RIBOTYPING TO DETERMINE THE SOURCE OF FECAL COLIFORM CONTAMINATION IN THREE HOUSEHOLD WELLS NEAR COCHRAN, GEORGIA

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Abstract. The Bleckley County Health Department reported that three households near Cochran, Georgia tested positive for fecal coliforms in their wells. Fecal coliforms are bacteria that are a measure of fecal contamination and are typically found in the intestines of warm-blooded animals, including humans. We were asked to isolate one bacterial species of these fecal coliforms, *Escherichia coli*, and to determine the source of the isolates by ribotyping. Ribotyping is a method to identify a subspecies of a bacterium by comparing differences in their DNA. We quantified the number of *E. coli* in water samples from one pond, two streams, a sinkhole, and three household wells and their accompanying septic systems. The pond, streams, and sinkhole are all connected. We ribotyped 51 *E. coli* isolates. Twelve different ribotypes were observed among the water sources and the household wells with their accompanying septic systems. Two ribotype patterns were observed from the septic systems, ten patterns among the pond, sinkhole, and two streams, and six patterns among the three household wells. At 100% similarity, all the ribotype patterns of *E. coli* from the household wells were associated with patterns from the pond, sinkhole, or two streams. The similarity of *E. coli* ribotype patterns from the household wells with the septic systems were only 80 and 86%. The results suggest that the point source of the *E. coli* contamination was the pond, sinkhole, or two streams, and not the septic systems.

INTRODUCTION

One of the most serious water quality problems in Georgia is the presence of pathogenic bacteria in drinking and recreational waters. The sources of these disease-causing bacteria are the feces of warm-blooded animals, typically from septic drainfields, animal wastes applied to land, and domesticated and wild animals. For this reason, every state tests their waters for the presence of pathogenic bacteria. However, because pathogenic bacteria pose a health risk to those persons who try to isolate them, people who monitor these waters test instead for *non*pathogenic "indicator" bacteria. One of the most common groups of "indicator" bacteria recognized by the American Public Health Association (Clesceri et al., 1998) is the fecal coliforms.

Before the advent of modern molecular genetics, the only way to potentially identify the host origin of a fecal coliform was to rely on the characteristics expressed by the bacterium (phenotypic characteristics like antibiotic resistance). Unfortunately, these characteristics were not sufficiently discriminatory. With the advent of molecular genetics, it is now possible to discriminate among different subtypes of the same bacterial species using DNA-based (or genotypic) methods. One of the most common genotypic methods for identifying host species is ribotyping.

Ribotyping is based on an examination of the DNA that encodes for production of ribosomal RNA (rRNA). This portion of DNA is important because it is present in all bacteria and higher organisms and does not mutate readily. For ribotyping to work, a single bacterial species must be selected. Of the four species that comprise almost all of the fecal coliforms, the best one to use for ribotyping is *E. coli* because there is good scientific evidence that specific strains of *E. coli* are associated with different host species (Faith et al., 1996).

In ribotyping, the DNA is obtained from each bacterial isolate and cut with a special enzyme (a restriction enzyme) that only recognizes certain DNA sequences. The different lengths of DNA are separated in a gel and transferred to a nylon membrane. The membrane is tested against a luminescent copy of the

DNA that codes for rRNA, and when properly treated, the membrane gives a banding pattern. These bands are imaged and analyzed for their similarity. If a library of ribotypes is established for each animal species, then the bacteria from an environmental sample can be matched to the animal source. The same matching can be done for environmental sources. For example, if *E. coli* is found in a drinking water well, then the location of the source of the contamination can be identified by isolating *E. coli* from likely environmental sources and matching them with the *E. coli* in the well. This is currently called "microbial source tracking" or "bacterial source tracking."

BACKGROUND AND RELATED WORK

Most ribotyping studies to determine the host origin of fecal coliforms have been conducted with *E. coli*. Samadpour and Chechowitz (1995) identified 421 of 589 *E. coli* ribotype patterns (71%) from Little Soos Creek (in Washington State) to cows, deer, dogs, duck, horses, humans, llama, swine, and poultry. Subsequent studies in U. S. national parks and recreational areas also linked *E. coli* to various animal hosts (Berghoff, 1998; Farag and Goldstein, 1998). Ribotyping has identified the host origin of *E. coli* isolates in oyster beds (Simmons et al., 1995) and swimming areas (Simmons and Herbein, 1998) as well as differences between human and nonhuman sources of *E. coli* under conditions of a saltwater to freshwater gradient (Parveen et al., 1999).

In this study, we investigated the possible sources of *E. coli* contamination in three household wells located in Bleckley County, approximately 5 km northeast of Cochran, Georgia (Fig. 1). The Health Department found that the household wells of each of these three families contained fecal coliforms. The wells were tested several times and fecal coliforms were consistently present. Although the wells were shock-chlorinated, this eliminated fecal coliforms for a only short period of time.

The University of Georgia Extension Service was asked to help. The source of the contamination was not clear but believed to come from a nearby sinkhole. Because Bleckley County is located in the upper Georgia coastal plain, the soils are primarily sands and sandy loams with limestone outcroppings. The weathering of the limestone outcroppings can result in sinkholes, which can provide direct channels for movement of surface water to subsurface groundwater.

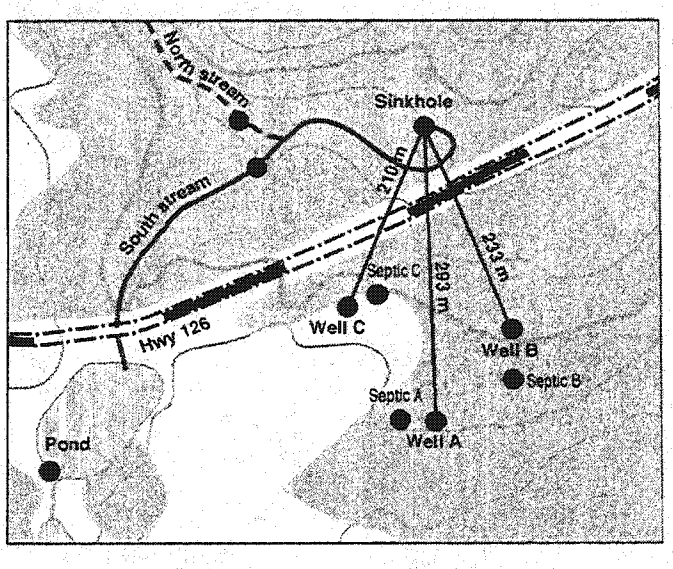

Fig. 1. Map showing the sampling sites (closed circles) and the distance of the sinkhole to the three household wells. The depths of Wells A, B, and C were 21, 22, and 25 m, respectively. Two streams drain into the sinkhole, an intermittent stream to the north (here called the north stream) and a perennial stream (here called the south stream) that drains a pond. The elevation difference between contour lines is 3 meters (10 feet).

The water from the pond, north and south streams, and the three homeowner wells was sampled by the University of Georgia Extension Service and sent to the Soil Microbiology Laboratory for testing on May 4, 2000. There were 1, 13, 10, and 60 *E. coli* per 100 mL of water for the pond, north stream, south stream, and sinkhole, respectively; for the household wells, there were 1, 3, and 1 *E. coli* per 100 mL of well water for Wells A, B, and C, respectively.

Because the septic systems were not previously considered as potential sources of contamination, the pond, the north and south streams, the three homeowner wells, and the septic tank drainfields were sampled on June 20, 2000. At this point, the wells had been shock-chlorinated again and no *E. coli* were observed in the household wells. Numbers of *E. coli* from the pond, north and south streams, and sinkhole were similar to the first sampling. Numbers of *E. coli* from each septic drainfield were all "too numerous to count." All *E. coli* isolates from the septic drainfields and two isolates from the pond were obtained from this sampling.

The objective of the study was to determine the source of fecal contamination in the homeowner wells.

METHODS

Water samples (>200 mL) were collected aseptically in sterile polypropylene bottles and kept on ice until tested. All samples were processed within 3 hours. Except for septic tank samples, 10- and 100-mL samples of water were filtered through separate 0.45-µm membranes. Septic tank samples were serially diluted to 10^{-4} before a 10-mL sample was filtered through a membrane. Each membrane was placed on mTEC medium (Difco Laboratories, Sparks, MD) and incubated at 44.5±0.2°C for 1 day. Yellow and yellow-brown bacterial colonies on the membranes were counted (these are the counts noted in the Background and Related Work section).

A total of 51 *E. coli* isolates were selected from the mTEC plates for ribotyping (number): pond (3), north stream (3), south stream (3), sinkhole (26), Household Well A (1) and septic system (2), Household Well B (8) and septic system (2), Household Well C (1) and septic system (2). To confirm that the isolates were *E. coli*, each isolate was streaked on tryptic soy agar (Difco) and incubated at 37°C for 1 day. The streaking was repeated twice to ensure the purity of each isolate. Each isolate was inoculated into a multiwell plate containing separate slants of Simmons citrate (Difco) and urea agar (Difco). In addition, an oxidase test was performed. Bacterial type species *Citrobacter freundii* ATCC #8090, *Enterobacter aerogenes* ATCC #13048, *Escherichia coli* ATCC #11775, and *Klebsiella pneumoniae* ATCC #13883 were used as controls. Isolates that were oxidase-, citrate-, and urea hydrolysis-negative were considered to be *E. coli*.

Each isolate of *E. coli* was inoculated into Luria-Bertani broth contained in a test tube and placed on a rotating shaker at 75 rpm at 35°C. After 18 hours, a sample of the culture was removed and the DNA extracted with a commercial kit (Qiagen DNeasy, Valencia, CA). The DNA was quantified with a fluorometer. DNA from *E. coli* was used as a standard.

A 1-µg sample of DNA from each isolate was digested overnight with the restriction enzyme *Pvu*II according to the manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, IN). The digested DNA was stained and was electrophoresed in a 1.0% agarose gel at 58 volts for 3 hours. Digoxigenin-labeled (DIG-labeled) Marker III (Roche) was the molecular weight marker and occupied every fifth lane of the gel. Additional lanes contained no DNA (control) and DNA from *E. coli* ATCC #11775. DNA was transferred by Southern blotting to a nylon membrane with a vacuum blotting system (VacuGene, Pharmacia, Piscataway, NJ). The DNA on the membrane was crosslinked with UV light. The DNA on the membrane was placed in prehybridization buffer at 42°C for 2 h. Following prehybridization, the membrane was hybridized at 42°C overnight to DIG-labeled cDNA from total ribosomal RNA. Membranes were prepared for chemiluminescence by a series of washing steps before a chemiluminescent substrate for alkaline phosphatase was added (Roche). Membranes were imaged with a FluorChem 8000 imager (Alpha Innotech, San Leandro, CA) equipped with a digital color printer and AlphaEase (Alpha Innotech) software. The images were saved as TIFF files and were imported into GelComparII (Applied Maths, Kortrijk, Belgium) for analysis. The lanes were analyzed at optimization and tolerance percentages of 4 and 3%, respectively. Lanes were normalized within the gel with DIG-labeled Molecular Weight Marker III and variations among the gels were assessed with the known *E. coli* strain.

CONCLUSIONS

The pond, the stream draining the pond (south stream), the stream draining the nearby land (north stream), a sinkhole draining both streams, and three household wells and their accompanying septic systems showed 12 different ribotypes with 100% similarity (Fig. 2). Two ribotype patterns were observed from the septic systems, 10 patterns among the pond, sinkhole, and two streams, and 6 patterns among the three household wells. At 100% similarity, the ribotype patterns of *E. coli* from the household wells were associated with the 6 of the 10 ribotype patterns from the pond, sinkhole, and two streams. The similarity of *E. coli* ribotype patterns from the household wells with the septic systems were 80 and 86%.

DISCUSSION

The data suggest that the *E. coli* observed in the household wells come from the pond, sinkhole, or two streams. The septic systems are an unlikely source because the ribotypes did not show a 100% match to the ribotypes of *E. coli* from the household wells.

The warm-blooded animal(s) responsible for the fecal contamination are unknown. It would be helpful to know the specific animal species that was the host of the *E. coli*. We are presently constructing a host origin database for the State of Georgia to determine this.

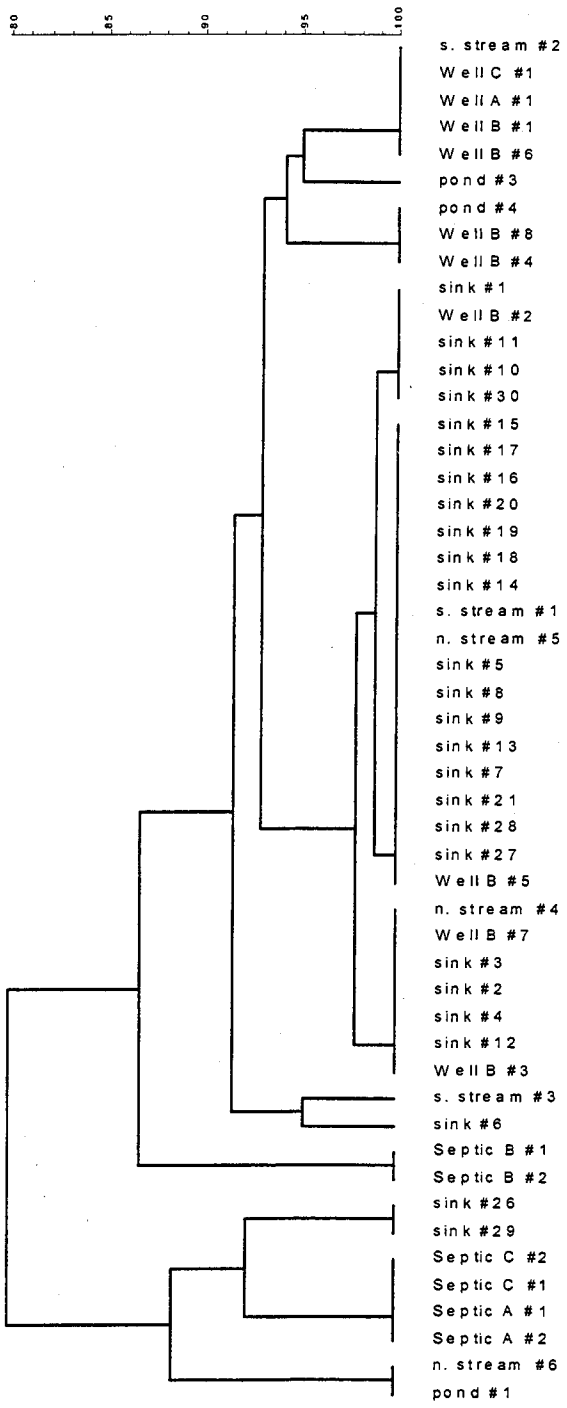

Fig. 2. Dendrogram of the ribotype patterns of 51 *E. coli* isolates from Cochran, Georgia. The isolates came from a pond, one stream draining the pond (south stream), one intermittent stream draining nearby land (north stream), a sinkhole draining both streams (sink), and three households (Well A, B, and C) and their accompanying septic systems (Septic A, B, and C). The percentage similarity among ribotype patterns is given on the top scale.

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