin is located along the border of Georgia, South Carolina and North Carolina. Cattle and poultry operations with some row crops are the major agricultural activities in this watershed. SFBR is listed on

the state of Georgia's 303(d) list for impaired waters, with pathogens being one of the top causes of contamination. Runoff is the major mode of transport for pollutants entering streams in this area. The runoff problems in the Broad River are attributed to effluent from septic systems, landfill leachate, riverbank erosion, destruction of the vegetative buffer, lack of tributary protection and, more importantly, non-point source agricultural practices. Livestock drinking water directly from the river contribute to water pollution, sedimentation, degradation and destabilization of riverbanks (USEPA, 2002).

METHODS

Water samples were collected from headwater streams crossing two beef cattle farms (Farm A and Farm B) located in Madison County, Northeast Georgia. These streams are tributaries of the SFBR. Seven sampling locations were located along the stream within each farm covering a distance of 2.3 and 0.85 km in Farm A and Farm B, respectively. One liter of water was collected at each location once a month from September 2003 through February 2005.

A modification of EPA Method 1600 was used to count, isolate and verify enterococci from the environmental samples. Briefly, 1, 5, 10, and 50 ml of stream water was filtered through 0.45um cellulose membranes and incubated on membrane-*Enterococcus* Indoxyl –β-D-Glucoside (mEI) agar plates at 41 ± 0.5˚C for 24 hours. After incubation, all colonies having a blue halo were considered to be presumptive enterococci. Five colonies from each location were isolated on brain-heart infusion agar (BHIA) slants.

All polymerase chain reactions (PCR) were conducted using currently established EPA Quality Assurance/ Quality Control guidelines. Speciation of enterococci isolated from the stream water samples was performed using a multiplex PCR procedure based on the superoxide dismutase gene as described by Jackson et al. (2004). All isolates were verified as Enterococcus using the PCR procedure by including a genus 16S-marker in each reaction. Whole cell templates were prepared in molecular grade sterilized water. These templates were used for up to three weeks. Seven master mixes were used to identify up to 23 species of *Enterococcus.* The procedure was performed testing the isolates with the master mixes in the following order: 1, 2, 6, 4, 3, 5, and 7. The majority of the isolates could be speciated by applying only the first three master mixes in the sequence, thereby achieving the best use of resources and the most time and cost effective approach. PCR products were separated and identified using a 2% 1X TAE agarose gel containing 2 µg/ ml ethidium bromide. Gel analysis was performed using a EpiChemi Darkroom BioImaging System (UVP, Inc.) equipped with a transilluminator, and fitted with Labworks 4.5 software. Band sizes were identified by comparing the sample DNA to the positive controls included with each run, and by comparing the band size to a 100 bp DNA ladder.

Statistical Analysis. A hierarchical cluster analysis was performed (using Minitab v.12 statistical software) on the isolates taken from upstream-of-the-farm stream water, and on-the-farm stream water. The objective of this analysis was to group together samples that showed similar relative abundances of the most common species of *Enterococcus*. Enterococcal species that were found in only a few of the samples were not included in the analysis. The following five species were seen frequently enough to be included: *E. faecalis, E. casseliflavus, E. flavescens, E. faecium* and *E. hirae.* Non-frequent species were collapsed into a category called "other". "Entero" was used for isolates for which the species could not be determined. We used the cluster designations for each of the samples to perform tests for differences in the clusters found for upstream-of-the-farm stream water, and on-the-farm stream water as well as changes in the seasonal occurrence of the clusters.

RESULTS AND DISCUSSION

Enterococcal abundance exhibited high variability throughout the year in both farms at the upstream and within-the-farm sites. Enterococcal counts in the farm were up to an order of magnitude higher than at the upstream sites. In Farm A, the average enterococcal abundance at the cattle crossing site was 1994.5 ±1541 CFU/100ml and at Farm B, it was 922.7 ± 610 CFU/100ml, while the numbers were 625.5 ± 899 and 118.9 ± 148 CFU/100 ml at the upstream sites for Farm A and B, respectively. The difference between the two types of sampling locations was larger in Farm A than in Farm B, which suggest that even though both upstream locations were located at the stream headwaters, the stream in Farm A seems to be impacted by an additional input of contamination. Because wildlife is expected to affect both streams, it is possible that contamination from some chicken houses located at the top of the stream A sub-basin, in fairly close proximity to the water, was affecting the water quality. Regardless of the high monthly variability, the enterococcal counts through out the year in the farm sites were higher than the GA limit for fecal enterococci which is either a geometric mean of

Table 1. Average frequency (%) of enterococcal species isolated from seven locations in streams under direct cattle impact.

Farm	Enterococcus species						
	casselifl.	hirae	flavesc.	faecalis	faecium	Entero	Other
A	17.4	9.9	18.5	37.7	*	*	11.2
B	25.1	5.6	*	56.1	10.7	*	2.5

* Collapsed into the "other" category due to a low number of individual observations.

33 CFU per 100 ml for freshwater or 104 CFU per 100 ml for a grab sample. Moreover, the water leaving the farms had enterococcal densities that were from 1.5 to 20 times higher than the enterococcal density in the stream water entering the farm. The upstream site in Farm B was above the maximum allowable density only four times through out the sampling period with the highest densities being 340 and 440 CFU/100 ml, for the months of April 2004 and January 2005, respectively. Reduced water quality on these farms was expected considering that the cattle have unrestricted access to the stream at all times and therefore, the streams are receiving direct fecal inputs. The results also indicate the high possible level of impact that this type of cattle farming operation may have on downstream locations.

A total of nine enterococcal species were identified in the stream water samples from both farms. These included *E. casseliflavus, E. faecalis, E. faecium, E. flavescens, E. hirae, E. mundtii*, *E. sulfureous*, *E. gallinarum* and *durans*. The first five species were present through out the year in most of the stations sampled. In contrast, the last four species were observed sparingly through out the year. *E. faecalis* and *E. casseliflavus* were the most abundant species identified in both farms with abundances of 38 and 35 %, respectively in Farm A and 56 and 25%, respectively in Farm B (Table 1). In Farm A, 35% reflects the combination of *E. casseliflavus* and *E. flavescens* because these two species have been reclassified as being the same species (Gilmore 2002). The next most abundant species were *E. hirae* (10%) in Farm A and *E. faecium* (11%) in Farm B (Table 1). Analysis of the combined data indicated that However, when the data were examined from month to month, most species did not show strong temporal trends, but rather randomness. The only significant temporal, month to month trend was observed in Farm B with *E. faecalis*, which showed a high frequency from April to November, and very low frequency from December to March. In Farm A, a low occurrence of *E. faecium* and *E. hirae* was observed during the summer.

Because of the high variability observed at the individual species level, the data were combined to determine whether a community approach (via cluster analysis) showed stronger seasonal trends. This analysis produced eight clusters of enterococcal communities. Although all eight clusters were present throughout the year, the relative proportions of some clusters were significantly higher during certain seasons. Cluster 1 was the only cluster significantly higher during winter (25%) than during two other seasons (Figure 1). Cluster 2 was significantly higher during spring (32%) when compared to fall, while Cluster 6 exhibited the inverse relationship (higher during fall than during spring), Cluster 7 was also higher during fall. Cluster 4 was higher during summer (29%) than during spring, while Cluster 5 was higher during spring (42%) than during summer. All other clusters were not significantly different through out the different seasons (Figure 1).

Given the cluster's species compositions, it can be concluded that clusters with high proportions of *E. casseliflavus* and *E. faecalis* dominated the enterococcal community during spring and fall, respectively. There is certain degree of overlap during summer where both *E. casseliflavus*- and *E. faecalis*- dominated clusters were in high abundance. During winter, despite a high degree of cluster overlap, the cluster dominated by *E. faecium* seems to increase. Nevertheless, because each cluster represents a community, the four other species of enterococci will also be present at all times at various relative proportions. Statistical analysis of the distribution of individual species resulted in an interpretation of randomness rather than significant differences. In contrast, cluster analysis seems to be a good approach to identify groups of enterococcal communities that are characteristic of a season or even a location and suggests that a community fingerprint as opposed to individual species information could be an efficacious approach to trace back fecal contamination. The changing temporal distributions of enterococcal clusters/communities has implications for the type of indicators that water quality managers should monitor when drawing conclusions about possible agricultural sources of contamination across different seasons.

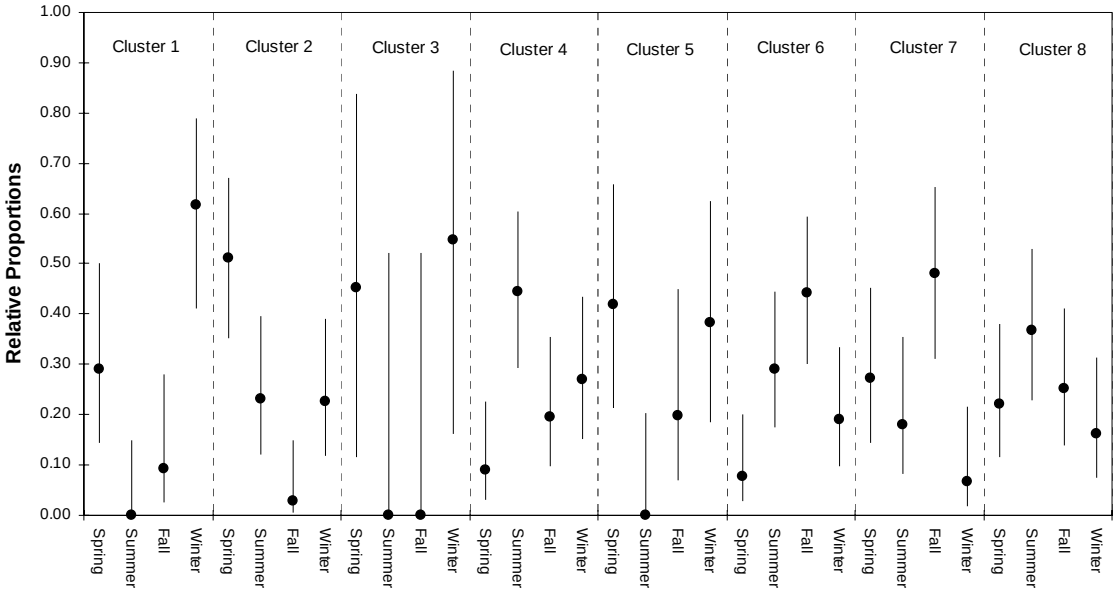

Figure 1. Seasonal trends of the occurrence of enterococcal clusters in samples collected from streams directly impacted by cattle contamination. Within each of the eight clusters, the occurrence in spring, summer, fall, and winter were compared using relative proportions. The mean relative proportion in each season is indicated by a dot, and the vertical lines indicate the 90% confidence interval for that mean.

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