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Use of Barcoded Pyrosequencing and Shared OTUs To Determine Sources of Fecal Bacteria in Watersheds

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While many current microbial source tracking (MST) methods rely on the use of specific molecular marker genes to identify sources of fecal contamination, these methods often fail to determine all point and nonpoint contributors of fecal inputs into waterways. In this study, we developed a new library-dependent MST method that uses pyrosequencing-derived shared operational taxonomy units (OTUs) to define sources of fecal contamination in waterways. A total 56,841 pyrosequencing reads of 16S rDNA obtained from the feces of humans and animals were evaluated and used to compare fecal microbial diversity in three freshwater samples obtained from the Yeongsan river basin in Jeonnam Province, South Korea. Sites included an urbanized agricultural area (Y1) (*Escherichia coli* counts ≥1600 CFU/100 mL), an open area (Y2) with no major industrial activities (940 CFU/100 mL), and a typical agricultural area (Y3) (≥1600 CFU/100 mL). Data analyses indicated that the majority of bacteria in the feces of humans and domesticated animals were comprised of members of the phyla *Bacteroidetes* or *Firmicutes*, whereas the majority of bacteria in wild goose feces and freshwater samples were classified to the phylum *Proteobacteria*. Analysis of OTUs shared between the fecal and environmental samples suggested that the potential sources of the fecal contamination at the sites were of human and swine origin. Quantification of fecal contamination was also examined by comparing the density of pyrosequencing reads in each fecal sample within shared OTUs. Taken together, our results indicated that analysis of shared OTUs derived from barcoded pyrosequencing reads provide the necessary resolution and discrimination to be useful as a next generation platform for microbial source tracking studies.

Introduction

According to the World Health Organization (WHO), diarrhea kills over 2 million people globally each year, and fecal contamination of water is one of the major contributing factors to disease incidence. Determining the sources of fecal contaminants in waterways using microbial source tracking methodologies has attracted much attention in recent years. However, current technologies often fail to consistently identify fecal contaminants in watersheds, prompting development of new and more sensitive methods (*1, 2*).

The application of 16S rDNA as a genetic marker for microbial source tracking has received recent attention, and markers purportedly specific for fecal bacteria (mainly *Bacteroidales*) originating from humans, bovine, and other animals have been developed and evaluated (*3*−*5*). Recently, however, the use of these marker genes has been questioned since there were large discrepancies in the performance of primers across different bovine populations, the lowest specificity was about 47%, and marker genes for all potential contamination sources are not available. In addition, several of the markers have been reported to have low sensitivity when used in quantitative PCR (qPCR) assays with environmental DNA (*6*), and there is generally a lack of correlation between the numbers of *Bacteriodales* present and counts of fecal indicator bacteria.

Ley et al. (*7*) used a 16S rDNA sequence library approach to examine how diet and phylogeny influences bacterial diversity. They reported that microbial diversity increases from carnivory to omnivory to herbivory, and these results indirectly support the contention that there is significant discrepancy in diversity and types of fecal bacteria in humans (omnivores) and livestock animals (herbivores). To date, several other studies have reported the use of culture-based 16S rDNA libraries to identify host specific microbial species in other animals, including *C. marimammalium* in gull feces (*8*), and *Lactobacillus sobrius/amylovorus* in pig manure (*9*).

While some of the identified marker genes hold great promise for assessing sources of fecal bacteria or feces in the environment, others have not proven useful due to issues of lack of sensitivity and cross-reactivity. This may in part be due to our lack of understanding of the broad microbial diversity present in intestinal environments, which is due, in part, to inherent deficiencies in using traditional culture based 16S rDNA library approaches. Studies of microbial diversity using reassociation kinetics for bacterial community DNA from pristine soils revealed that bacterial species diversity is more than 2 orders of magnitude greater than estimates based on using traditional culture dependent method (*10, 11*).

Pyrosequencing, a next generation sequencing technology that determines DNA sequences by using a "sequencing by synthesis" approach, has revolutionized the study of microbial diversity (*12*). While the first pyrosequencers, such as the Roche 454 FLX system, only produced average read-lengths of 100 bp, more recent advances have increased read lengths to about 400 bp, with considerable cost reduction (*13*). Pyrosequencing has been further improved by allowing for the simultaneous analysis of 1544 samples by using error-correcting barcoded primers (*14*). Short 16S rDNA pyrosequencing reads have proven useful for microbial community analysis (*15*) and has been widely applied to study microbial

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