WHO'S RESPONSIBLE: FECAL SOURCE TRACKING WITH *BACTEROIDES*

Katharine Field (kate.field@orst.edu), Assistant Professor, Department of Microbiology, Oregon State University, 220 Nash Hall, Corvallis, Oregon 97331, Phone: (541) 737-1837; **T.J. Brodeur, L.K. Dick, M. Simonich, T.E. Jones, Jr. and C. Bracken**, Oregon State University; **A.E. Bernhard**, University of Washington

Introduction

Standard methods of measuring fecal pollution in water do not distinguish its source. Non-point sources such as manure from dairy pastures, failing septic systems, and overloads at sewage treatment facilities may all contribute to fecal pollution problems. Coliforms, the traditional indicators of fecal pollution, have the advantage of being easy to grow, but coliform species are common to many hosts and thus do not identify the source of the pollution.

Several methods of discriminating the source of fecal pollution have been proposed (e.g.see 4, 5, 7, 8, 10), but each has drawbacks. Often the level of correct identification is unacceptably low, or the methods require lengthy collection and culturing steps and large numbers of reference strains for comparison.

Our purpose was to develop an alternative indicator for fecal source tracking, based on molecular genetic markers. Ideally, such a method would be quick and reliable, would not require culturing bacterial isolates, would not require a library of reference strains, would be flexible for easy field handling and storage of samples, and would require a minimum of specialized equipment.

We chose the fecal anaerobic genus *Bacteroides* as an indicator because this group, common to all warm-blooded animals, contains far more genetic diversity than coliform bacteria, and *Bacteroides* species have been reported to show host-specific distributions. Up to a third of fecal bacteria are in the *Bacteroides* group. Because these bacteria are restricted to gut habitats, their presence in water always indicates fecal pollution. Molecular detection circumvents the complexities of growing anaerobic bacteria.

Methods

We initially targeted human and cow feces to identify *Bacteroides* genetic markers that would distinguish between human and cow fecal pollution in water. We chose to use the small-subunit ribosomal RNA gene (16S rDNA) as a marker, because this gene is widely used in evolutionary and ecological studies of bacteria, and many gene sequences are available through international databases.

Any test for fecal pollution must first increase the indicators to a level sufficient for detection. Coliforms tests do this by growing the bacteria. To amplify our genetic markers to a detectable level, we utilized the polymerase chain reaction (PCR), a method of making many copies of a target gene sequence in a test tube. In PCR, specific gene sequences are "selected" for amplification by the use of "primers" specific for the desired sequences.

Using available gene sequences, we designed PCR primers specific for *Bacteroides* 16S rDNA (2). We extracted DNAs from fresh human and cow feces collected from a variety of locales (2). By means of our *Bacteroides*-specific PCR primers, we amplified *Bacteroides* 16S rDNA gene fragments from the human and cow fecal DNAs. We separated the amplified gene fragments by

size, using LH-PCR (9) and T-RFLP (6). These two techniques use a DNA automated sequencer and GeneScan software to discriminate between gene fragment lengths. We looked for unique host-specific patterns or sizes among the amplified fragments.

Once we had identified PCR fragments specific to cow or human feces, we analyzed fresh- and saltwater samples from the watershed of the Tillamook Estuary, Oregon, to see if these *Bacteroides* markers were present. Fecal coliforms in these samples ranged from 0 to 120 CFU/100mls. We filtered the samples through 0.2 micron nucleopore filters and extracted DNA from the filters. We amplified *Bacteroides* rDNA with specific primers as above and analyzed the fragments using LH-PCR and T-RFLP.

To verify the identities of the genetic markers, we constructed 16S rDNA clone libraries from cow and human fecal DNAs and Tillamook Bay water samples using *Bacteroides*-specific primers. We screened 192 clones from each host and sequenced clones that had the LH-PCR or T-RFLP pattern of interest. We also constructed *Bacteroides* rDNA clone libraries from Tillamook Bay water samples (3). We analyzed the resulting gene sequences and constructed phylogenetic trees.

To develop a PCR assay for identifying sources of fecal bacteria in water, we designed primers specific for each of the unique phylogenetic groups of sequences we identified (3). We established specificity and optimal annealing temperatures for all primers using plasmid DNAs from target and closely related non-target sequences as well as *Bacteroides* DNA from cultures. Specificity of the primers was further tested by amplifying fecal DNAs from target hosts and other animal species.

We tested the sensitivity of our host-specific primers using serial dilutions of cow feces or raw sewage. DNAs from each dilution were tested for the markers by PCR. We measured fecal coliforms in each dilution according to standard methods (1).

Results

We identified several *Bacteroides* 16S rDNA gene fragments that were unique to either cow or human fecal DNAs (2). LH-PCR identified a 276 base pair (bp) cow-specific fragment. T-RFLP analysis revealed cow-specific peaks at 227 and 222 bp and a 119 bp human-specific fragment.

We found six different clones in the library from human feces corresponding to the 119 bp human-specific marker. These sequences were related to, but not identical to, *B. vulgatus* or *B. uniformis.* We recovered seven clones from cow feces that produced the 227 bp size fragment. Sequence analysis of the clones corresponding to the 222 bp (T-RFLP) and the 276 bp (LH-PCR) cow-specific markers revealed that these markers represented the same sequences. We found four clones representing three different sequences that corresponded to these two markers. None of the cow-specific clones were closely related to any characterized microorganisms. They formed two distinct gene clusters within the genus *Bacteroides*.

The new PCR primers could reliably discriminate fecal pollution from cows and humans. Sensitivity of detection was similar to sensitivity of fecal coliform testing. However, we found that the cow fecal markers also occur in other ruminant species, although not in other hoofed mammals such as pigs and horses.

Our current research is focused on developing markers for more species. We have tested markers for pig, elk, dog and cat, and are developing markers for various wildlife species. All of the animal species that we have tested have contained previously-unknown *Bacteroides* strains,

pointing out how little is known about the fecal bacteria of non-humans. Interestingly, we found that although most kinds of animals have many unique *Bacteroides* types, dogs and cats share quite a few types with humans. We are also testing the parameters of this method of fecal source discrimination, to establish the best sample size, sample storage conditions, and similar variables.

Conclusions

PCR-based tracking of species-specific genetic markers from *Bacteroides* bacteria shows promise as a quick and reliable method of fecal source discrimination. Our ongoing studies in Tillamook and Coos Bay, Oregon, are using this method of fecal source tracking in conjunction with standard public health indicators. The procedure takes one or two days, does not require growing bacteria, is as sensitive as coliform-based assays, and is highly specific. This method is comparable in complexity to standard tests carried out in food safety and public health diagnostic labs, and lends itself to automation and high-throughput analysis.

References

APHA 1992. Standard Methods for the Examination of Waste and Wastewater, 18th ed. American Public Health Association, Washington.

Bernhard, A. E., and K. G. Field 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes Appl. Environ. Microbiol. 66:1587-1594.

Bernhard, A. E., and K. G. Field 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA Appl. Environ. Microbiol. 66:4571-4574.

Dombek, P. E., L. K. Johnson, S. T. Zimmerley, and M. J. Sadowsky 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources Appl. Environ. Microbiol. 66:2572-2577.

Hsu, F. C., Y. S. Shieh, J. van Duin, M. J. Beekwilder, and M. D. Sobsey 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes Appl Environ Microbiol. 61:3960-6.

Liu, W.-T., T. L. Marsh, H. Cheng, and L. J. Forney 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA Appl. Environ. Microbiol. 63:4516-4522.

Parveen, S., N. C. Hodge, R. E. Stall, S. R. Farah, and M. L. Tamplin 2001. Phenotypic and genotypic characterization of human and nonhuman *Escherichia coli* Water Res. 35:379-86.

Sinton, L. W., A. M. Donnison, and C. M. Hastie 1993. Faecal streptococci as faecal pollution indicators: a review. Part II: Sanitary significance, survival, and use New Zealand J. Marine Freshwater Res. 27:117-137.

Suzuki, M., M. S. Rappé, and S. J. Giovannoni 1998. Kinetic bias in estimation of coastal picoplankton community structure obtained by measurements of small subunit rRNA gene PCR amplicon length heterogeneity Appl. Environ. Microbiol. 64:4522-4529.

Wiggins, B. A., R. W. Andrews, R. A. Conway, C. L. Corr, E. J. Dobratz, D. P. Dougherty, J. R. Eppard, S. R. Knupp, M. C. Limjoco, J. M. Mettenburg, J. M. Rinehardt, J. Sonsino, R. L. Torrijos, and M. E. Zimmerman 1999. Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution Appl. Environ. Microbiol. 65:3483-3486.