VIRULENCE FACTORS DETECTED IN ESCHERICHIA COLI ISOLATED FROM THE CUMBERLAND AND RED RIVERS, MONTGOMERY COUNTY, TENNESSEE

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Virulence Factors Detected in Escherichia coli Isolated from the Cumberland and Red Rivers, Montgomery County, Tennessee

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ABSTRACT

Waterborne outbreaks of disease are on the rise in the United States. Recreational waters are excellent sources of bacterial contamination and may be responsible for the increase. This research investigated the prevalence and distribution of pathogenic Escherichia coli in the Cumberland and Red Rivers. Three sites along each river were chosen, water collected and filtered, and E. coli enumerated and identified. A total of 2325 E. coli isolates were subjected to biochemical and genetic tests to identify diarrheagenic types. Forty-seven isolates of enterotoxigenic E. coli were detected. No other pathogenic biotypes were found. Random isolates were then examined for antibiotic sensitivity and all were found to be susceptible to a wide range of antibiotics. The data show that all sites contained higher denisites of E. coli than what is recommended by the Environmental Protection Agency for recreational water use. Although risk of infection by diarrheagenic E. coli is minimal, bacterial contamination levels indicate that risks to recreational water users exist.

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Introduction

Escherichia coli is the predominant gram negative facultative anaerobic bacterium of the human intestinal tract where it helps maintain intestinal physiology (13). Escherichia coli is relatively easy to isolate and identify by colony morphology and by standard biochemical tests. The numerous strains of E. coli are classified as either non-pathogenic or pathogenic based on the potential of the strain to cause disease. In general, the pathogenicity of the bacteria depends upon: i) possession of virulence determinants by the bacteria, ii) infectious dose of bacteria required, iii) portal of entry into the host and iv) the susceptibility of the host (9). Most strains of E. coli are considered avirulent or non-pathogenic because they lack virulence determinants (28). Those strains of E. coli that do possess virulence determinants are responsible for a wide variety of diseases including diarrhea, dysentery, hemolytic uremic syndrome, bladder infections, pneumonia and meningitis (9,13,28).

Food and water are known routes of transmission in the dissemination of E. coli and represent a major portal of entry into the human host. Pathogenic E. coli are not aquatic bacteria and are only present in water systems when fecal contamination of the water occurs. Water flow and tidal action typically suppress bacterial multiplication and physically wash bacteria downstream (17,32). Detection of large numbers of bacteria in water is therefore an indicator of recent contamination (17). Recreational areas located near effluents of sewage treatment plants could serve as significant sources of pathogenic

E. coli.

In addition to E. coli, many pathogenic bacteria are also in the feces of humans and other warm-blooded animals, making detection of fecally contaminated water extremely important (16). Vibrio cholereae, Shigella sp. and Salmonella sp. are just a few of the pathogenic bacteria that may be present. Escherichia coli is used as the indicator of fecal contamination for several reasons: i) ease in isolation and identification, ii) ability to grow at elevated temperatures that suppress most bacterial growth and iii) biochemical reactions will distinguish it from other bacteria capable of growth at elevated temperatures (16). Escherichia coli can easily be identified by colony morphology and color on various culture media (9). For example, on MacConkey Agar, E. coli colonies are flat and pink to dark red while on Eosin-Methylene Blue Agar (EMB) colonies are red black with a metallic sheen. The standard biochemical test, IMViC (indole, methyl red, Vogues-Proskauer, and Simmons Citrate), will further identify E. coli. All E. coli are indole positive, methyl red positive, Vogues-Proskauer negative and citrate negative (9). The Environmental Protection Agency (EPA) lists acceptable E. coli levels as 1 bacteria in 100 milliliters (ml) of drinking water and no more than 1000 bacteria in 100 ml of recreational water (16).

There are at least four categories of diarrheagenic E. coli that can be transmitted through contaminated water: enterotoxigenic, enteroinvasive, enteropathogenic and enterohemorrhagic (9,13,28). The categories are distinguished by virulence properties, interactions with intestinal mucosa, clinical syndromes, epidemiology and unique biochemical and serological reactions (13).

Enterotoxigenic E. coli (ETEC) are toxin elaborating strains responsible for a cholera-like illness. The most common symptoms are watery diarrhea, vomiting, cramps and a low-grade fever. The bacteria contain 140-220 megadalton (MDa) plasmids that encode fimbrial colonization factors. These factors promote colonization of the proximal small intestine. These factors are species specific preventing animal strains from causing disease in humans (27).

The plasmids also encode one or more enterotoxins that play key roles in the development of disease. There are two heat-labile toxins (LT-I, LT-II) and a single heat-stable toxin (ST)(1,7). The bacteria producing enterotoxin LT-II primarily cause disease in livestock and are insignificant in human disease. A few differences exist between LT-I and ST. The LT-I enterotoxin is inactivated at temperatures above 44.5° C while the ST toxin is active even at temperatures above 100° C (15). LT-I is structurally and genetically similar to the cholera toxin, showing about 75% DNA and protein sequence homology (1,2,32,35). Unlike the cholera toxin, LT-I is periplasmically retained and appears to "leak" out under high salt or low iron conditions (13). The receptor for LT-I is the protein, ganglioside GM1 on the surface of the intestinal cells. Upon binding, a series of biochemical reactions occurs leading to elevated cyclic adenosine monophosphate levels which causes hypersecretion of fluids and electrolytes in the intestinal lumen (13,27). The ST toxin is a family of small toxins that are secreted directly into the small intestine. The receptor for ST is guanylate cyclase in the apical membrane of the cells of the small intestinal. Binding to the receptor creates elevated cyclic guanidine monophosphate levels and the same hypersecretion of fluids as seen with

LT-I (13). The most severe form of disease is seen with E. coli strains containing plasmids that encode both ST and LT-I (27). The disease can be fatal in infants and young children as the result of rapid dehydration. ETEC illness is known by such common names as Traveller's diarrhea and Montezuma's Revenge as it is hyperepidemic in Mexico and Brazil (5,13,28).

Enteroinvasive E. coli (EIEC) cause an invasive, dysenteric form of diarrhea that is initially watery with blood, mucus and/or pus appearing after three days (28). Other symptoms include fever, severe cramps and malaise. The bacteria invade the epithelial cells of the colon, spreading laterally to adjacent cells. The invasion and proliferation by EIEC eventually induces cell death. A large, 140- MDa plasmid, coding for several outer membrane proteins promotes the bacteria's ability to invade host tissues. Production of a toxin is not a part of EIEC pathogenicity. Disease resulting from EIEC is generally not fatal (13,28).

Enteropathogenic E. coli (EPEC) are responsible for one of the more clinically severe forms of diarrhea. The symptoms include diarrhea with mucus or blood, vomiting and fever. These symptoms may persist for as long as two weeks and can eventually lead to dehydration and death (13,28). The bacteria exhibit a "patchy" pattern of adherence to the epithelial cells of the small intestine. Adherence produces dramatic alterations in the structure of the host mucosal cells, leading to localized destruction of microvilli on the intestinal epithelial cells. This event, known as attaching and effacing, is caused by adherence factors encoded on a 60 MDa plasmid. Production of a cytotoxin is seen but disease will occur in its absence, indicating a possible secondary role to the attaching and

effacing lesions (13,28). The exact mechanism by which EPEC causes diarrhea is unknown, but the attaching and effacing lesions appear to be the crucial component (11).

Enterohemorrhagic E. coli (EHEC) is a recently well documented category of diarrheagenic E. coli that is more prevalent in developed countries such as the United States. The increase in infection and death rates due to EHEC has prompted the Centers for Disease Control (CDC) to classify EHEC as an emerging infection (7,14,23). Infected persons typically have hemolytic uremic syndrome (HUS) with symptoms including cramps, bloody diarrhea and fever. Hemolytic uremic syndrome is often fatal due to acute kidney failure (1,3). The bacteria use plasmid encoded proteins to adhere to the epithelial cells of the small intestine, and like EPEC bacteria, cause attaching and effacing lesions. Production of a toxin similar to the Shigella toxin is seen in EHEC bacteria containing a 60 MDa plasmid (13,28). The toxin, rather than the invasiveness, is thought to cause the pathogenicity, although little is known about the mechanism of action of the toxin.

The World Health Organization states that more people die annually from waterborne outbreaks of disease than cancer or AIDS (23). The vast majority of disease occurs in underdeveloped countries, although many outbreaks occur in the United States. From 1971 to 1983, 423 cases of waterborne disease were reported in the United States. Twenty-one of these cases (involving 4062 people) were due to ETEC contaminated drinking and recreational water. Nineteen other cases (involving 7363 people) were due to contamination of recreational water with an unidentifiable bacterium (18,22). In 1993, more than 500 people in four western states were infected with EHEC, leading to fifty-six cases of HUS and four deaths (23). In that same year, the Council of State and Territorial

Epidemiologists passed a resolution recommending that EHEC outbreaks be reported by all states and territories (23). The EPA believes that the case numbers for all waterborne outbreaks of E. coli are low. Most outbreaks of diarrheagenic E. coli are not reported because symptoms are usually mild and short-lived (18,22). In many instances, the disease causing bacteria cannot be easily identified, further decreasing case numbers. It is reasonable to infer that the incidence of E. coli outbreaks is actually much greater than what is being reported. Accurate assessment of the prevalence of waterborne E. coli would require recreational water testing. The testing procedures would involve collections at various locations and would need to be done at regular intervals. Possible tests to be performed would include bacteria densities, types of bacteria being isolated and detection of other waterborne microorganisms such as protozoa.

Until recently, scientists and doctors were only able to identify disease-causing bacteria by standard biochemical tests and colony morphology. These tests are time consuming, costly and slow. Often, only the genus could be determined without any strain designation. This was true for the diarrheagenic bacteria. Classification of the diarrheagenic biotypes was limited to an understanding of the symptoms reported for the patients. In EHEC and ETEC illness, antisera to the Shigella and cholera toxins, respectively, were developed and used to presumptively identify these biotypes. These anitsera unfortunately were not reliable (27). Recently, genetic studies have resulted in the development of reliable genetic probes that allow highly specific and rapid strain identification (27.)

Genetic probes are fragments of DNA or RNA that are labelled either

radioactively (32P) or non-radioactively (biotin) and used to detect complementary sequences in a sample (21). If a sequence that is complementary to that of the probe is present in the sample, the probe will hybridize or "bind" that sequence. This hybridization event is then detected by autoradiography for radioactive labels or by addition of a chromogenic substrate if a nonradioactive label is used. A positive signal indicates the presence of complementary DNA sequence in the sample.

These techniques have been used to study clinical and environmental samples (15). Many laboratories today, including those of Martins (1992), Jerse (1990) and Levine (1987), use DNA probes for detection of diarrheagenic E. coli. The probes are synthesized based on plasmid sequences encoding toxins, colonization factors or attaching and effacing factors, depending on the biotype. This type of detection is extremely specific and more rapid than the standard biochemical or serological testing procedures (15,21,27).

Positive identification of pathogenic bacteria enables one to effectively treat the disease with antibiotics. Treatment is complicated by the presence of antibiotic resistant strains of the offending bacteria. Antibiotic resistance can be intrinsic or acquired. Intrinsic resistance is a fundamental property of the organism and was present long before antibiotic use by humans. Escherichia coli is not known to contain any intrinsic resistance genes (9).

Acquired antibiotic resistance is the result of chromosomal mutation or acquisition of extrachromosomal elements like plasmids or transposons. Bacteria acquiring plasmids and becoming resistant to antibiotics are increasing in incidence. The

mechanism of plasmid acquisition in E. coli is usually conjugation, or DNA transfer as a result of contact between two bacteria (9). A bacterium containing an antibiotic resistance plasmid (R plasmid) will attempt conjugation with any bacteria it contacts resulting in widespread antibiotic resistance in the immediate environment. Plasmid transfers such as these are known to occur in a wide variety of habitats including river water (12,30). The transfers can occur between human and animal strains increasing the likelihood of spreading the resistance (12,30). Studies of the ability of diarrheagenic E. coli to acquire R plasmids under environmental conditions have been performed and indicate that acquisition is common (12).

Escherichia coli infections are typically treated with ampicillin (Amp) or tetracycline (Tet) (9). As plasmid mediated Tet resistance is common, Amp is usually the antibiotic of choice (9). Transferable antibiotic resistance obviously poses a serious threat to infectious disease treatment necessitating that all pathogenic bacteria isolated from clinical specimens be tested for antibiotic resistance.

The goal of this project was to determine the prevalence and distribution of diarrheagenic E. coli in the Cumberland and Red Rivers of Montgomery County, Tennessee. One previous fecal coliform study of the Cumberland River was done by Davis, et al in 1968. The results indicated that the river was contaminated at many of the chosen sites. The one site in common between these two studies is mile 125.2 or the sewage effluent site. No previous studies of the Red River have been reported. The objectives were to 1) enumerate and identify E. coli, 2) subject E. coli isolates to biochemical and genetic tests to determine the prevalence of pathogenic biotypes and 3)

determine antibiotic susceptibility of the isolates. The results are discussed as risk assessment data for recreational water users.

MATERIALS AND METHODS

I. Water collections

Six sites were chosen for water collections (Figure 1) Fairgrounds Park lies along the Cumberland River just prior to entry into Clarksville McGregor Park is just downstream from a sewage effluent and is a common boating area The confluence of the Cumberland and Red Rivers serves as two sites with water being taken from both rivers A major sewage effluent discharges into the Red River near this site Trice's Landing, a fifth site, lies downstream from this effluent The final site on the Red River is along Interstate 24 prior to the river's entry into Clarksville

Approximately 150 ml of water was collected from each of the six sites The water was collected in sterile, screw-cap bottles using Standard Methods techniques (33) To prevent bacterial multiplication in the collected water, bottles were stored on ice and transported to the lab Transport time was minimal resulting in samples being processed within 30 minutes of collection

Three sets of collections were taken a winter collection in February and March, a spring collection in April and May and a summer collection in June and July The sets involved biweekly collections that continued until approximately 100 to 150 isolates of E coli were obtained per site The total number of colonies collected for testing were Trice's Landing (428), Fairgrounds (341), McGregor Park (417), Cumberland River (434), Red River (405) and Interstate 24 (300) Colonies were randomly selected so the data could be used to test the seasonal distribution of diarrheagenic biotypes

II. Filtration, enumeration and identification

The water was filtered using a sterile vacuum apparatus onto 47 mm Metricel Membrane filters with a 0.45 μm pore size. The water was filtered in 0.1, 1 and 10 ml aliquots. The 0.1 ml and 1 ml samples were adjusted to 10 ml with sterile peptone water. All aliquots were filtered in triplicate. The filters were incubated overnight at 44.5°C on either Fecal Coliform (FC) media or Endo Agar. Both media are selective for the coliform group of bacteria. Escherichia coli grow as blue colonies on FC and deep red colonies with a metallic sheen on Endo Agar (26). Incubation of the filters at 44.5°C further increases the selectivity of the assay, as most bacteria will not replicate at this temperature.

Following incubation, the E. coli were identified by colony morphology and enumerated. Standard biochemical tests were used to confirm the positive identification of the E. coli (9).

III. Replicate plating

Colonies of E. coli isolated and identified above, were replicate plated onto Luria-Bertani (LB) agar (21). Following overnight incubation at 37°C, one set of the plates was stored at 4°C as stock cultures while the other set of plates was used for colony lifts in the molecular tests. Each plate contained a maximum of fifty colonies from a single site.

IV. Strain determination

The isolated bacteria were subjected to a series of genetic and biochemical tests to identify diarrheagenic strains.

A). Genetic analysis by colony-hybridization

Colonies that were identified as E. coli were screened for the presence of genetic

sequences unique to pathogenic biotypes. One of the replicate plates was overlayed with a nitrocellulose filter and incubated 30 minutes at 37°C. The filters were carefully removed and the colonies lysed by an alkaline hydrolysis procedure. The filter paper, colony-side up, was placed sequentially onto a series of four pads saturated with 0.5 N NaOH, 1.5 M Tris-HCl (pH 7.5), 2X SSC and 70% ethanol, respectively. As controls, E. coli cells known to harbor the correct gene sequence and cells known to contain no virulence genes were included on each filter. The filters were allowed to air dry before hybridization experiments.

The following molecular probes were used to identify the pathogenic biotypes. pCVD419, a plasmid encoding fimbrial genes specific to EHEC bacteria and pEWD299, a plasmid encoding genes for the heat-labile toxin of ETEC. Plasmid DNA was purified as described below (section IV B). The BRL BluGene Kit (Life Technologies, Gaithersburg, MD) was used to label the genetic probes by primer extension with biotinylated nucleotides.

The dried nitrocellulose filters were prehybridized in a solution of 50% deionized formamide, 2X SSC and 10 mM EDTA (pH 8.0) for 2 hours at 42°C. The prehybridization solution was removed and replaced with a hybridization solution (50% deionized formamide, 2X SSC, and 10 mM EDTA, pH 8.0) containing the labeled DNA probe which had been heated 3 minutes at 100°C immediately prior to adding to the buffer. The filters were hybridized 12-18 hours at 42°C. After hybridization, the filters were washed twice at room temperature for 15 minutes each time in 2X SSC containing 0.1% SDS. The final wash was incubated 30 minutes at 56°C in a solution of 0.1 X SSC

and 0.1% SDS. The biotinylated probe was detected with a strepavidin-alkaline phosphatase conjugate. Positive hybridization was visualized by detection of the alkaline phosphatase with the chromogenic substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. All colonies showing color development were retested to confirm reactivity.

B). Medium scale preparation of plasmid DNA

Medium scale plasmid DNA isolation was performed on bacterial cells isolated from 50 ml LB broth cultures The bacterial cells were collected by centrifugation at 3,000 x g for 10 minutes The supernatant was discarded and the pellet was resuspended in 5 ml of solution I (50 mM Tris-HCl, pH 7 5, 10 mM EDTA pH 8 0, 25 mM glucose) After incubation at room temperature for 10 minutes, the cells were lysed by the addition of 20 ml freshly prepared solution II (1% SDS, 0 2 N NaOH) The solution was gently swirled and placed on ice for 10 minutes after which 10 ml of 3 M sodium acetate (pH 4 8) was added and incubation continued for another 15 minutes The solution was clarified by centrifugation at 3,000 x g for 15 minutes The supernatant was then transferred to a clean tube and 20 ml of isopropanol added to precipitate DNA After incubation for 15 minutes at room temperature, the precipitate was collected by centrifugation at 10,000 x g for 10 minutes at 4 °C The supernatant was drained from the pellet and the pellet resuspended in 5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Then, 5 ml of 7.5 M ammonium acetate was added to the resuspended pellet The tubes were inverted several times and incubated on ice for 20 minutes Debris was pelleted by centrifugation for 10 minutes after which time the supernatant was transferred

to a new tube. Plasmid DNA was precipitated by addition of 0.9 ml of isopropanol and incubation on ice for 20 minutes. The DNA was collected by centrifugation for 15 minutes in the eppendorf centrifuge. After draining the supernatant from the pellet, the pellet was rinsed with cold 95% ethanol and air dried. The dried plasmid DNA was resuspended in 0.05 ml TE buffer and stored at -20°C.

C). Small scale isolation of plasmid DNA

Colonies testing positive for hybridization in part A were randomly selected for plasmid isolation. Plasmid DNA was isolated from 2 ml cultures of the bacteria. The procedure follows that of the medium scale isolation, adjusting only the amounts of solutions added.

D). Agarose gel electrophoresis of DNA fragments.

Agarose gels were prepared by dissolving 0.7 g of agarose (5510UB, Bethesda Research Laboratories, electrophoresis grade, ultra pure) in 100 ml of TAE buffer (0.04 M Tris-acetate, 1 mM EDTA) by heating to 100°C. The agarose solution was cooled and poured into an agarose gel forming apparatus. After the gel solidified, TAE buffer (containing 0.5 μg/ml ethidium bromide) was added to a depth of 1 mm over the gel and the comb removed. DNA samples were mixed with 6X gel-loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol) and loaded into the wells of the submerged gel. Electrophoresis was conducted at 4°C at 5 V/cm for appropriate lengths of time. The DNA fragments were visualized by UV- illumination.

E). Southern analysis of DNA fragments separated on agarose gels.

DNA samples to be analyzed by Southern analysis were separated on standard 0.7% agarose gels and then transferred to nitrocellulose membranes by standard methods (29). After electrophoresis, the DNA in the gel was partially depurinated by soaking the gel in two gel volumes of 0.35 M HCl for 15 minutes. The depurination was repeated and the gel rinsed twice in distilled water. The DNA was then denatured by incubating the gel twice in two volumes of 0.5 M NaOH and 1.5 M NaCl for 15 minutes. The DNA was then neutralized by soaking the gel 60 minutes in two changes of two gel volumes of 1 M Tris-HCl and 1.5 M NaCl. A nitrocellulose filter was cut to the size of the gel, wetted with water and soaked in a solution of 2X SSC along with 3 sheets of Whatman 3mm paper cut to the same dimensions. The gel was inverted and placed on a wick on a solid support in a dish of 2X SSC. The nitrocellulose was placed onto the gel followed by the 3 sheets of Whatman filter paper. An 8cm high stack of paper toweling was added and transfer allowed to proceed for 12 hours at room temperature. Care was taken at each step to insure that no air bubbles were trapped next to the gel. After the transfer was complete, the sandwich was disassembled and the nitrocellulose filters dipped in 2X SSC and allowed to air dry. The dried nitrocellulose was prehybridized and hybridized as detailed above for genetic probing of the colony lifts (section IV.A).

F). Antibiotic susceptibility testing

The diarrheagenic E. coli may contain plasmid DNA that confers resistance to antibiotics. Genetic and biologic tests were performed to determine if any strains

identified in this study possessed antibiotic resistance markers. A plasmid probe containing Ampicillin (AMP), Tetracycline (TET) and Kanamycin (KAN) resistance genes was use for hybridization studies as detailed in part IV.A. In addition, a standard broth dilution assay was used to determine the minimum inhibitory concentration (MIC) of these antibiotics for all isolates.

The E. coli isolates were cultured overnight in Mueller-Hinton (MH) broth (Difco Laboratories). The culture was adjusted to a standard optical density and used as the inoculum. Stock solutions of the antibiotics were prepared in water and serial two-fold dilutions were performed in MH broth. A standardized inoculum of 0.1 ml was added to each antibiotic dilution. Following overnight incubation at 37°C, the cultures were visually examined for turbidity. The MIC was recorded as the lowest concentration of antibiotic that prevented growth as determined by the absence of turbidity.

Results

A total of eleven collections were taken: four in the winter, four in the spring and three in the summer. The collections were performed in a variety of weather conditions (eg. rain, snow, sun) to monitor the effects of weather on prevalence and distribution of pathogenic biotypes of E. coli. The data were organized to assess seasonal distribution. Table 1 provides weather condition information and numbers of E. coli per 100 ml for each collection at each site.

The EPA guidelines for fecal coliforms in recreational waters are: 1) five samples over a thirty day period are not to exceed 200 bacteria per 100 ml and 2) no more than ten percent of the total samples over a thirty day period should exceed 400 bacteria per 100 ml (25). As can be seen in Table 1, the water in the chosen sites does not meet these guidelines. Over the six month collection period, each site conformed to the standards differently. The worst site was the Red River, at the location of sewage discharge. This area complied with guidelines only once in the eleven collections. Three sites (Trice's Landing, McGregor Park and the Cumberland River) met the standards three times out of eleven. The Fairgrounds conformed four times out of ten and the Interstate site twice out of seven collections. The highest numbers of bacteria were first observed on 3-8-95 during a long period of rain, 4-10-95 during a sewage line break in the Red River and 5-16-95 throughout an extended period of heavy rain. These results indicate that sewage discharge and storm run-off have a profound effect on bacterial densities.

A total of 2325 E. coli colonies were obtained during the eleven collections. The isolates were submitted to genetic and biochemical tests to determine if any of the isolates

could be categorized as one of the diarrheagenic biotypes.

All isolates were screened for hybridization to three genetic probes: pCVD419, pEWD299 and the probe containing the antibiotic genes. No hybridization was observed with pCVD419. The reliability of these data are supported by the positive hybridization of the pCVD419 probe to the positive control. In addition, no EHEC bacteria were identified by the biochemical analyses. All isolates exhibited Beta-glucuronidase (GUR) activity and fermented sorbitol within a twenty-four hour period; both properties are negative in EHEC bacteria.

A total of 47 colonies or 2% of the entire sample of E. coli isolates were positive for hybridization to pEWD299 (ETEC gene probe). Figures 2-4 depict the percentages of positive colonies tested per site per collection. Trice's Landing was the highest with 26 positive colonies out of 428 tested. The data for the other sites were as follows: McGregor Park 10 positive out of 417, the Cumberland River 9 positive isolates out of 438, the Red River 2 positive isolates out of 402 and the Fairgrounds 1 positive isolate out of 331. The Interstate site had no positive isolates in the 300 colonies tested. Seasonal distributions showed 5 out of 6 sites tested positive for 17 ETEC during the winter collection, 2 out of 6 sites tested positive for 12 ETEC during the spring collection and 4 out of 6 sites tested positive for 19 ETEC during the summer collection. More than half of the ETEC found in the spring and summer collections were isolated from the Trice's Landing site.

The ETEC results were supported by the following data. First, specific hybridization of the pEWD299 probe to control E. coli containing the LT-gene sequence.

Second, biochemical analysis demonstrated the isolates were GUR positive and sorbitol positive. All of these results are consistent with ETEC bacteria. Finally, Southern Blot analysis was performed on the plasmid DNA's purified from the hybridization positive isolates. DNA purified from the positive colonies exhibited hybridization to pEWD299, indicating that the first set of hybridizations were accurate. These three conformational steps prove that ETEC bacteria were present within the samples.

All isolates were screened for hybridization with a probe containing genes for Amp, Kan and Tet resistance. None of the isolates were positive for hybridization to this probe. To confirm this data, MIC's were obtained for the isolates using these antibiotics. Reference strain E. coli ATCC 25922 was used to standardize the MIC data. The reference strain has a MIC for Amp of 8ug/ml and 4ug/ml for Kan and Tet. The ranges of MIC's obtained for the isolates obtained from this study were: Amp = 4-8ug/ml, Kan = 1-2 ug/ml, and Tet = 1-2 ug/ml. Since all of these MIC ranges are equal to or below the MIC for the reference strain, all isolates were classified as antibiotic susceptible.

Discussion

The increase in outbreaks of waterborne disease demonstrate the need for rapid monitoring of water contamination and improved water quality regulations. Previous studies on Tennessee recreational waters show that some of the more important water systems do not conform to the EPA guidelines (3,24). The Cumberland River was found to be one of the most polluted systems. This water system is heavily impacted by commercial, industrial, farm and residential pollution. As society grows, the risk of disease associated with this contamination increases. . The sewage effluent at the junction of the Cumberland and Red Rivers in Clarksville is an example of such a health risk. This effluent releases water that can be contaminated with fecal bacteria, including E. coli. This study was done to determine the risk of infection by pathogenic E. coli to recreational water users.

The large numbers of E. coli found in the water samples demonstrates an existing contamination problem. The concentration of fecal coliforms in any individual sample shall not exceed 1000 colony forming units/100ml according to the EPA standard for recreational water (24). Any count exceeding this standard is considered to be polluted and is not suitable for recreational swimming or other bodily contact (24). McGregor Park and the Cumberland River sites exceeded this guideline five times, Trice's Landing four times, the Red River three times, Interstate 24 twice and the Fairgrounds once (Table 1). McGregor Park is a popular boating and skiing site which lies downstream from an unmarked and supposedly unused sewage effluent. The Cumberland River and Trice's Landing are also popular recreational sites. Trice's Landing lies downstream from a

marked sewage effluent discharge site. The lower fecal coliform counts for the Red River site are probably the result of increased water flow in this area. The strong current would wash the sewage effluent downstream towards Trice's Landing.

The numbers of E. coli obtained varied by as much as 6000 bacteria between collections. The study performed by Davis, et al (1968) also found large variations in numbers between collections. These fluctuations in bacterial densities may be explained by the observations that bacterial populations along river banks respond to increased nutrients from land run-off, sewage, industrial discharge and vertical mixing (22). Each of the highest densities of E. coli were observed during or immediately after long periods of rain (Table 1). Rain would cause tremendous amounts of land run-off. These effects can best be seen at locations such as the site at the Fairgrounds and at Interstate 24 which are more impacted by farmland. In addition, many common soil bacteria, such as Klebsiella, Pseudomonas, and Proteus, were observed at these locations, further implicating the effects of land run-off in the data fluctuations. During these long periods of rain, the sewage effluent at the confluence of the Cumberland and Red Rivers, will discharge. Sewage discharge would definitely lead to an increase in bacteria numbers, especially fecal bacteria. Some of the more extreme numbers of fecal bacteria were detected during the sewage line break (Table 1, 4-10-95). The large numbers of bacteria isolated in this study indicate the need for improved water standards and their enforcement. The potential for the spread of disease is high along both the Cumberland and Red Rivers, especially at points inside Clarksville, TN. Regular water testing is encouraged to control for variations that occur between collections.

Variation in E. coli numbers was not closely correlated to differences in temperature. Air temperatures ranged from -1°C to 24°C while water temperatures ranged from 4°C to 15 °C. Consecutive collections with temperatures differing by only five degrees showed extreme variation in bacteria numbers (Table 1). Seasonal difference in numbers are not easily detected either. The spring and summer collections had larger numbers of bacteria which can be primarily attributed to larger amounts of rainfall. A larger sample size is needed to complete an evaluation of variation in numbers of fecal coliforms as a result of climatic changes.

The only diarrheagenic biotype found was ETEC bacteria The incidence of ETEC bacteria was low, comprising only 2% of the total sample (47 out of 2325 isolates). Other studies using DNA probes to detect diarrheagenic E coli in water samples have found substantially different numbers. Martins, et.al. (1992) detected 1 ETEC in 237 E. coli isolates (0.4%) and Echeverria (1982) detected 31 in 350 isolates (9%). Two plausible explanations exist for the variation in data. First, the low numbers seen in this project and Martins could be attributed to the incubation of the bacteria at 44.5°C. The LT toxin is inactivated at this temperature and it is possible that the high temperature can cause instability of the plasmid (15). Echeverria's tests were done on bacteria incubated at 37°C. No comparison between the two incubation temperatures has been performed to assess this hypothesis. Therefore, incubations at 44.5°C selectively enriches for fecal coliform enumeration but may hamper detection of enterotoxigenic strains. The second possibility for the differences is technique. Many advances in recombinant DNA technology have been made since Echeverria's study. It is possible that conditions being

used in that study were not stringent enough or the probes were not completely specific for the LT toxin. The low numbers of ETEC seen in this project and Martins may truly be indicative of the proportion of natural ETEC in water systems of the United States.

The distribution of diarrheagenic biotypes indicated that Trice's Landing had the highest count of 26 ETEC, McGregor Park had 10 ETEC, Cumberland River had 9 ETEC, Red River had 2 ETEC, the Fairgrounds 1 ETEC and the Interstate no ETEC. Trice's Landing, as stated previously, lies directly downstream of the sewage effluent at the junction of the Red and Cumberland Rivers. Martins(1992) and Shakoori (1993) found ETEC only in sewage discharges supporting the idea that sewage discharge is a prime source of fecal coliforms and water contamination. Interestingly, the Red River area had lower numbers than expected. The increased flow of water observed at this site may again explain the data. The data indicate that the primary contaminant of the rivers, within Clarksville, TN, is the marked sewage effluent. Collections need to be taken in the water immediately downstream from the discharge to determine the bacterial content and densities within the sewage discharge.

Seasonal distributions of ETEC are, once again, less apparent. Each season contained samples testing positive for the presence of ETEC. The variation in number of ETEC detected in each season is not substantially different. A larger sample size would need to be taken to accurately assess any seasonal distributions.

Enterohemorrhagic biotypes were not detected using either DNA probes or biochemical tests. Martins and Echeverria found zero in 237 isolates and one in 350 isolates, respectively (4,15). Although EHEC illness is on the rise in the United States, the

majority of the outbreaks are from undercooked food rather than water contamination (9).

No antibiotic resistance could be detected in the bacteria isolated. Ampicillin, Tetracycline and Kanamycin were the antibiotics tested as they would be the most likely antibiotics used in disease treatment. It is possible that resistance to other antibiotics exist. The incubation of the bacteria at 44.5°C could have again altered the data due to decreased stability of plasmid DNA at this temperature. If the plasmid encoding the resistance is undergoing denaturation or is being released from the cell, antibiotic resistance would be compromised. Studies on plasmid stability at 44.5°C need to be completed to determine if loss of antibiotic resistance could occur at these temperatures.

A couple of problems with this experiment need to be addressed. First, the only diarrheagenic biotypes examined were ETEC and EHEC. The lack of probes to EIEC and EPEC prevented their detection. Biochemical testing would not specifically identify either of these bioytpes. Although these two biotypes are much less common in the United States, they still exist and could be located within the study sites. The project seems incomplete without EIEC or EPEC data. Second, G testing is controversial. Martins claims that Methylumbelliferyl (MUG) media is only 50% accurate in detecting diarrheagenic E. coli while Frampton states that it is 97% accurate (6,15). All MUG examinations done for this project were followed by random biochemical testing of both MUG negative and positive strains. All MUG positive colonies were biochemically identified as E. coli while all MUG negative colonies belonged to other genera. The data from the MUG testing support that of Frampton (6).

The final aspect of this project is risk assessment to recreational water users. The

EPA states that 15-90ml of water is ingested by the typical water user each outing (25). Based on the lowest and highest numbers of bacteria collected in this project, an average swimmer would ingest a minimum of 90 bacteria per 90ml and up to 5490 bacteria per 90ml when swimming in the Cumberland River. An infectious dose of ETEC bacteria is 100,000,000 while the infectious dose for EHEC is 10-200 bacteria (9). The risk of infection by ETEC is minimal. Infection by EHEC is considered minimal since our tests were unable to detect this pathogenic biotype.

The aquatic life must also be considered. Fish have been documented carriers of human E. coli (17). The fish can carry the bacteria for 9-14 days before excreting it into the water. Potentially pathogenic biotypes can thus be carried to other areas not being tested. Poorly cooked fish containing these bacteria could easily cause infection. It is also known that transfer of R plasmids from animal to human strains occurs. This transfer can be performed either in the water or within the gut of the animal. The animal R plasmids confer antibiotic resistance to the human bacteria. This drastically effects the treatment of disease, possibly requiring that new antibiotics be developed. Cumberland River recreational water users need to be cautious of both the aquatic life and the water itself.

Regular recreational water testing needs to be considered. As can be seen in this project, bacteria levels vary greatly from day to day. As these levels rise, pathogenic biotypes need to be identified as possible risk factors for recreational water users. Further testing needs to be completed on the marked sewage effluent. The levels of fecal coliforms released by the effluent needs to be determined. Improved regulations may need to be considered to lower levels of fecal coliforms being released. Recreational water

users need to understand the potential risks they take every time they enter the water. Public warnings stating that the river is contaminated could prevent large numbers of infections.

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APPENDIX

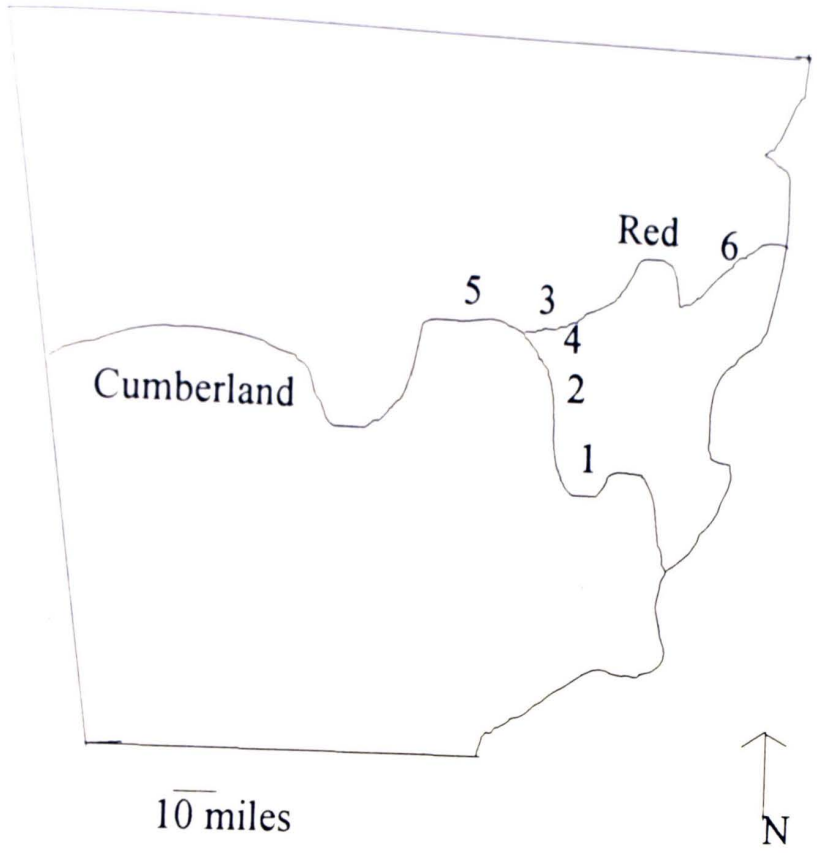


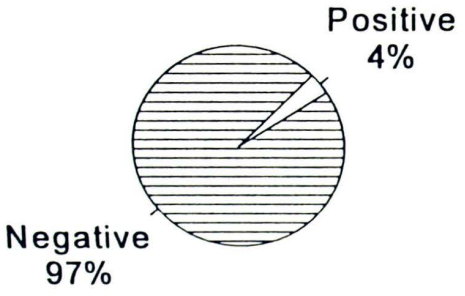
Figure 1. Map of sites chosen for collections along the Cumberland and Red Rivers in Montgomery Co. Tennessee. 1 = Fairgrounds, 2= McGregor Park, 3= Red River, 4 = Cumberland River, 5 = Trice's Landing and 6 = Interstate 24.

Table 1. Average number of colonies per 100 ml of water collected from the Cumberland and Red River in Montgomery County, Tennessee. The asterisk indicates water collected during a sewage line break within the Red River. Abbreviations: (T) = Trice's Landing, (F) = Fairgrounds (M) = McGregor Park, (C) = Cumberland, (R) = Red, (I) = Interstate 24, (N/D) = not done due to physical barriers preventing water collection.

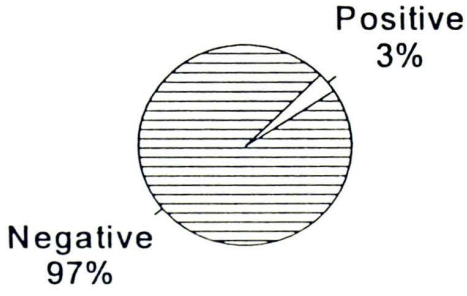
Number of Bacteria per 100 ml per site

Date and Weather	Trice	Fairgrounds	McGregor	Cumberland	Red	Interstate
1-23-95;Cold	100	300	300	300	300	200
2-16-95;Rain	900	100	1400	1000	900	300
3-1-95;Cold	300	100	100	200	300	100
3-8-95; Rain	2700	1300	1400	1200	3100	1600
4-4-95;Sun	100	100	100	600	400	100
4-10-95;Sun	1800	100	2800	6100	3100	N/A
4-26-95;Sun	600	N/A	600	600	400	N/A
5-16-95;Rain	2000	1700	4900	4400	3600	N/A
5-30-95;Rain	1400	800	2800	1100	700	1000
6-12-95;Sun	200	400	300	100	100	N/A
6-28-95;Sun	600	300	200	200	400	400

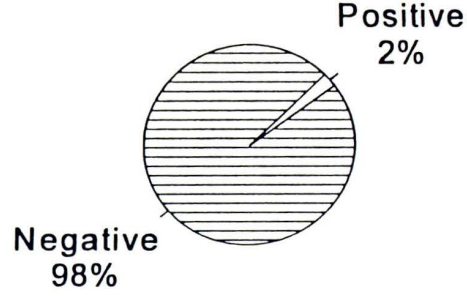
Percent ETEC Bacteria per site for Set I



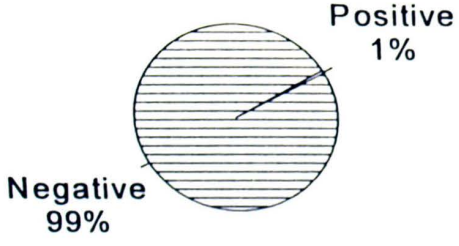
Trice's Landing(169)



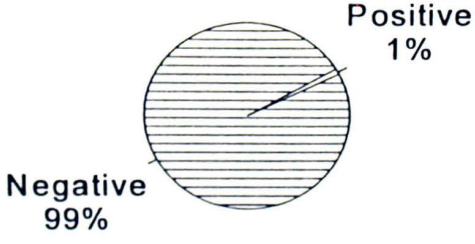
McGregor(150)



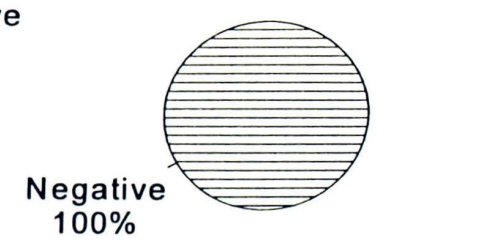
Cumberland(175)



Red(177)

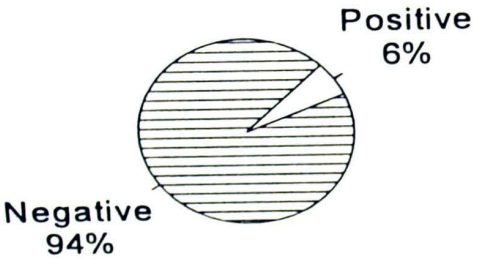


Fairgrounds(107)



Interstate(138)

Percent ETEC Bacteria per site for Set II



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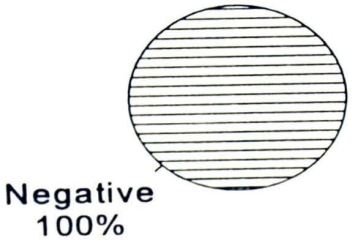
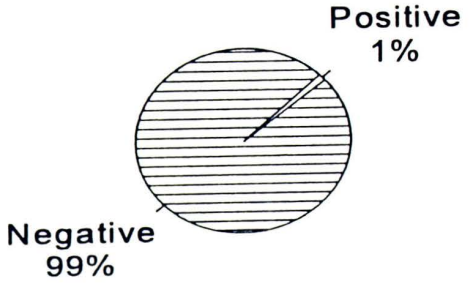
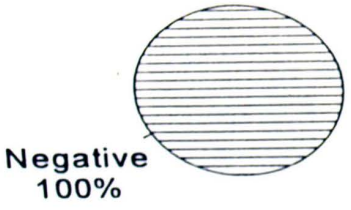


McGregor(151)



Cumberland(166)



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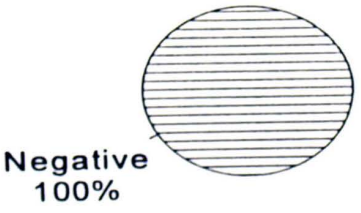

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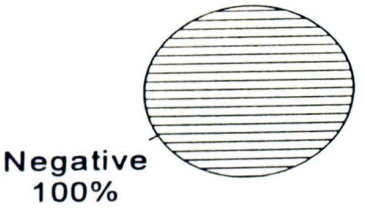
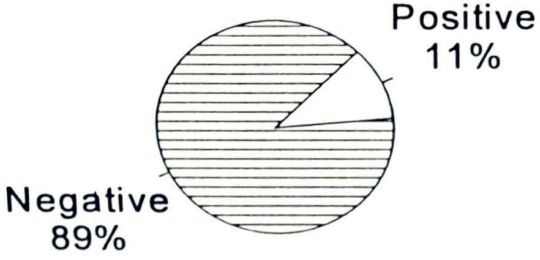
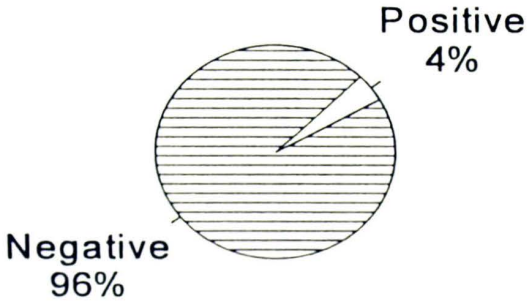


Interstate(100)

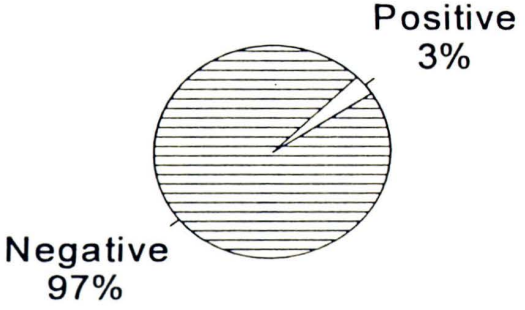
Percent ETEC bacteria per site for Set III



Trice's Landing(100)



McGregor(116)



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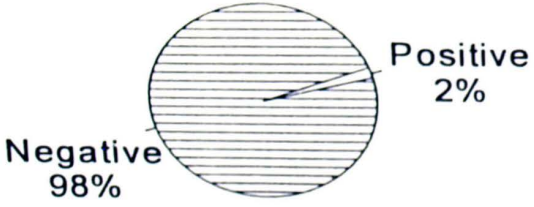

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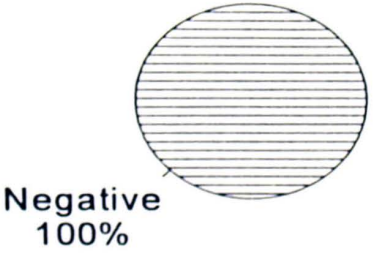

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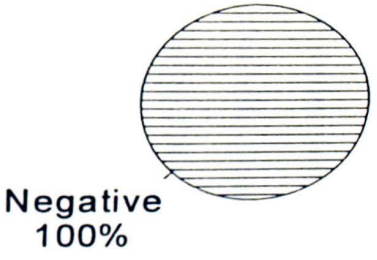

Interstate(129)

VITA

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