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Preliminary Survey of Antibiotic-Resistant Fecal Indicator Bacteria and Pathogenic *Escherichia coli* from River-Water Samples Collected in Oakland County, Mich., 2003

Scientific Investigations Report 2005-5058

U.S. Department of the Interior
U.S. Geological Survey

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Conversion Factors and Abbreviated Water-Quality Units

Multiply	By	To obtain
square mile (mi^2)	2.590	square kilometer (km^2)
gallon (gal)	3.785	liter (L)
ounce, fluid (fl. oz)	29.57	milliliter (mL)
microliter (µL)	0.001	milliliter (mL)
cubic foot per second (ft^3/s)	0.02832	cubic meter per second (m^3/s)
ounce, avoirdupois (oz)	28.35	gram (g)
milligram per liter (mg/L)	1000	micrograms per microliter (µg/µL)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

°F = (1.8 x °C) + 32

Concentration of bacteria in water is given in colony forming units per 100 milliliters (CFU/100 mL).

Concentrations of reagents are given in millimolar (mM) or micromolar (µM).

Preliminary Survey of Antibiotic-Resistant Fecal Indicator Bacteria and Pathogenic *Escherichia coli* from River-Water Samples Collected in Oakland County, Mich., 2003

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Abstract

A preliminary study was done in Oakland County, Michigan, to determine the concentration of fecal indicator bacteria (fecal coliform bacteria and enterococci), antibiotic resistance patterns of these two groups, and the presence of potentially pathogenic *Escherichia coli* (*E. coli*). For selected sites, specific members of these groups [*E. coli*, *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*)] were isolated and tested for levels of resistance to specific antibiotics used to treat human infections by pathogens in these groups and for their potential to transfer these resistances. In addition, water samples from all sites were tested for indicators of potentially pathogenic *E. coli* by three assays: a growth-based assay for sorbitol-negative *E. coli*, an immunological assay for *E. coli* O157, and a molecular assay for three virulence and two serotype genes. Samples were also collected from two non-urbanized sites outside of Oakland County. Results from the urbanized Oakland County area were compared to those from these two non-urbanized sites.

Fecal indicator bacteria concentrations exceeded State of Michigan recreational water-quality standards and (or) recommended U.S. Environmental Protection Agency (USEPA) standards in samples from all but two Oakland County sites. Multiple-antibiotic-resistant fecal coliform bacteria were found at all sites, including two reference sites from outside the county. Two sites (Stony Creek and Paint Creek) yielded fecal coliform isolates resistant to all tested antibiotics. Patterns indicative of extended-spectrum-β-lactamase (ESBL)-producing fecal coliform bacteria were found at eight sites in Oakland County and *E. coli* resistant to clinically significant antibiotics were recovered from the River Rouge, Clinton River, and Paint Creek. Vancomycin-resistant presumptive enterococci were found at six sites in Oakland County and were not found at the reference sites. Evidence of acquired antibiotic resistances was detected in bacteria from multiple sites in Oakland County but not detected in bacteria from the reference sites. Integrons capable of transferring resistance were detected in isolates from the River Rouge and Clinton River. *E. faecium* and *E. faecalis* identified in samples collected from Kearsley Creek and Evans Ditch were resistant to high levels of vancomycin and carried transferable genes responsible for resistance.

Several sites in Oakland County had indicators of pathogenic *E. coli* in August and (or) September 2003. Two samples from the Clinton River in August tested positive for all three *E. coli* O157 tests. Both the August and September samples from one River Rouge site were positive for the immunological and molecular assay for *E. coli* O157. A combination of virulence genes commonly associated with human illness was detected at five sites in August and seven sites in September. Antibiotic-resistance profiles of clinical concern along with genes capable of transferring the resistance were found at several sites throughout Oakland County; samples from many of these sites also contained potentially pathogenic *E. coli*.

Introduction

Many rivers and streams in Michigan are affected by fecal contamination (Harrison, 2003). Pathogenic bacteria associated with fecal contamination can cause mild to serious illness. **Pathogenic bacteria**[1] that may be associated with fecal contamination include: pathogenic strains of *Escherichia coli* (*E. coli*), *Campylobacter*, *Salmonella* species, *Shigella*, and *Enterococcus* species. In addition to these organisms causing human illnesses, resistance to antibiotics has made treatment more difficult.

[1] Terms defined in the glossary are in **bold print** where first used in the main body of the report.

As part of a larger study to assess the quality and quantity of waters in Oakland County, the U.S. Geological Survey (USGS), in cooperation with the Oakland County Department of Human Services, collaborated on a study to evaluate the microbiological quality of waters within the county.

Purpose and scope

The purpose of this report is to describe the results of a preliminary study on the presence of antibiotic-resistant fecal indicator bacteria and potentially pathogenic *E. coli* in waters collected in Oakland County, Mich. This report includes (1) concentrations of fecal indicator bacteria (fecal coliform bacteria, *E. coli*, and enterococci) in river-water samples collected in August and September 2003, (2) fecal coliform and enterococci antibiotic resistances to selected antibiotics, (3) detection of transferable genetic elements (integrons and vancomycin resistance genes), and (4) indications of the presence of presumptive pathogenic *E. coli*. This report also includes a comparison to results obtained from two reference sites outside of Oakland County that represent very different land uses.

Description of study area

Oakland County, Michigan, includes 910 mi^2 in southeastern Michigan. The county is largely suburban, with a population of approximately 1.2 million in 2000. Oakland County is in the headwaters of five major rivers: the Shiawassee and Flint Rivers draining north towards Saginaw Bay, and the Clinton Rivers draining east towards Lake St. Clair, and the Huron and Rouge draining south towards Lake Erie. Developed land ranges from approximately 30 percent to almost 100 percent in some watersheds. Agricultural land use is typically less than 20 percent of total watershed area, and no known confined-feeding or other large-scale livestock producers operate in the county. Study sites include 14 sites in watersheds draining Oakland County (fig. 1, table 1); 13 sites were within the county, and 1 site was just outside of the county in the Clinton River watershed. These sites will be referred to as "Oakland County sites" hereafter. In addition to the Oakland County sites, two reference sites were also included: the Au Sable River in a national forest and Washington Creek in Isle Royale, Mich. (table 1).

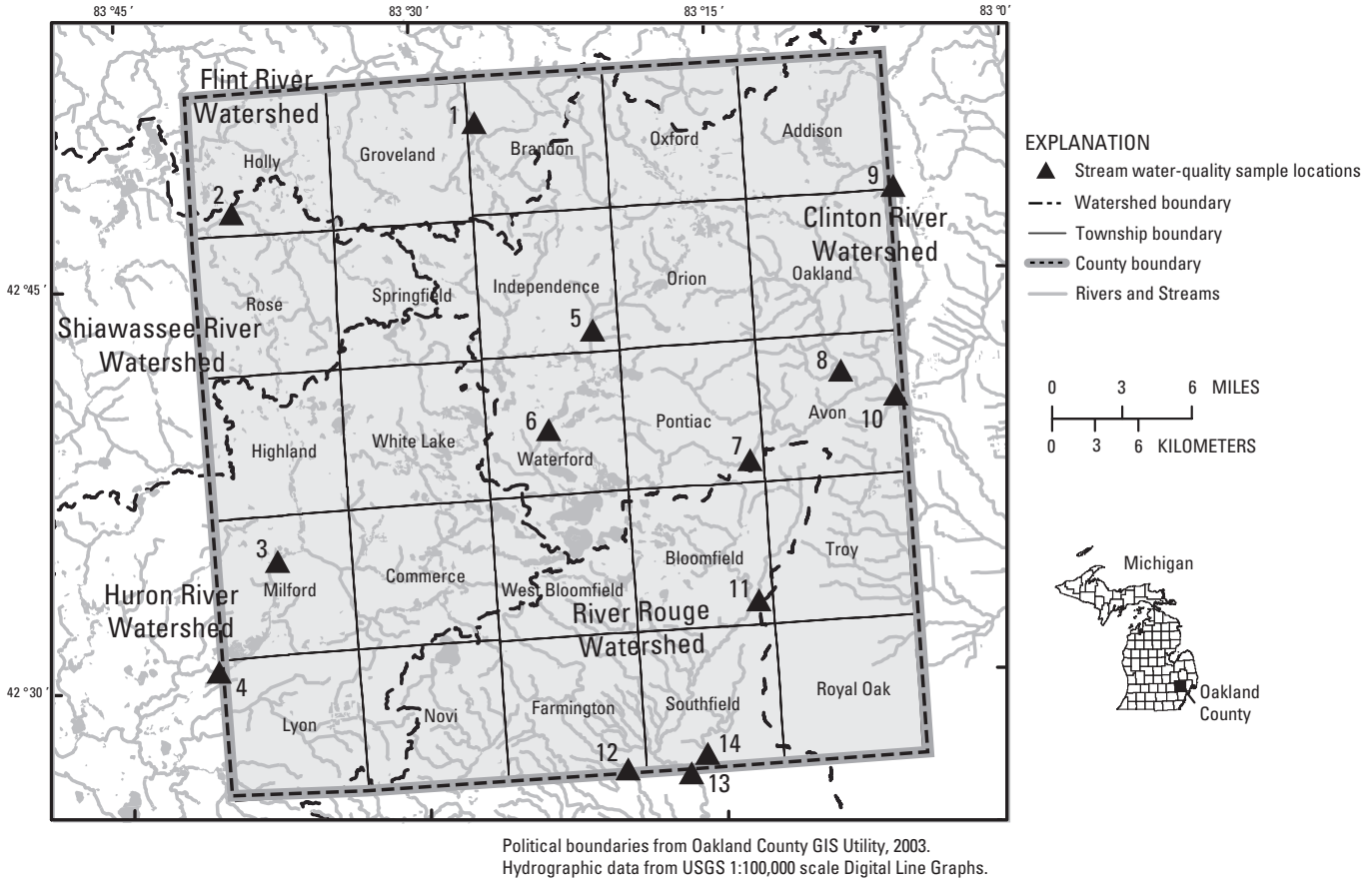

Figure 1. Locations of Oakland County, Mich., study sites. Map numbers correspond to U.S. Geological Survey station numbers in table 1.

Table 1. Sample-collection sites.

[USGS, U.S. Geological Survey]

Map number	USGS station number	Station name	County	Watershed
1	04148035	Kearsley Creek at Mill Street at Ortonville, Mich.	Oakland	Flint
2	04143830	Shiawassee River at Holly, Mich.	Oakland	Shiawassee
3	04170000	Huron River at Milford, Mich.	Oakland	Huron
4	04170500	Huron River near New Hudson, Mich.	Oakland	Huron
5	04160800	Sashabaw Creek near Drayton Plains, Mich.	Oakland	Clinton
6	04160900	Clinton River near Drayton Plains, Mich.	Oakland	Clinton
7	04161000	Clinton River at Auburn Hills, Mich.	Oakland	Clinton
8	04161540	Paint Creek at Rochester, Mich.	Oakland	Clinton
9	04161580	Stony Creek near Romeo, Mich.	Macomb	Clinton
10	04161810	Clinton River at Yates, Mich.	Oakland	Clinton
11	04166000	River Rouge at Birmingham, Mich.	Oakland	Rouge
12	04166315	Upper River Rouge at Clarenceville, Mich.	Oakland	Rouge
13	04166100	River Rouge at Southfield, Mich.	Oakland	Rouge
14	04166200	Evans Ditch at Southfield, Mich.	Oakland	Rouge
Reference sites				
	04135500	Au Sable River near Grayling, Mich.	Crawford	Au Sable
	04001000	Washington Creek at Windigo, Mich. (Isle Royale)	Keweenaw	Lake Superior

Background

Bacteria groups studied and their relation to pathogens

Because pathogens are often difficult to detect in the environment, fecal indicator bacteria are commonly used to measure the sanitary quality of water (Rose and Grimes, 2001; U.S. Environmental Protection Agency, 1986, 2000). This study focuses on the fecal indicators fecal coliform bacteria, including *E. coli*, and enterococci.

Fecal coliform bacteria and *Escherichia coli*

Fecal coliform bacteria are a group of gram negative bacteria that ferment lactose at 44.5 degrees Celsius. They are usually present in high concentration in feces from humans and other warmblooded animals. Many fecal coliform bacteria are members of the Enterobacteriaceae family composed of many pathogens and nonpathogens, which are genetically similar and capable of exchanging genes. *E. coli* is a member of both the Enterobacteriaceae and fecal coliform bacteria groups. *E. coli* has been recommended by the U. S. Environmental Protection Agency (USEPA, 2000) as an indicator of sanitary water quality for surface waters. *E. coli* is part of the natural human intestinal flora, and most strains do not cause human illness. Certain pathogenic strains of *E. coli*, however, are capable of causing mild to serious human illnesses.

Pathogenic *E. coli* (in particular the **serotype** *E. coli* O157:H7) have been identified as the causative agents in several recreational waterborne-disease outbreaks (Lee and others, 2002). Most pathogenic *E. coli* belong to one of four major groups: enterhemorrhagic *E. coli* (EHEC), which include shiga toxin-producing *E. coli* (STEC); enterotoxigenic *E. coli* (ETEC); enteropathogenic *E. coli* (EPEC); and enteroinvasive *E. coli* (EIEC) (Nataro and Kaper, 1998). The most commonly seen pathogenic *E. coli* strains isolated in the United States belong to the EHEC/STEC group (Bopp and others, 2003). EHEC/STEC are defined by the severity of the disease they cause, and they commonly share similar genes responsible for their virulence. These genes include the intimin gene (*eae*A), which is associated with close attachment of *E. coli* to intestinal cells, and the shiga toxin producing genes (*stx*1 and *stx*2) responsible for cell destruction. STEC strains usually have the *eae*A gene and, in addition, carry one or both of the shiga toxin genes (*stx*1 and *stx*2). Recent findings suggest that strains of *E. coli* O157 with *eae*A and *stx*2 are more virulent than those strains with only *stx*1 (Boerlin and others,

1999). More than 200 STEC serotypes have been isolated from persons with diarrhea and (or) hemolytic-uremic syndrome. In the United States, *E. coli* O157:H7 is the most frequently isolated STEC in clinical samples. However, increasingly, other non-O157 STEC strains are identified in outbreaks and sporadic illness (Bopp and others, 2003). STEC and EHEC strains have been found in fecal samples from humans with severe disease, farm animals, bird species (gulls, chickens, turkeys, and pigeons), and sewage from human and animal waste (Nataro and Kaper, 1998).

Isolating and identifying *E. coli* O157:H7 and other pathogenic organisms from environmental waters is often difficult because of the low expected concentrations of the pathogen and the high concentrations of other bacteria commonly found in environmental waters. Surface waters contain a wide range of environmental bacteria, many of which have not been identified. When water also contains fecal contamination, a very diverse set of bacteria, some similar to the target bacterium, may be present to interfere with assays. Most diagnostic techniques have been developed for clinical diagnosis of disease and for detection of contamination in food. These samples contain a limited and usually a known range of other bacteria species. As a result, when one uses techniques developed for food or clinical diagnosis, the limitations of the approach and the influence of environmental bacteria must be considered before interpreting the results.

Commonly used methods for identifying STEC strains are based on the growth characteristics of STEC organisms on selective media, the presence of antigens specific to the strain, and the detection of genes responsible for virulence and pathogenicity. To isolate *E. coli* O157, cefixime and tellurite are added to sorbitol MacConkey growth medium (CT-SMAC) (Bopp and others, 2003). *E. coli* O157 ferments the sorbitol in this medium much more slowly than other *E. coli*, resulting in a colorless colony in comparison to pink colonies for those isolates that ferment sorbitol much more efficiently. The cefixime and tellurite are added to inhibit the growth of many fecal bacteria, decreasing the amount of background growth. However, there are still fecal coliform bacterial species other than *E. coli* O157 that can produce a similar non-sorbitol-fermenting CT-SMAC result. These bacteria include: *Hafnia* and *Shigella* (Zadik and others, 1993; Fujisaw and others, 2000).

Immunological assays are used to identify specific antigens present on *E. coli* O157. Commercial tests are available that use antibodies designed for the detection of *E. coli* O157 (for example, the *E. coli* O157:H7 8 hour Reveal test; Neogen, Lansing, Mich.). This is a presence/absence test and is not quantifiable. This kit was designed for use in the food industry, and previous studies have shown other non-*E. coli* O157 bacteria are capable of cross-reacting with the O157 antibodies used, specifically, *Citrobacter freundii*, *Yersinia enterocolitica*, *Pseudomonas maltophilia*, *Brucella abortus*, *Escherichia hermannii*, *Hafnia alvei*, *Morganella morganii,* and the *Salmonella* group N (Power and others, 2000). As a confirmatory test, molecular methods can be used to identify a gene specific for *E. coli* O157 (rfb_{O157}). To identify samples that may contain STEC, polymerase chain reaction (PCR) is used to detect genes commonly associated with STEC (*eae*A, *stx*1, and *stx*2) that are responsible for virulence.

Enterococci

Enterococci are a group of gram-positive bacteria commonly associated with fecal material from mammals and birds. They are also found in the soil and associated with plant material. Although they are common members of the human bacterial flora, they are opportunistic pathogens that can cause disease particularly in elderly and immunocompromised patients (Aarestrup and others, 2002; Malani and others, 2002). Enterococci have been identified as the second leading cause of **nosocomial** urinary tract and wound infections and the third leading cause of nosocomial bacteremia in the United States (Teixeria and Facklam, 2003). *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) account for more than 95 percent of clinical enterococci isolates (Teixeira and Facklam, 2003). The other enterococci species are less frequently identified in human infections.

Overview of antibiotic resistance in bacteria

Antibiotics are used in human and veterinary medicine, animal husbandry, and in the treatment of agricultural crops. Recent studies have shown the presence of antibiotics in waters across the United States, but in very low concentrations (Kolpin and others, 2002). Antibiotics and antibiotic-resistant bacteria may enter the environment in discharge from wastewater-treatment plants, pharmaceutical companies, or hospital waste; runoff from animal-feeding operations or land manure application; infiltration from agricultural application; or from landfill leachate. Antibiotic resistance is of increasing concern because of the emergence of **multiple antibiotic resistant** pathogens, such as multiple-drug-resistant *E. coli* and *Salmonella* spp. (Walsh, 2003).

Intrinsic versus acquired resistance

Bacterial resistance to antibiotics can be either **intrinsic** or **acquired**. Intrinsic antibiotic resistance is due to an inherent feature of the organism that prevents the antibiotic mode of action. This type of resistance is determined by chromosomal genes and is not due to the acquisition of new genes. For example, most fecal coliform bacteria are intrinsically resistant to vancomycin because their cellular structure consists of an outer membrane that prevents the vancomycin molecule from attacking its target (Rice and others, 2003). Intrinsic resistance is not transferable to other bacteria. Acquired antibiotic resistance may be transferred to other organisms. Resistance is acquired through a mutation in the chromosomal genes or through the acquisition of new genes responsible for antibiotic resistance. Genes responsible for acquired antibiotic resistance are often carried on genetic elements that can easily be trans-

ferred among bacteria (plasmids, transposons, and integrons; Roy, 1999). In many cases these genetic elements may carry several antibiotic resistance genes, thus transferring multiple antibiotic resistances to other organisms. The genetic elements can pick up new genes or lose genes as they transfer between organisms, and they are responsible for widespread antibiotic resistance in clinical **isolates**. Additionally, they have been frequently found in environmental bacterial isolates (Ash and others, 2002; Park and others, 2003; Roe and others, 2003). In some cases these genetic elements may also carry genes for resistance to metals or other toxic compounds (Ug and Ceylan, 2003; Bass and others, 1999). Therefore, the transfer of these genetic elements under selective pressure of antibiotics, metals, or toxic chemicals may result in the transfer of several types of resistance genes.

Without species identification, determining whether antibiotic resistance is acquired or intrinsic can be difficult. Several factors can indicate that the resistance is acquired. The most obvious is the presence of **transferable genetic elements** that are transferred among organisms. There are also antibiotic resistance patterns that are more indicative of acquired resistance. Both of these factors are described in more detail throughout this report. Antibiotic-resistant fecal coliform bacteria and enterococci in surface waters may pose a threat to human health because of the potential for transfer of antibiotic-resistance genes to pathogens.

Environmental versus clinical bacteria antibiotic resistance

Much of our knowledge of antibiotic resistance comes from the clinical setting in which antibiotic-resistant pathogens have made treatment very difficult. Only a small selection of antibiotics are actually used to treat human infections, and pathogens that are resistant to these select few antibiotics are very difficult to treat. Antibiotic-resistant bacteria have previously been isolated from surface waters (McArthur and Tuckfield, 2000; Ash and others, 2002; Roe and others, 2003; Yang and Carlson, 2003) but few studies have investigated the environmental occurrence of **clinically significant antibiotic resistances** such as resistance to second and third generation cephalosporins or vancomycin. In a study by Roe and others (2003), *E. coli* resistant to ceftriaxone, cephalothin, ampicillin, gentamicin, and streptomycin were isolated from the Rio Grande; of these, 32 percent were resistant to multiple antibiotics. In addition, **integrons**–genetic elements that can transfer antibiotic resistance genes to other organisms–were found in 13 percent of the *E. coli* isolates in that study. In another study by Ash and others (2002), antibiotic-resistant bacteria were isolated from 13 rivers across the United States at 22 locations. All 22 sites sampled contained bacteria resistant to one or more cephalosporin antibiotics. Sixteen sites contained bacteria resistant to third-generation cephalosporins (ceftazidime and cefotaxime). In addition, 40 percent of the isolates resistant to ampicillin and at least one other tested antibiotic harbored plasmids (genetic elements that may carry multiple antibiotic resistance genes). Most of the bacteria identified by Ash in this study were from the following genera: *Acinetobacter*, *Alcalingenes*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, and *Serratia*. These findings suggest that bacteria resistant to antibiotics of clinical concern are present in surface waters; however, very little is known about the distribution of antibiotic-resistant bacteria in urban settings in the United States. Antibiotic-resistant fecal and nonfecal bacteria have been found in surface waters outside the United States. (Goñi-Urriza and others, 2000; Reinthaler and others, 2003; Park and others, 2003).

Because many organisms typically found in surface water have intrinsic antibiotic resistances (Farmer, 2003) or may not be pathogens, identification of the resistant bacteria is required to interpret the health significance. Pathogenic bacteria with resistance to an antibiotic used to treat the infection caused by that organism are an obvious concern. Less obvious is the significance of environmental (nonpathogenic) bacteria with antibiotic resistances. Of most concern is the ability of environmental bacteria to transfer antibiotic resistance genes to human pathogens. Therefore, identification of transferable genetic elements also is necessary.

In a collaborative effort of numerous federal and state governmental agencies including the Food and Drug Administration (FDA), U.S. Department of Agriculture (USDA), and the Centers for Disease Control and Prevention (CDC), the National Antimicrobial Resistance Monitoring System (NARMS) has been established to track antibiotic-resistance patterns of enteric and foodborne pathogens (National Antimicrobial Resistance Monitoring System, 2005). Enteric pathogens including *E. coli* and *Salmonella* isolated from human and veterinary clinical samples, healthy farm animals, and raw meat from food animals are tested for resistance to various antibiotics including (but not limited to) ampicillin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, gentamicin, streptomycin, and tetracycline. A select group of these results taken from CDC, (2003) and USDA (2004) is listed in table 2. Analysis by the CDC was done only for *E. coli* serotype O157:H7 human clinical isolates, whereas data collected by the USDA for nonhuman samples are for *E. coli* in general. These isolates were not identified to the serotype level. The results of the NARMS studies provide a framework to compare *E. coli* antibiotic-resistance profiles from this study to antibiotic-resistance profiles of *E. coli* from clinical and animal sources.

Clinically significant antibiotic resistance

Most of the antibiotics chosen for this study represent antibiotic resistances that are of concern for each respective bacterial group. Not all antibiotics are used to treat infections by all bacterial species. Therefore, this study focuses on two major antibiotic resistances that have become a major health concern for the two groups of bacteria included in this study: (1) extended-spectrum-β-lactamase-producing Enterobacteriaceae and (2) vancomycin-resistant *Enterococcus*.

Table 2. Selected data from the National Antimicrobial Resistance Monitoring System (NARMS): Percentage of antibiotic-resistant *E. coli* O157:H7 from human clinical samples and *E. coli* from diagnostic veterinary, dairy cattle, fruit and vegetables, and environmental samples.

[CDC, Centers for Disease Control; USDA, United States Department of Agriculture. *E. coli*; *Escherichia coli*; all data in percent]

Antibiotic	CDC *E. coli* O157:H7 human clinical isolates: 1996-2001[a] (n=1,651)	USDA *E. coli* isolates-2002[b]			
		Diagnostic veterinary sources (n=343)	Dairy cattle on farm (n=1,389)	Fruit and vegetables (n=736)	Environment (n=130)
Ampicillin	2.0	53.90	21.0	3.5	80.0
Cefoxitin	0.4	20.10	0.4	1.0	0.0
Ceftriaxone	0.0	0.30	0.0	0.0	0.0
Cephalothin	1.4	28.30	4.0	1.9	2.3
Ceftiofur	1.0	13.40	0.6	0.7	0.0
Gentamicin	20.0	21.80	0.1	0.5	0.8
Streptomycin	3.0	28.50	4.0	4.9	0.8
Tetracycline	5.0	65.90	10.7	5.0	6.9

[a] Data from CDC, 2003, National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) 2001 annual report.

[b] Data from USDA, 2002 *E. coli* report, accessed January 2005 at http://www.arru.saa.ars.usda.gov/ECOLI/2002reports.htm.

Extended-spectrum-β-lactamase (ESBL)-producing Enterobacteriaceae

Of increasing concern in the clinical setting are Enterobacteriaceae that produce extended-spectrum-β-lactamases (ESBL) (Rice, 2001; Diekema and others, 2004; McGowan and Tenover, 2004). ESBL-producing bacteria are typically resistant to all cephalosporins (except for cephamycins) and other β-lactam antibiotics such as the penicillins (ampicillin). ESBL-producing Enterobacteriaeceae are frequently resistant to other non-β-lactam antibiotics, such as the aminoglycocides and tetracylines, because plasmids that contain ESBL genes also carry other antibiotic-resistance genes, such as genes responsible for gentamicin, streptomycin, and/or tetracycline resistance (Nathisuwan and others, 2001). Being plasmid-borne, these resistances can be transferred from one organism to another; such transfer has led to ESBL-producing pathogens including *E. coli*, *Klebsiella*, *Pseudomonas*, *Enterobacter*, and *Salmonella* species (Bradford, 2001; Pérez- Pérez and Hanson, 2002; Philippon and others, 2002; Preston and other, 2003).

Vancomycin-resistant enterococci (VRE)

In the United States, the number of reported vancomycin-resistant enterococci (VRE) from clinical samples has increased (Huycke and others, 1998; Cetinkaya and others, 2000). Vancomycin is restricted to human medicine in the United States and the prevalence of VRE are rare outside the clinical setting (Cetinkaya and others, 2000); however, VRE have been found in hospital wastes (Harwood and others, 2000). A few known species of enterococci are intrinsically resistant to vancomycin at levels less than 16 µg/mL (Kak and Chow, 2002). Higher levels of resistance to vancomycin (greater than 16 µg/mL) indicate acquired resistance, a result of vancomycin-resistance genes (*van*A and *van*B) carried on plasmids (Kak and Chow, 2002) that can be transferred to pathogenic *Enterococcus* or *Staphylococcus* species.

Using antibiotic resistances to determine sources of fecal contamination

Recent studies have suggested using antibiotic-resistance patterns of fecal coliform bacteria and enterococci to determine sources of fecal contamination in surface waters (Wiggins and others, 1999; Harwood and others, 2000). This study was not designed to determine a source of fecal contamination; however, resistance patterns to antibiotics commonly used in particular practices may give some indication of the source of fecal contamination. Cephalosporin antibiotics are commonly used in human medicine but the first and second generation cephalosporin antibiotics are becoming more frequently used in veterinary medicine and livestock production. Ceftriaxone has remained primarily an antibiotic for human medicine. This premise is supported by Harwood and others (2000), in which they reported that fecal coliform bacteria resistant to the antibiotics cephalothin and ampicillin were most often associated with humans, whereas fecal coliform bacteria resistant to the antibiotics tetracycline and streptomycin were most commonly associated with animals.

Enterococci resistant to low to intermediate levels of vancomycin have been reported to be more associated with animal feces than wastewater, most likely because of the greater prevalence of enterococci species with intrinsic resistance in animal feces (Harwood and others, 2000). Enterococci with

high levels of resistance to vancomycin, resulting from antibiotic-resistance genes carried on plasmids, are exceptionally rare in animal species in the United States (McDonald and others, 1997) where vancomycin has been restricted for human medicine.

Methods and Approach

To determine the sanitary water quality of the sampled streams, fecal coliform bacteria, *E. coli*, and enterococci were quantified, and resulting fecal coliform bacteria and enterococci isolates from those samples were tested for resistance to a selected group of antibiotics. Using molecular techniques, genetic elements responsible for antibiotic resistance were analyzed in *E. coli* and enterococci isolates. Three different methods were used to determine the presence of presumptive pathogenic *E. coli* for these sites.

Sample collection

Water samples were collected August 5, 2003, as 1-L, grab samples from 14 streams in Oakland County, Mich. To determine whether results obtained in August were reproducible and to overcome difficulties with the methods applied in August, additional samples were collected September 8–9, 2003, from these same sites. Reference samples were collected from Washington Creek (August 26, 2003) and the Au Sable River in Grayling, Mich. [August 4 (referred to as August sampling), and August 26 (referred to as the September sampling), 2003]. Station numbers and locations are listed in table 1. Samples collected in August were held on ice for 24 hours before processing. Samples collected in September were processed immediately in the field for fecal indicator bacteria. Field parameters (water temperature, specific conductance, pH, dissolved oxygen, and stream discharge) and water-quality constituents were collected and measured at these same sites (Aichele and others, 2004).

Quantification of fecal indicator bacteria

Fecal coliform bacteria, *E. coli*, and enterococci were isolated from all samples by means of membrane filtration techniques (American Public Health Association 1998; U.S. Environmental Protection Agency, 2000). Sample volumes of 100, 10, 1, and 0.1, mL and sterile buffered saline control were passed through individual sterile 0.45-µm-pore-size gridded cellulose nitrate membrane filters (Advantec MFS, Inc., Pleasanton, Calif.). Fecal coliform bacteria were identified on mFC agar LES medium (DIFCO Laboratories, Detroit, Mich.). For *E. coli* identification, membranes with 15–50 well-separated fecal coliform colonies were transferred to Nutrient Agar containing 4-methylumbelliferyl-β-D-glucuronide (NA-MUG; DIFCO). Colonies that fluoresced blue under UV light were identified presumptively as *E. coli*. Fecal coliform cultures on the 100-mL filter plate were preserved at -80°C in Luria Bertani broth and 20 percent glycerol and will be referred to hereafter as **FC cultures**. *Enterococcus* isolates were identified by means of membrane filtration on mEI agar as described by U.S. Environmental Protection Agency (2000). Colonies with blue halos were presumed enterococci. The mEI cultures were preserved at -80°C in tryptic soy broth and 20 percent glycerol and will be referred to as **ENT cultures**.

Determination of antibiotic resistance

For samples collected in August 2003 (after quantification), mFC filters that produced between 20 and 60 colonies were tested for clinically significant antibiotic resistance by means of replica-plating techniques (Carlton and Brown, 1981). Antibiotic media were made by adding one of the following antibiotics to Mueller-Hinton (MH) agar in concentrations suggested by the National Committee for Clinical Laboratory Standards (NCCLS, 2002): cefoxitin (32 µg/mL), cephalothin (32 µg/mL), ceftriaxone (64 µg/mL), ampicillin (32 µg/mL), tetracycline (8 µg/mL), streptomycin (64 µg/mL), and gentamicin (16 µg/mL). These antibiotics were chosen to help define resistance that may be a threat to human health and also help to determine whether the sources of bacterial populations were different among the different sites. The colonies from the mFC plate, which included both fecal coliform and non-fecal-coliform colonies, were transferred on sterile velvet cloth to each of the MH-plus-antibiotic plates. Resistant colonies were not confirmed to be fecal coliform bacteria; rather, they were described as capable of growing at 44.5ºC on mFC media and therefore might include non-fecal-coliform bacteria. Some plates were very overgrown, and colonies could not be differentiated. In these cases, results were reported as positive or negative resistance for that sample rather than a number of resistant colonies. Similarly, enterococci were transferred by replica-plating to MH agar with one of the following antibiotics in concentrations suggested by the NCCLS (2002): vancomycin (8 µg/mL), vancomycin (16 µg/mL), gentamicin (100 µg/mL), and streptomycin (1,000 µg/mL), and tetracycline (16 µg/mL). Again, these antibiotics represent clinically significant antibiotic resistances or resistances that may differ with sources of contamination. The plates were incubated for 18–24 hours at 37ºC. Growth was recorded as a resistant colony.

Because of the amount of overgrowth seen on the antibiotic plates from August, replica-plating was abandoned; instead, fecal coliform and enterococci colonies from the September sampling were picked individually and transferred with a sterile toothpick to each of the MH-plus-antibiotic plates (described above). Tetracycline was omitted for September enterococci isolates. For plates with 50 or more colonies, only 50 representative colonies were picked. For plates with fewer than 50 colonies, all colonies were picked and transferred.

For antibiotic-resistance tests, control bacterial strains were tested alongside study samples as recommended by the NCCLS (2002). For fecal coliform bacteria analyses, *E. coli*

25922 (American Type Culture Collection, ATCC) was tested for sensitivity to cephalothin, cefoxitin, ceftriaxone, gentamicin, streptomycin, and tetracycline. For enterococci analyses, *E. faecalis* 29212 (ATCC) was tested for sensitivity to vancomycin, gentamicin, streptomycin, and tetracycline.

Determination of the presence of antibiotic-resistant *E. coli*

FC cultures which were successfully obtained from September samples were screened for isolates that would grow on MH medium with one of the cephalosporin antibiotics (cephalothin, cefoxitin, and ceftriaxone). Resistant isolates were then grown on mFC and further tested on NA-MUG to identify presumptive *E. coli*. Presumptive *E. coli* were confirmed by a negative oxidase reaction, a positive indol reaction, a positive o-nitrophenyl-β-D-galactopyranoside (ONPG) reaction, and a gram-negative morphology (Holt and others, 1994). ATCC control strain *E. coli* 25922 was used as a positive control for each reaction. Intermediate and high-level antibiotic resistance to cephalothin, cefoxitin, ceftriaxone, gentamicin, and ampicillin was determined for confirmed *E. coli* by means of microdilutions (National Committee for Clinical Laboratory Standards, 2002). Again, *E. coli* 25922 (ATCC) was used as a bacterial control for antibiotic resistance tests.

Determination of the presence of vancomycin-resistant enterococci

Because vancomycin-resistant enterococci pose a significant health threat, vancomycin-resistant enterococci were isolated by spread culturing from the ENT cultures (that originally contained presumptive vancomycin-resistant enterococci) on MH agar containing vancomycin (16 µg/ml). Colonies that grew on this medium were confirmed as enterococci by (1) growth on mEI medium, (2) bile esculin hydrolysis on bile esculin agar (BEA), (3) growth in brain heart infusion broth containing 6.5 percent NaCl, (4) negative catalase reaction, (5) gram positive reaction, and (6) cell morphology. To better understand the threat to human health, presumptive enterococci were then identified to the species level by use of BioMérieux Strep 20 API test strips (BioMérieux; Hazelwood, Mo.) which test a series of biochemical reactions. ATCC control strain *E. faecalis* 29212 was used as a positive control tested alongside study isolates. Levels of resistance to vancomycin were determined for confirmed enterococci isolates that were vancomycin resistant at 16 µg/mL (four isolates from site 04148035 in September, six isolates from site 04161540 in September, and one isolate from site 04166200 in August) by use of a microdilution technique established by the NCCLS. Again, *E. faecalis* 29212 (ATCC) was used a bacterial control for vancomycin tests.

Determination of the presence of antibiotic-resistance genetic elements

By means of polymerase chain reaction (PCR), phenotypically confirmed *E. coli* were tested for the presence of integrons; protocols used were similar to those described by Lévesque and others, (1995). A 1-µL loop of overnight culture was suspended in 100 µL of phosphate-buffered saline, and 1µL of this suspension was added to the PCR reaction. The PCR reaction consisted of a final concentration of 1X PCR buffer, 1.5 mM, MgCl2, 0.2 mM dNTPs, 0.2 µg/µL, bovine serum albumin (BSA), 0.2 µM of each primer (table 3), and 1.25 units Taq polymerase (Promega, Madison, Wis.) in a final volume of 25 µL. A negative control was run with each reaction by omitting the addition of DNA to the reaction mix.

DNA was amplified using a PerkinElmer GeneAmp PCR System 2400 Thermal Cycler with the conditions listed in table 3. The amplified PCR product was visualized on a 1.5-percent agarose ethidium bromide stained gel under UV light.

Table 3. Primers and polymerase chain reaction conditions for integrons and vancomycin resistance genes.

[5´, five prime end; 3´, three prime end; F, forward primer; R, reverse primer; G, guanidine; C, cytosine; A, adenosine; T, tyrosine; min, minute; sec, second; °C, degrees Celsius]

Gene	Primers 5´-3´	Thermocycler conditions	Reference
Integron	F: GGC ATC CAA GCA FCA AG R: AAG CAG ACT TGA CCT GA	5 min 94°C 35 cycles: 1 min 94°C 1 min 95°C 2 min 72°C 10 min at 72°C	Lévesque and other, 1995
*van*A	F: GGG AAA ACG ACA ATT GC R: GTA CAA TGC GGC CGT TA	3 min 96°C 40 cycles: 20 sec 95°C 40 sec 55°C 1.5 min 72°C 5 min 72°C	Dutka-Malen, 1995
*van*B	F:ATG GGA AGC CGA TAG TC R: GAT TTC GTT CCT CGA CC		

A 100 base pair DNA size ladder was used to determine the size of DNA fragments. Gels were visualized with a foto/prep transilluminator (Fotodyne), imaged with a Kodak EDAS 290 Zoom digital camera, and analyzed with the Kodak 1D-gel image analysis software.

The genes responsible for acquired high-level vancomycin resistance (*van*A) and low- to intermediate-level resistance (*van*B) were detected in identified enterococci isolates by means of protocols similar to those described by Dutka-Malen and others (1995). A 1-µL loop of overnight culture was suspended in 50 µL of 0.05N NaOH. Cells were lysed by heating the suspension at 95°C for 10 minutes. Cellular debris was pelleted before 1µL of supernatant was added to the following PCR reaction: 1X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µg/µl BSA, 0.3 µM of each primer (table 3), and 2.5 units Taq polymerase (Promega) in a final volume of 25 µL. DNA was amplified, and resulting PCR products were visualized and photographed as described above. Positive control strains also were tested along with a negative (non-DNA) control and study samples. Positive controls for these experiments included vancomycin-resistant *E. faecium* ATCC 700221 containing the *van*A gene, vancomycin-resistant *E. faecalis* ATCC 51299 containing the *van*B gene, and vancomycin-susceptible *E. faecalis* ATCC 29212 lacking both *van*A and *van*B.

Determination of the presence of pathogenic *E. coli*

Three different assays were used to detect the presence of potential pathogenic strains of *E. coli*. The first, a growth assay (**GA**), was used to determine whether *E. coli* O157 was present. The FC culture was serially diluted in sterile phosphate buffered saline solution and grown on sorbitol MacConkey medium containing cefixime and tellurite (CT-SMAC). CT-SMAC is commonly used in clinical settings to isolate and differentiate the *E. coli* O157 group (Zadik and others, 1993). Samples containing non-sorbitol-fermenting colonies (white or colorless) were recorded as presumptive *E. coli* O157 and will be hereafter referred to as GA positive. However, because of the background growth of sorbitol-fermenting pink colonies in some samples, white colonies may not have been detectable if they were in very low concentrations compared to pink colonies. In the second assay, Reveal O157:H7 8 hour *E. coli* test kits (Neogen Inc., Lansing, Mich.) were used to detect *E. coli* O157 by immunological methods. This test was used to detect specific surface antigens that are expressed on *E. coli* O157 cells (Bird and Hoerner, 2001; Power and others, 2000). Because this test was designed for detection of *E. coli* O157:H7 in meat and had not previously been evaluated for surface-water samples, it was used with slight modifications conducive to the samples to determine whether these antigens were present in the FC cultures. Briefly, from the FC culture, 100 µL was added to 45 mL of the Reveal enrichment media. The sample was incubated at 42°C for 8 hours, and a portion of the enrichment was applied to a Reveal test device. Reveal positive results will hereafter be referred to as **IA** positive. Because of the amount of FC sample that could be used for this method and the ability to selectively enrich for the desired organisms, this resulted in our most sensitive detection of *E. coli* O157. Because certain non-*E. coli* O157 bacteria can cross-react with the antibodies used in this method, it is possible to obtain false-positive results. Therefore, samples were tested by means of a third assay to detect the *E. coli* O157-specific gene rfb_{0157}. Owing to the constraints of this method, the amount of FC culture used for the rfb_{0157} analysis was often much less than that used for the IA test, resulting in a disagreement between immunological and gene-based methods reflective of detection-limit and test-sensitivity issues.

In the third assay, PCR was used to identify the genes rfb_{0157}, *stx*1, *stx*2, and *eae*A. DNA was extracted from 1 to 250 µL (dependent on number of fecal coliforms detected in the original water sample) of FC culture by use of the Qiagen DNeasy DNA extraction kit (Qiagen, Valencia, Calf.). The manufacturer's instructions were followed for DNA extraction from bacterial cultures. DNA was collected into two 200-µL volumes of elution buffer and stored at -20°C until analysis.

For the detection of the gene rfb_{0157}, a 25-µL PCR reaction was set up with 5 µL of extracted DNA added to a final concentration of 1X PCR buffer, 5 mM MgCl2, 0.2 mM dNTP's, 0.1 µM primers (table 4), and 2.5 units of Taq polymerase (Promega) in buffer B. DNA was amplified, and resulting PCR products were visualized and photographed as described previously.

A multiplex PCR was used to detect the presence of the *eae*A, *stx*1, and *stx*2 genes by methods similar to those described by Fagan and others (1999). Each 25-µL PCR reaction mixture consisted of 1µL of extracted DNA and a final concentration of 1X buffer II PCR reaction buffer, 3 mM MgCl2, 0.2 mM dNTP's, 0.1 µg/µL BSA, 0.05 µM of *eae*A, *stx*2 and *stx*1 primers (table 4), 0.025 µM of the internal-control *E. coli* specific primers (EC; table 4), and 2.5 units AmpliTaq Gold Polymerase. DNA was amplified, and resulting PCR products were visualized and photographed as described above. For both the rfb_{0157} and multiplex PCR, DNA extracted from a known strain of *E. coli* O157:H7 was added to one reaction as a positive control, and no DNA was added to another reaction for a negative control. The 16S rDNA gene specific for *E. coli* (EC, table 4) was included as a positive-control run with every sample. This control was an amplification of a gene specific for all strains of *E. coli*. To be sure the sample was amplifiable, this product must be visible. If it was not visible the sample was repeated.

Results of Bacterial Analyses

Bacterial analysis of river water collected from 14 locations in Oakland County in August and September 2003 revealed high fecal indicator concentrations including exceedences of recreational water-quality standards throughout the county. Antibiotic-resistant fecal indicator bacteria

Table 4. Primers and polymerase chain reaction (PCR) conditions for the rfb_{0157} gene, and multiplex PCR for the virulence genes.

[5´, five prime end; 3´, three prime end; F, forward primer; R, reverse primer; G, guanidine; C, cytosine; A, adenosine; T, tyrosine; min, minute; sec, second; °C, degrees Celsius]

Gene	Primers 5´-3´	Thermocycler conditions	Reference
rfb_{O157}	F: CGT GAT GAT GTT GAG TTG R: AGA TTG GGT TGG CAT TAC TG	5 min 94°C 30 cycles: 1 min 94°C 1 min 53°C 1 min 72°C 5 min 72°C	Osek, 2003 Maurer and others, 1999
Multiplex PCR			
*stx*1	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG		
*stx*2	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA GCA CTT TG	10 min 95°C 35 cycles: 30 sec 95°C 40 sec 56°C 1 min 72°C 5 min 72°C	Gannon and others, 1992 Fagan and others, 1999 Sabat and others, 2000
*eae*A	F: GTG GCG AAT ACT GGC GAG ACT R: CCC CAT TCT TTT TCA CCG TCG		
EC	F: GGA AGA AGC TTG CTT CTT TGC TGA C R: AGC CCG GGG ATT TCA CAT CTG ACT TA		

were isolated from all samples collected and tests indicative of pathogenic *E. coli* were positive in samples with high-level antibiotic resistances.

Fecal indicator bacteria enumeration

Fecal indicator bacteria (fecal coliforms, *E. coli*, and enterococci) were quantified in the samples from the 14 Oakland County streams and two samples from reference sites collected in August and September 2003 (appendix A); results are shown in figure 2. *E. coli* concentrations ranged from 3 colony forming units (CFU) per 100 mL (August and September at site 04170500) to a high of 25,000 CFU/100 mL (site 04166200 in August) and a high of 1,300 CFU/100 mL (site 04166315) in September. The *E. coli* concentrations were compared to the Michigan water-quality standards for recreational waters (Michigan Water Quality Standard Rule 62 of the Michigan Water Quality Standards: 130 *E. coli*/100 mL of water as a 30-day geometric mean and 300 *E. coli*/100 mL of water at any one sampling). Seven of the sites in August and 8 of the 14 sites in September exceeded the single sample criterion of 300 *E. coli*/100 mL of water (fig. 2). All seven of these samples in August and one in September had *E. coli* concentrations greater than 1,000 CFU/100 mL. *E. coli* concentrations were greater in August than in September at nine of the sites and greater in September than August at just four sites. In August, the mean number of *E. coli* was 2,497 CFU/100 mL with a median of 650 CFU/100 mL, compared to a mean of 375 CFU/100 mL in September with a median of 315 CFU/100 mL. None of the three reference samples collected (2 from site 04135500 and one from site 04001000) had concentrations that exceeded 300 *E. coli*/100 mL.

Enterococci and fecal coliform bacteria concentrations also were determined in August and September (figs. 3, 4, and appendix A). Although Michigan does not have water-quality standards for enterococci or fecal coliform bacteria, USEPA recommends that enterococci concentrations do not exceed 61 CFU/100 mL water in a single sample for recreational waters (U.S. Environmental Protection Agency, 2000). In August, 13 of 14 samples exceeded this recommendation, and 12 of 14 samples exceeded the recommendation in September. In August, the mean number of enterococci was 464 CFU/100 mL (median of 316 CFU/100 mL) and in September, 466 CFU/100 mL (median of 370 CFU/100 mL). At seven sites, enterococci concentrations were greater in September than in August, in contrast to the *E. coli* concentrations which were more frequently greater in August. None of the reference samples exceeded the USEPA recommendations for enterococci. The highest concentration for a reference site was only 14 enterococci/100 mL at site 04135500. Fecal coliforms are not currently a recommended indicator of water quality; however, prior to 1986, USEPA recommended that fecal coliforms not exceed 400 CFU/100 mL for a single sample for primary contact waters (U.S. Environmental Protection Agency, 1976). This criterion was exceeded in 12 out of 14 Oakland County sites in August and 9 sites in September (fig. 1 and appendix A). No reference samples exceeded this criterion. In August fecal coliform bacteria concentrations ranged from 6 CFU/100 mL at site 04170500 to 126,000 CFU/100 mL at site 04166200. In September, concentrations ranged from 3 CFU/100 mL (site 04170500) to 3,300 CFU/100 mL (site

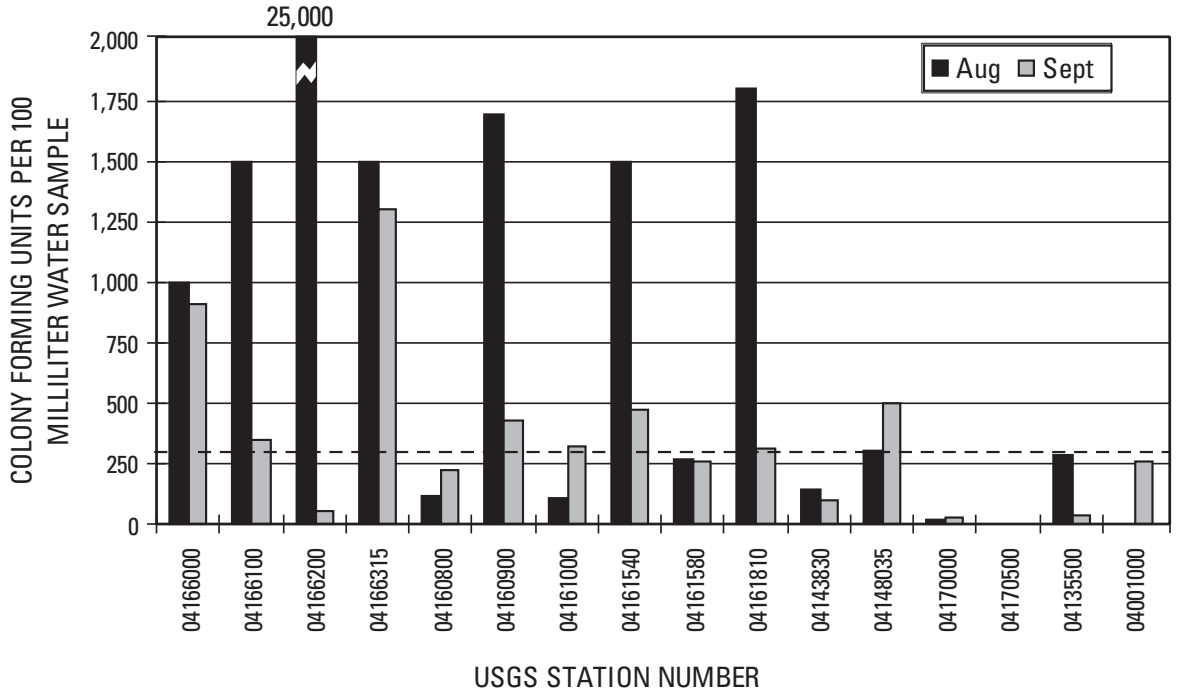

Figure 2. *Escherichia coli* concentrations for Oakland County and reference samples collected in August and September 2003. Dotted line represents Michigan Water Quality Standard for single sample, limit of 300 colony forming units /100 mL for recreational waters.

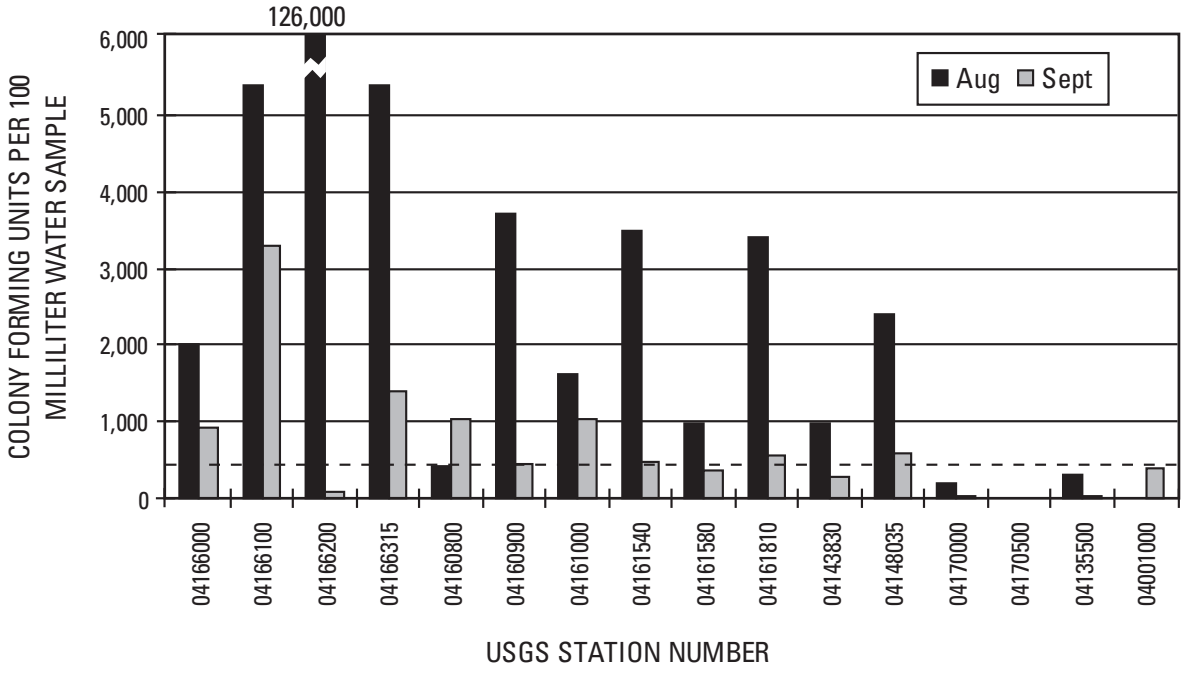

Figure 3. Fecal coliform concentrations for Oakland County and reference samples collected in August and September 2003. Dotted line
units /100 mL for primary contact waters.

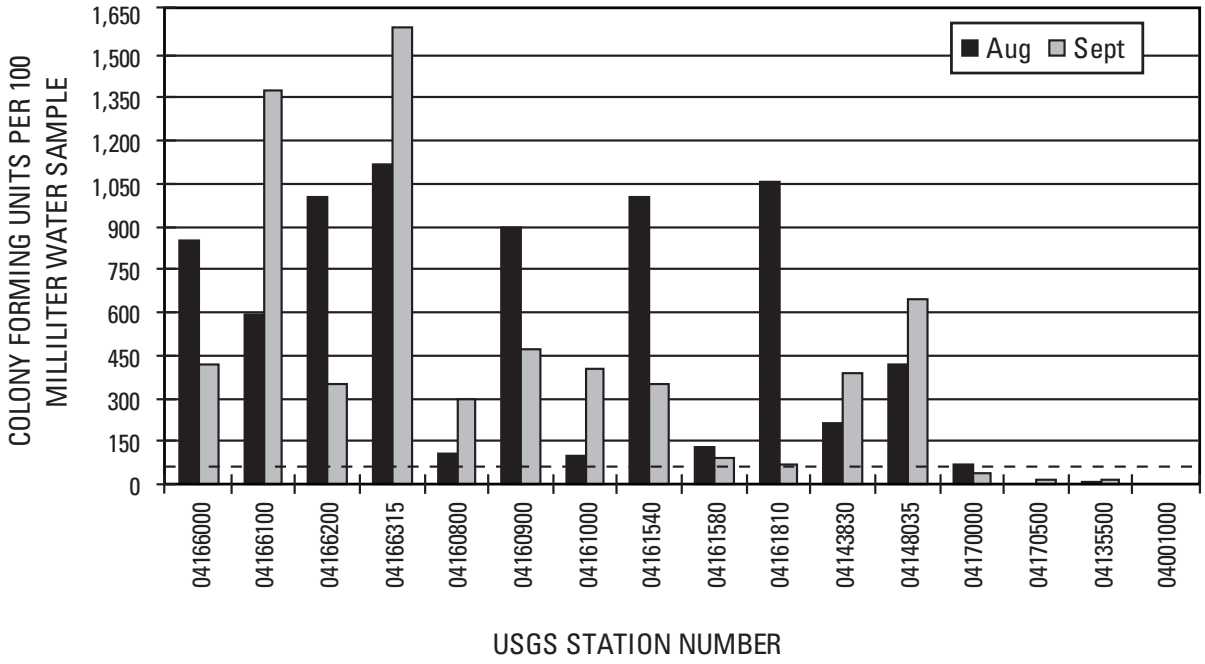

Figure 4 . Enterococci concentrations for Oakland County and reference samples collected in August and September 2003. Dotted line repr
waters.

04166100). In nine Oakland County samples in August and four in September, fecal coliform concentrations were greater than 1,000 CFU/100 mL. At all but one site, fecal coliform concentrations were greater in August than September. The median Oakland County fecal coliform number in August was 2,200 CFU/100 mL and in September was 525 CFU/100 mL. In August, fecal coliform concentration for the tested reference site (04135500) was 320 CFU/100 mL. In September, fecal coliform bacteria concentrations were highest at site 04001000 at 380 CFU/100 mL (fig. 2).

Samples collected in August were held beyond the recommended 6-hour holding time. In addition, stream water temperature was on average 3°C warmer in August. Both August and September samples were collected at low flow; however, streamflows were on average 5.3 ft^3/s greater during the August sampling, which took place after a small rainstorm in the county. Field-measured characteristics corresponding to the September and August samplings are listed in appendix B.

Antibiotic resistances

Widespread antibiotic resistance was found among the fecal coliform and enterococci groups in both August and September. Multiple antibiotic resistant fecal coliform bacteria were found at all sites sampled. *E. coli* and enterococci resistant to antibiotics of clinical concern were recovered from several Oakland County sites. In addition, genetic elements that could serve as a vector for the transfer of antibiotic resistance also were found at several Oakland County sites.

Antibiotic-resistant fecal coliform bacteria

All 14 Oakland County samples contained fecal coliform isolates resistant to at least one of six tested antibiotics (ampicillin, cephalothin, cefoxitin, ceftriaxone, gentamicin, streptomycin) in both August and September 2003. Tetracycline was not included because results were not interpretable for all samples. Appendix C and D list the percentage or number of fecal coliform bacteria resistant to the tested antibiotics. Because of the methods used in August, antibiotic-resistant fecal coliform bacteria were difficult to identify. The percentage of fecal coliform bacteria resistant to each antibiotic could not be verified. In many cases, overgrowth of bacteria on the plate limited results to a finding of resistance to that particular antibiotic for that sample; quantifying the number of resistant isolates and verifying that the resistant isolates of fecal coliforms were precluded. In September, isolates were individually tested, and resistant fecal coliform bacteria were quantified. Every sample collected in September contained fecal coliform isolates resistant to multiple antibiotics (table 5). Out of a total of 518 fecal coliform isolates tested from Oakland County watersheds, only 30 of them did not show resistance to any of the 6 tested antibiotics, and only 149 were resistant to only 1 antibiotic. The

Table 5. Percentage of fecal coliform bacteria resistant to multiple antibiotics in September 2003.

USGS station number	Total fecal coliform bacteria tested	Percentage of isolates sensitive to all antibiotics	Antibiotic resistances (percent)[1]					
			1	2	3	4	5[a]	6[a]
Rouge River watershed								
04166000	50	44	40	10	6	0	0	0
04166100	50	0	82	14	2	2	0	0
04166200	8	0	0	50	38	13	0	0
04166315	14	0	21	36	29	7	**7[a]**	0
Clinton River watershed								
04161580	36	0	0	0	3	25	**50[a]**	**22[a]**
04160900	45	0	42	36	18	4	0	0
04161540	48	0	27	31	25	8	**4[a]**	**4[a]**
04161810	50	2	10	20	40	20	**8[a]**	0
04161000	50	0	16	28	56	0	0	0
04160800	50	0	0	30	36	20	**14[a]**	0
Other Oakland County watersheds								
04148035	50	6	32	38	18	6	0	0
04143830	29	0	3	7	69	17	**3[a]**	0
04170500	5	0	0	100	0	0	0	0
04170000	33	6	30	15	36	12	0	0
Reference sites								
04001000	30	0	37	33	27	0	**3[a]**	0
04135500	5	40	40	20	0	0	0	0

[1] Percentage of isolates resistant to 1, 2, 3, 4, 5, or 6, different antibiotics.

[a] Bold samples represent isolates in which multiple antibiotic resistances may be a result of an acquired resistance.

remaining 339 fecal coliform isolates were resistant to 2 or more antibiotics.

At Stony Creek near Romeo (04161580), 22 percent of the fecal coliform isolates were resistant to all six antibiotics. Similarly, 4 percent of the fecal coliform isolates from Paint Creek at Rochester (04161540) also were resistant to all 6 antibiotics. Both sites are within the Clinton River watershed. In addition, 50 percent and 4 percent of the fecal coliforms from these two sites (04161580 and 04161540, respectively) were resistant to five of the tested antibiotics. Two other Clinton River watershed samples also contained fecal coliform isolates resistant to 5 of the 6 tested antibiotics: Sashabaw River near Drayton Plains (04160800), 14 percent and Clinton River at Yates (04161810), 8 percent. Two other Oakland County sites also had fecal coliform isolates that were resistant to 5 of the 6 antibiotics: Shiawassee River at Holly (04143830), 3 percent; and Upper River Rouge at Clarenceville (04166315), 7 percent. Neither the reference sample from Washington Creek (04001000) nor that from Au Sable River at Grayling (04135500) contained any fecal coliform isolates that were resistant to all six tested antibiotics. However, at site 04001000, 3 percent of the fecal coliform isolates were resistant to five antibiotics.

Fecal coliform bacteria resistant to cephalosporins were found in all samples collected from Oakland County (table 6). In the Clinton River watershed, fecal coliform bacteria with antibiotic-resistance profiles indicative of ESBLs were found at five out of six sites (04161580, 04161540, 04160800, 04161810, and 04160900; fig. 5). These isolates were resistant to cephalothin, ceftriaxone, and ampicillin and were susceptible to cefoxitin. In addition, isolates from four of these sites (04161580, 04161540, 04161810, and 04160800) not only had this ESBL profile but also were resistant to other non-β-lactam antibiotics tested. Of most interest were isolates from sites 04161580, 04161540, and 04161810 in which 2, 1, and 1 isolate, respectively, were resistant to cephalothin, ceftriaxone, ampicillin, tetracycline, gentamicin, and streptomycin. This is a strong indication that this resistance may be due to a plasmid-carrying multiple antibiotic-resistance genes. In contrast, only one of the four River Rouge sites had isolates resistant to

cephalothin, ceftriaxone, and ampicillin with a susceptibility to cefoxitin (04166100; fig. 5). Sites 04166000 and 04166315 in this same watershed had isolates resistant to cephalothin and cefoxitin but susceptible to ampicillin. The remaining River Rouge watershed sample did not have any isolate resistant to cephalothin and ceftriaxone. Other sites sampled in Oakland County also resulted in fecal coliform isolates with an ESBL profile, but none of these were resistant to the non-β-lactam antibiotics (table 6 and fig. 5).

The fecal coliform isolates in this study were not identified to the genus or species level and were not tested for antibiotic-resistance genes; intrinsic resistance, therefore, cannot be ruled out. However, many of fecal coliform bacteria in this study showed resistance patterns indicative of acquired resistance, such as multiple resistances to antibiotics in different groups and resistance patterns indicative of ESBL production. Two Clinton River watershed sites, 04161580 and 04161540, had isolates resistant to all tested antibiotics and isolates with ESBL patterns of resistance. By use of molecular techniques, resistant organisms could be identified and genes responsible for resistance could be detected; such an analysis would yield more information as to the significance of these results.

Antibiotic-resistant *E. coli*

From the FC cultures, presumptive *E. coli* (isolates that grew on mFC at 44.5°C and fluoresced under UV light on nutrient agar with MUG) that were resistant to one of the cephalosporin antibiotics were isolated from seven Oakland County sites (04166000, 04160900, 04148035, 04161540, 04160800, 04161580, and 04161810). By means of a set of additional biochemical tests, isolates phenotypically confirmed to be *E. coli* were obtained from only three sites (04166000, 04160900, and 04161540). The remainder were determined

Table 6. Resistances of individual fecal coliform bacteria to cephalosporin antibiotics, September 2003.

[Bold samples represent resistance of clinical significance; CEP, cephalothin; FOX, cefoxitin; AXO, ceftriaxone]

USGS station number	Total fecal coliform isolates tested	CEP only	FOX only	AXO only	CEP + FOX	CEP + AXO[a]	FOX + AXO	CEP + FOX + AXO	No cephalosporin resistance
River Rouge watershed									
04166000	50	2	0	**1**	3	**1[a]**	0	0	7
04166100	50	46	0	0	2	**2[a]**	0	0	0
04166200	8	0	1	**1**	2	0	1	3	0
04166315	14	4	0	0	6	**2[a]**	0	2	0
Clinton River watershed									
04160800	50	22	0	0	14	**6[a]**	0	8	0
04160900	45	21	2	0	15	**4[a]**	0	3	0
04161000	50	17	0	0	32	0	0	0	1
04161540	48	18	0	0	4	**16[a]**	0	10	0
04161580	36	0	0	0	0	**5[a]**	0	31	0
04161810	50	14	1	0	23	**3[a]**	0	4	5
Other Oakland County watersheds									
04143830	29	5	0	0	23	0	0	1	0
04148035	50	30	1	**1**	9	**1[a]**	0	2	6
04170000	33	7	0	0	15	**2[a]**	0	0	9
04170500	3	0	0	0	0	0	3	0	0
Reference sites									
04001000	30	21	0	0	5	**1[a]**	0	2	1
04135500	5	2	1	0	0	0	0	0	2
Total	551	209	6	**3**	153	**43**	4	66	31

[a] Pattern indicative of extended-spectrum-β-lactamase (ESBL)-producing fecal coliform bacteria.

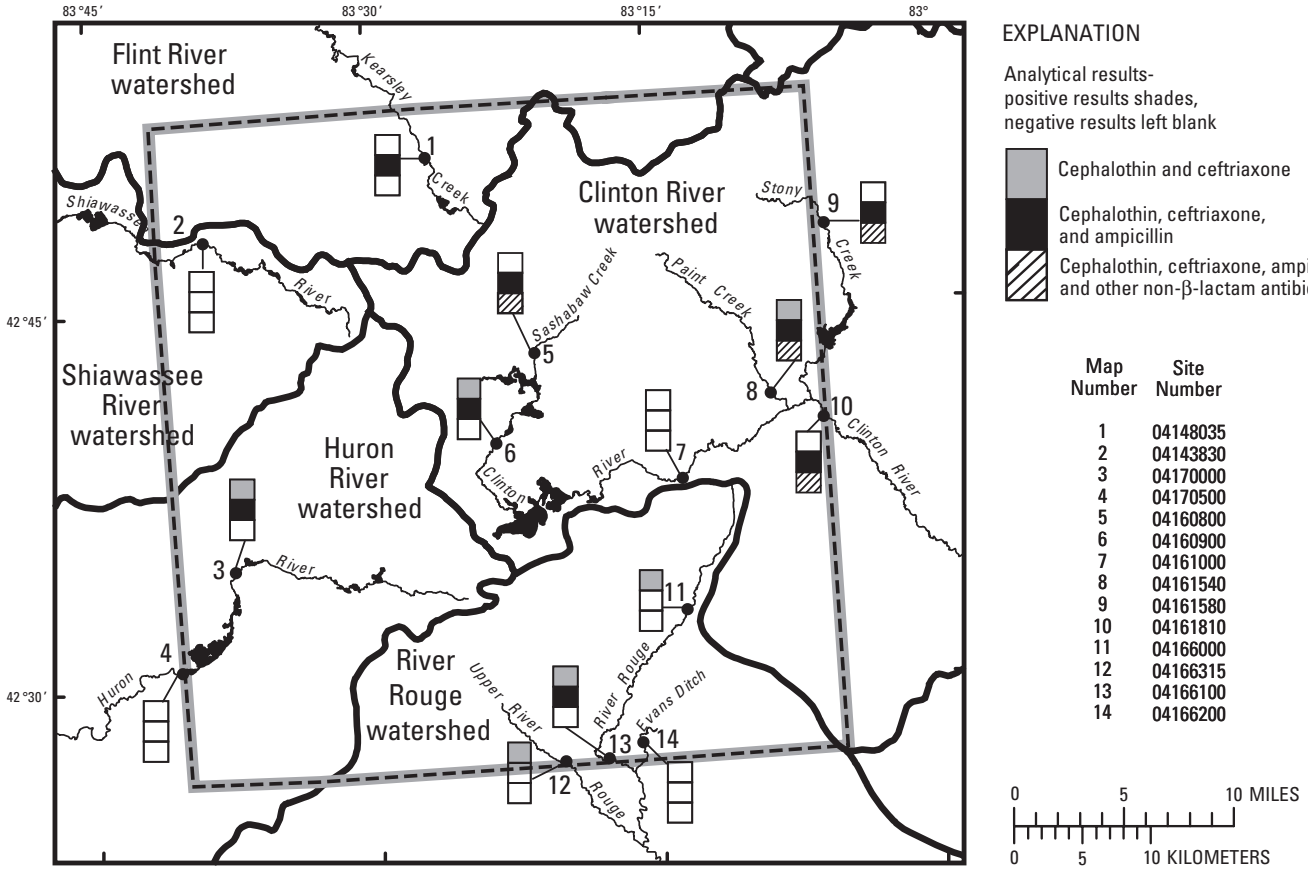

Figure 5. Oakland County, Mich., sites that contain fecal coliform bacteria with antibiotic resistance profiles indicative of extended-spectrum-β-lactamases (ESBLs) [cephalothin and ceftriaxone; cephalothin, ceftriaxone, and ampicillin; cephalothin, ceftriaxone, ampicillin, and one or more non-β-lactam antibiotics (tetracycline, streptomycin, and/or gentamicin)].

to be false positives on the basis of a negative indol reaction. Other studies have reported several bacterial genera that could result in a false positive for β-glucuronidase reaction including *Klebsiella*, *Enterobacter*, *Shigella*, and *Salmonella* (Sarhan and Foster, 1991; McDaniels and others, 1996; Geissler and others, 2000). All of these genera contain species that are potential pathogens of human health concern. More investigation is necessary to determine the identity of these non-*E. coli* isolates from Oakland County.

Phenotypically confirmed *E. coli* resistant to one of three tested cephalosporin antibiotics were tested for intermediate-level (I) and high-level (H) resistance to cephalothin (I=16 μg/mL, H=32 μg/mL), cefoxitin (I=16 μg/mL, H=32 μg/mL), ceftriaxone (I=16 μg/mL, H=64 μg/mL), gentamicin (I= 8 μg/mL, H=16 μg/mL), and ampicillin (I=16 μg/mL, H=32 μg/mL). Results are listed in table 7. One *E. coli* isolate obtained from site 04166000 showed a high level of resistance to cephalothin, cefoxitin, and ceftriaxone and intermediate resistance to gentamicin. Other *E. coli* isolates from this site also showed high-level resistance to cephalothin. *E. coli* isolates with high-level resistance to both cephalothin and ampicillin were also recovered from site 04160900. *E. coli* were recovered from site 04161540 with high-level of resistance to cephalothin.

Integrons were detected in *E. coli* isolated from sites 04160900 and 04166000 (table 7). Integrons are transferable among similar organisms. Further studies are needed to determine whether the antibiotic resistances seen in the *E. coli* isolates in this study are encoded by genes on the integrons that are present. Other genes responsible for resistance to the cephalosporin antibiotics and ampicillin were not evaluated in this study; however, these genes should not be overlooked as additional possibilities to explain the resistance patterns detected.

Antibiotic-resistant enterococci

Enterococci isolates from both August and September were tested for resistance to a low level (8 μg/mL) and intermediate level (16 μg/mL) of vancomycin. Low-level vancomycin resistance was found in enterococci from 8 of the 15 samples collected in August and 13 of the 15 samples with enterococci in September (table 8). Some enterococci species

Table 7. *Escherichia coli* antibiotic-resistance profiles and integron-detection results.

[AMP, ampicillin; CEP, cephalothin; FOX, cefoxitin; AXO, ceftriaxone; GEN, gentamicin; S, susceptible; I, intermediate level resistance; H, high level resistance, µg/mL, microgram per milliliter; +, positive result; -, negative result, ≥, greater than or equal to; ≤, less than or equal to]

USGS station number	Isolate number	AMP	CEP	FOX	AXO	GEN	Integron
04166000	1	S	R	R	R	I	-
	2	I	R	S	S	S	+
	3	S	R	S	S	S	-
	4	I	I	S	S	S	-
	5	S	I	S	S	S	-
04160900	1	R	R	S	S	S	+
	2	R	R	S	S	S	-
	3	S	R	S	S	S	-
	4	R	I	S	S	I	-
	5	S	I	S	S	S	-
04161540	1	S	R	I	S	S	-
	2	S	R	S	S	S	-
	3	S	I	S	S	S	-
S[1]		≤ 8 µg/mL	≤ 8 µg/mL	≤ 8 µg/mL	≤ 8 µg/mL	≤ 4 µg/mL	
I[1]		16 µg/mL	16 µg/mL	16 µg/mL	16-32 µg/mL	8 µg/mL	
R[1]		≥ 32 µg/mL	≥ 32 µg/mL	≥ 32 µg/mL	≥ 64 µg/mL	≥ 16 µg/mL	

[1] Resistance levels based on NCCLS minimum inhibitory concentration (MIC) interpretive standard for *Enterobacteriaceae* (National Committee for Clinical Laboratory Standards, 2002).

have an intrinsic resistance to low levels of vancomycin, but these species are usually not resistant to higher levels. Intermediate to high levels of vancomycin resistance are often a result of a resistance gene that is carried on a plasmid. Enterococci resistant to intermediate levels of vancomycin were found at one site in August (04166200) and at five sites in September (04170500, 04161540, 04148035, 04143830, and 04160800; table 8). For all but site 04170500, enterococci concentrations were at least 300 CFU/100 mL. Not all samples with concentration greater than 300 CFU/100 mL contained vancomycin-resistant colonies.

In addition to vancomycin, all August and September enterococci isolates were tested for their resistance to gentamicin and streptomycin. Gentamicin-resistant enterococci were isolated from 10 Oakland County sites in August and 3 sites in September (table 8). Only one site contained gentamicin-resistant enterococci on both dates (site 04160800). Streptomycin-resistant enterococci were isolated from 8 Oakland County sites in August and 5 sites in September; samples from only three of those sites contained streptomycin-resistant enterococci on both dates (table 8). Neither gentamicin nor streptomycin-resistant enterococci were found at the reference sites.

Three enterococci isolates resistant to high levels of vancomycin (≥ 64 µg/mL) were isolated from the ENT cultures from the August Evans Ditch in Southfield (04166200) and September Kearsley Creek at Ortonville (04148035) samples. The Evans Ditch isolate and two of the Kearsley Creek enterococci isolates were resistant to 128 µg/mL vancomycin. Api Strep20 identification strips identified these isolates as *E. faecium*. By use of PCR, the *van*A gene, which is responsible for acquired high-level vancomycin resistance (≥ 128 µg/mL), was detected in the isolate from Evans Ditch and in one of the isolates from Kearsley Creek. *E. faecium* is the most frequently isolated vancomycin-resistant *Enterococcus* in clinical settings (Kak and Chow, 2002). The *van*A gene is usually carried on plasmids, which are easily transferred among similar organisms. The transfer of this resistance is possible with the presence of the *van*A gene. Other isolates resistant to intermediate levels of vancomycin also were isolated from these two sites and from Paint Creek at Rochester (04161540); these isolates were identified as *E. faecium* and *E. faecalis*. Both of these enterococci species have potential to cause disease in humans. Vancomycin-resistant isolates would make treating such infection extremely difficult.

Table 8. Percentage of enterococci isolates resistant to tested antibiotics.

[VAN, vancomycin; STR, streptomycin; GEN, gentamicin; NA, not available; >, greater than]

	August 2003[a]					September 2003[b]				
USGS station number	Total entero-cocci iso-lates	VAN 8 µg/mL	VAN 16 µg/mL	STR 2000 µg/mL	GEN 100 µg/mL	Total entero-cocci isolates	VAN 8 µg/mL	VAN 16 µg/mL	STR 2000 µg/mL	GEN 100 µg/mL
River Rouge watershed										
04166000	844	6	0	0	0	42	3	0	0	0
04166100	596	0	0	8	0	50	0	0	0	2
04166200	>1,000	6	2	2	2	35	0	0	0	0
04166315	1,096	0	0	40	92	50	3	0	1	0
Clinton River watershed										
04160800	96	0	0	16	38	30	10	2	0	1
04160900	906	2	0	0	2	47	4	0	2	0
04161000	100	2	0	2	14	39	1	0	3	0
04161540	>1,000	0	0	14	2	35	7	3	2	0
04161580	118	4	0	0	8	50	1	0	1	0
04161810	1,032	0	0	2	36	50	4	0	0	0
Other Oakland County watersheds										
04143830	206	0	0	0	26	39	6	3	0	0
04148035	416	0	0	0	0	50	11	4	0	2
04170000	70	2	0	0	0	35	2	0	0	0
04170500	2	4	0	2	2	13	5	2	0	0
Reference sites										
04001000	NA	NA	NA	NA	NA	0	NA	NA	NA	NA
04135500	5	4	0	0	0	14	1	0	0	0

[a]August antibiotic resistance test included all mEI isolates which may include non-enterococci.

[b]September antibiotic resistance test was on individual mEI positive isolates, therefore, tested isolates are all presumptive enterococci.

Potential sources of resistance

In Oakland County, cephalothin resistance was seen in a large percentage of isolates from nearly all sites in September (appendixes C and D). The exceptions were the River Rouge site at Birmingham (04166000) and the Huron River site at Milford (04170500), where only 10 percent of the isolates were resistant to cephalothin at 04166000 and none of the isolates at site 04170500. At site 04170500, there were only three fecal coliform bacteria per 100 mL water to test, and all three were resistant to ceftriaxone and cefoxitin. Fecal coliform bacteria resistant to ampicillin were also found at every site in September; however, the percentage of the isolates resistant to ampicillin varied with watershed. Fecal coliform bacteria resistant to ceftriaxone were found throughout the county (appendixes C and D). Only at the Clinton River at Auburn Hills (04161000) were ceftriaxone-resistant fecal coliform bacteria absent. This site did have high levels of cephalothin-, cefoxitin-, and ampicillin-resistant fecal coliform bacteria. In contrast, the percentages of fecal coliform bacteria resistant to tetracycline, streptomycin, and gentamicin were lower than the percentages at the other Oakland County sites (appendixes C and D).

The two reference sites included in this study are areas with less human presence and activity then in Oakland County. The first is a site on the Au Sable River in Grayling (04135500) and the second, Washington Creek, is on Isle Royale (04001000). Only 5 CFU/100 mL fecal coliform bacteria were present in the September Au Sable River sample to test for antibiotic resistance. These fecal coliform bacteria were all susceptible to ceftriaxone, ampicillin, and gentamicin; 60 percent, 40 percent, 20 percent, and 20 percent of the

isolates were resistant to tetracycline, cephalothin, streptomycin, and cefoxitin, respectively. Antibiotic-resistance patterns in September at Washington Creek were similar to those for many of the samples collected in Oakland County (appendixes C and D); 97 percent of the Washington Creek fecal coliform isolates were resistant to cephalothin. Resistance for the other tested antibiotics ranged from 20 to 27 percent.

For the enterococci isolates, resistance to intermediate levels of vancomycin (16 µg/mL) was found at only one site in August, the Evans Ditch at Southfield site (04166200), and at five Oakland County sites in September. These sites include two from the Clinton River watershed (04160800 and 04161540), the Shiawassee River site (04143830), the Flint River watershed site (04148035), and the Huron River (04170500). No sample from the River Rouge watershed in September contained enterococci resistant to intermediate levels of vancomycin. Streptomycin-resistant enterococci were found at four out of the six Clinton River watershed sites (04160900, 04161000, 04161540, and 04161580), but at only one of the River Rouge watershed sites (04166315). The gene responsible for high-level vancomycin resistance commonly found on plasmids was found in *E. faecium* isolated from two Oakland County sites, Kearsley Creek (04148035) in September and at Evans Ditch (04166200) in August.

Although the source of the fecal contamination cannot be determined from these data, there is evidence that the fecal indicator bacteria communities are different among the various watersheds. The Huron River watershed samples had much less indication of fecal contamination than the other Oakland County sites. All fecal indicator bacteria concentrations were much smaller for Huron River samples than for samples from other watersheds (in most cases meeting water-quality standards) and in some cases lower than those at the reference sites (figs. 2-4). The Clinton River watershed samples tended to have more multiply-resistant isolates. For example, four of the six sites contained fecal coliform isolates resistant to five or six antibiotics, whereas only one River Rouge watershed sample had fecal coliform isolates resistant to many antibiotics (table 5). In addition, five of the Clinton River watershed samples had fecal coliform isolates with resistances to cephalothin, ceftriaxone, and ampicillin, in contrast to just one sample from the River Rouge watershed and one from the Huron River watershed (fig. 5). Finally, enterococci with intermediate- to high-level vancomycin resistance were not frequently detected in either the Clinton or River Rouge watersheds (two sites in the Clinton and one in the Rouge), but they were detected in all but one of the other Oakland County samples (table 8). The differences seen in both patterns of antibiotic resistance and concentrations of fecal indicator bacteria suggest the communities of fecal indicator bacteria are different between the major watersheds in the County.

Water-chemistry data for September 2003 also suggest sources may differ between samples and watersheds. Sodium, chloride, calcium, and phosphorus were all higher in the Rouge River watershed samples in September than in any other sample (Aichele and other, 2004). “Wastewater constituents” (a suites of analytes including pharmaceuticals, hormones, and household chemical products) were detected at all Oakland County sites (Aichele and others, 2004). Certain groups of these constituents were more commonly found in particular watersheds. For example, cholesterol and prometon (herbicide) were detected in three of the four River Rouge watershed samples, but were not detected in any other sample during this time period. The detergent-degradation compound, 4-tert-octylphenol was detected in five of the six Clinton River watershed samples and in only one River Rouge and one Huron River watershed sample.

Pathogenic bacteria

Based on previous studies (Boerlin and others, 1999), *E. coli* O157 that possess the *eae*A and *stx*2 genes pose a greater human health threat than other serotypes with other gene combinations. Multiple lines of evidence were used to identify *E. coli* O157 in this study, as previously mentioned. *E. coli* O157 is indicated by positive GA, IA, and/or rfb_{0157} results. Results of these analyses are shown in figure 6 and listed in table 9.

Indications of potentially pathogenic *E. coli* were detected throughout Oakland County watersheds. Positive IA and rfb_{0157} results were obtained for three sites: 04161000, 04161810, and 04166000 in August and 04166000 again in September. Only two of these sites (04161000 and 04161810) were GA positive. Three other sites (04143830 and 04166200 in August, and 04166315 in September) were GA and IA positive but rfb_{0157} negative. No Oakland County sites were rfb_{0157} positive and IA negative, but seven sites were GA positive and IA negative, all of which were from the September sampling (fig. 6 and table 9). Further evaluation would be necessary to positively identify these samples as carrying *E. coli* O157. However, the multiple lines of evidence used in this study do indicate the potential for these organisms to be present in waters within Oakland County. In contrast, the three samples from the reference sites were all IA and rfb_{0157} negative; one sample was GA positive.

Table 9. Results of pathogenic *Escherichia coli* tests.

[IA, immunological assay; GA, growth-based assay; CFU, colony forming units; mFC, fecal coliform media; µL, microliters; PCR, polymerase chain reaction; +, positive result; -, negative result; NT, not tested]

USGS station number	August 2003						September 2003					
			PCR amplified genes						PCR amplified genes			
	IA	GA (CFU/µL mFC stock)	*eae*A	*stx*1	*stx*2	rfb_{0157}	IA	GA (CFU/µL mFC stock)	*eae*A	*stx*1	*stx*2	rfb_{0157}
Rouge River watershed												
04166000	+	0	+	-	-	+	+	0	+	-	+	+
04166100	-	0	+	-	+	-	-	0	+	-	-	-
04166200	+	100	+	-	+	-	-	1,500	+	-	+	-
04166315	-	0	+	-	+	-	+	8	-	-	-	-
Clinton River watershed												
04160800	-	0	+	-	-	-	-	300	+	-	-	-
04160900	-	0	+	-	+	-	-	0	+	-	+	-
04161000	+	10,000	+	-	-	+	-	200	+	-	-	-
04161540	-	0	+	-	-	-	-	1	+	-	+	-
04161580	-	0	+	+	-	-	-	0	+	-	+	-
04161810	+	100	+	-	-	+	-	7	+	-	+	-
Other Oakland County watersheds												
04170000	-	0	+	-	-	-	NT	NT	NT	NT	NT	NT
04170500	-	0	-	-	-	-	NT	NT	NT	NT	NT	NT
04143830	+	100	+	-	+	-	-	6,000	+	-	+	-
04148035	+	0	+	-	-	-	-	8,000	+	-	-	-
Reference sites												
04135500	-	1	+	+	-	-	-	0	+	-	-	-
04001000	NT	NT	NT	NT	NT	NT	-	0	+	-	-	-

Three genes commonly associated with STEC were analyzed for this study: *eae*A, *stx*1, and *stx*2. As previously mentioned, the combination of *eae*A and *stx*2 genes poses the greatest threat to human health. This gene combination was detected in FC cultures from five Oakland County sites in August and seven sites in September (fig. 6 and table 9). Of these, only three sites had FC cultures that were also IA positive (sites 04166200 and 04143830 in August and 04166000 in September) and two that were also GA positive (sites 04166200 and 04143830 in August). In September, the FC culture from site 04166000 was IA and rfb_{0157} positive and had both the *eae*A and *stx*2 genes. No FC culture was positive for all three indicators of *E. coli* O157 as well as the *eae*A and *stx*2 genes. Only the FC culture from site 04161580 was positive for the *stx*1 gene (fig. 6 and table 9). The *eae*A gene was present in all three of the FC cultures from the reference samples, but *stx*2 was absent from all three samples. The *stx*1 gene was detected in one reference samples (table 9). Representative gel images resulting from the PCR for the toxin genes, *eae*A, *stx*1, and *stx*2 are shown in figs. 7 and 8. Results of the toxin gene PCR are listed in table 9.

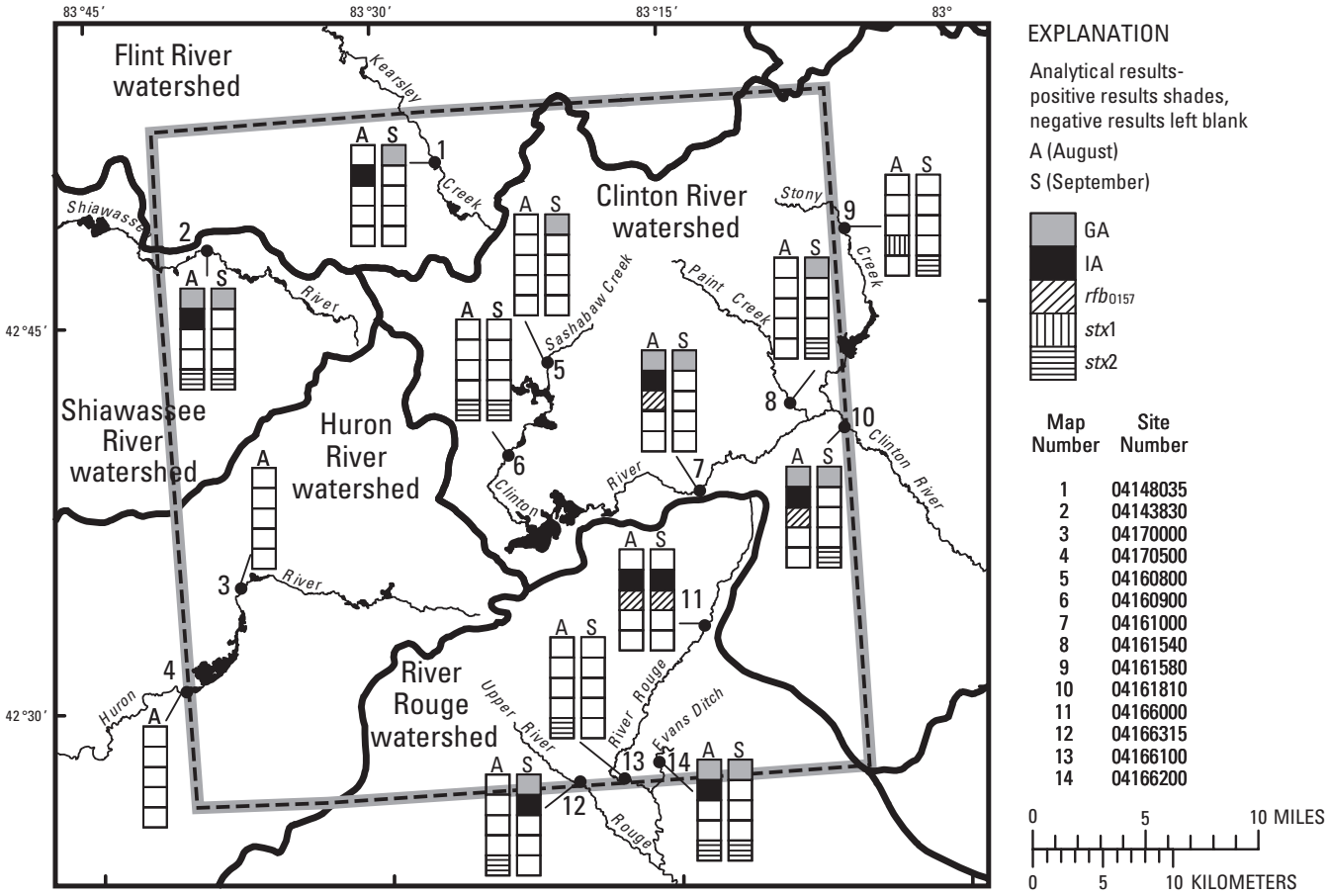

Figure 6. Oakland County, Mich., sites that contain positive results for the following: IA, GA and rfb_{0157} for the detection of *E. coli* O157; and genes (*eae*A, *stx*1, and *stx*2) responsible for pathogenicity.

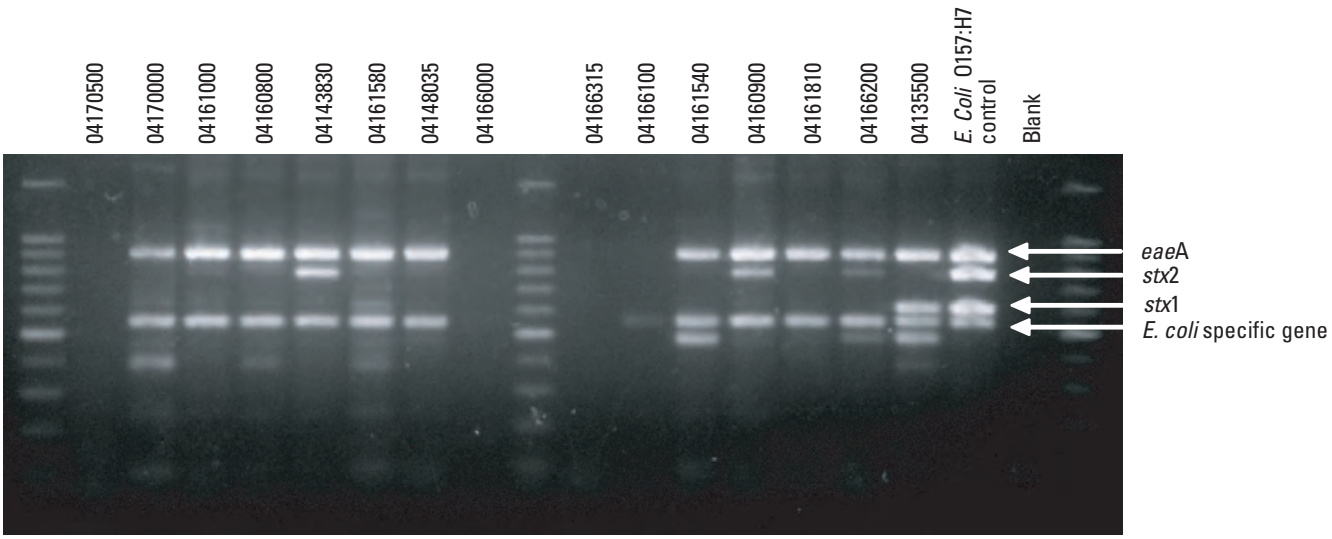

Figure 7. Polymerase chain reaction detection of toxin genes (*eae*A, *stx*1, and *stx*2) and an *Escherichia coli* (*E. coli*) specific gene, in samples collected August 2003 in Oakland County watersheds, Mich.

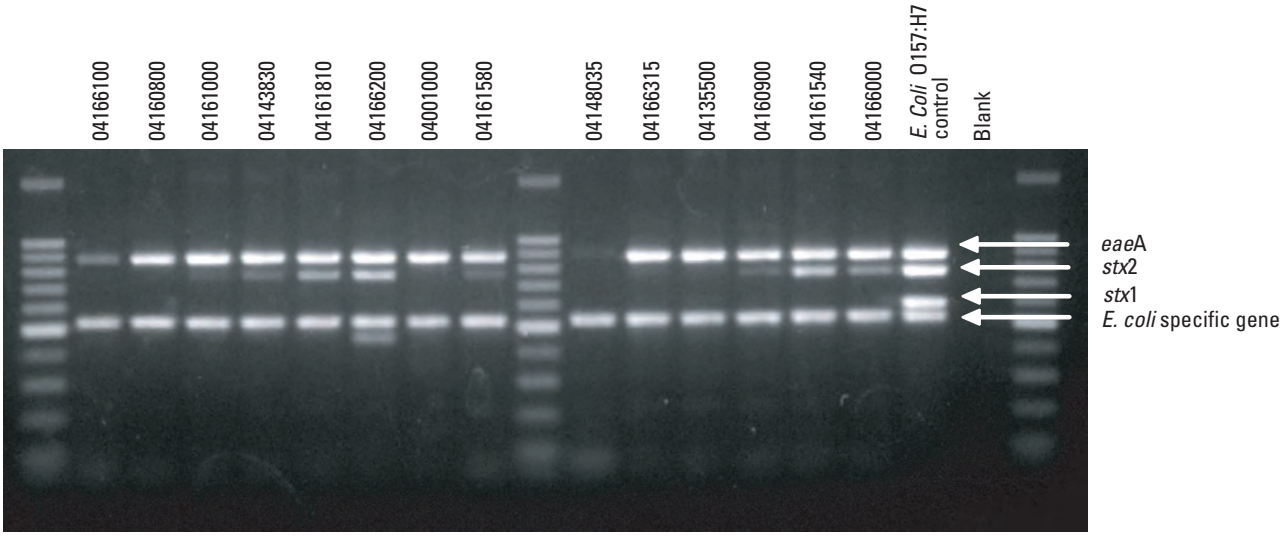

Figure 8. Polymerase chain reaction detection of toxin genes (*eae*A, *stx*1, and *stx*2) and an *Escherichia coli* (*E. coli*) specific gene, in samples collected September 2003 in Oakland County watersheds, Mich.

Summary and Conclusions

To better understand the microbial water quality of streams in Oakland County, Mich., the USGS in cooperation with Oakland County Department of Human Services conducted a preliminary study of streams in the County to determine if antibiotic resistances of clinical significance occurred in fecal indicator bacteria and if potentially pathogenic *E. coli* could be detected. Results of this study indicate several exceedences in fecal indicator concentrations, antibiotic resistances of clinical concern, indications of acquired antibiotic resistances, and evidence of potentially pathogenic *E. coli* from surface-water samples. Samples for this study were initially collected in August 2003 from 14 streams in Oakland County. Interpretations of the results from the August sampling are limited because of difficulties in sampling and processing of these samples. In particular, samples collected in August were held beyond the 8-hour recommended holding time and overgrowth of antibiotic-resistant bacteria from the fecal coliform bacteria plates prevented accurate quantification of resistant fecal coliform bacteria. To address these issues, samples were collected again in September from these same sites in Oakland County; these samples were processed within the recommended holding time, and a change in the technique testing antibiotic resistances allowed for accurate quantification of antibiotic-resistant fecal coliform bacteria. In addition, reference samples were also collected from two sites outside of Oakland County representing significantly different land uses.

E. coli and enterococci concentrations exceeded standards for recreational waters at several sites in August and September 2003. *E. coli* exceeded the Michigan single-sample standard for water quality at 7 of 14 sites in August and 8 of 14 sites in September. Enterococci concentrations exceeded USEPA standards for recreational waters at 13 of 14 sites in August and 12 of 14 sites in September. None of the reference samples collected exceeded the recommend *E. coli* standards.

Fecal coliform bacteria and *E. coli* with resistance to ampicillin, cephalothin, and ceftriaxone and sensitivity to cefoxitin may carry extended-spectrum-β-lactamases (ESBLs). This is significant because ESBL-producing *E. coli* and other Enterobacteriaceae have been cited as a critical resistance in the healthcare setting. In most cases this type of resistance is acquired and transferable. This pattern was found in fecal coliform bacteria isolated from the following sites: Sashabaw Creek near Drayton Plains (04160800), Clinton River near Drayton Plains (04160900) and at Yates (04161810), Paint Creek at Rochester (04161540), Stony Creek near Romeo (04161580), River Rouge at Southfield (04166100), Kearsley Creek at Ortonville (04148035), and Huron River at Milford (04170000). This pattern was not found in the reference samples.

The *van*A gene in enterococci and class I integrons in *E. coli* indicate an acquired resistance with the ability to transfer this resistance to similar organisms. The *van*A gene was detected in enterococci from Evans Ditch at Southfield (04166200) in August and Kearsley Creek at Ortonville (04148035) in September. Isolates with the *van*A gene were identified as *E. faecium*. One type of acquired resistance found in the Enterobacteriaceae family is due to the acquisition of an integron. Integrons were detected in the *E. coli* isolates recovered from September FC cultures from River Rouge at Birmingham

(04166000) and Clinton River near Drayton Plains (04160900). Integrons have the capability of carry multiple-antibiotic-resistance genes that could easily be transferred to other Enterobacteriaece organisms. These transferable genes (*van*A and integrons) were not found in the reference samples.

Multiple antibiotic resistances are often a result of transferable genetic elements. Multiple-antibiotic resistant fecal coliforms were isolated from every September sample. Only a selected number of *E. coli* isolates recovered from the FC culture were analyzed for the presence of integrons. However, fecal coliforms also may carry this element or other transferable elements that were not analyzed for in this study. In samples with isolates resistant to five or more antibiotics, the likelihood that this resistance is a result of a transferable genetic element is higher. There were six sites in Oakland County where fecal coliform isolates were resistant to five or six antibiotics: Upper River Rouge at Clarenceville (04166315), Stony Creek near Romeo (04161580), Paint Creek at Rochester (04161540), Clinton River at Yates (04161810), Sashabaw Creek near Drayton Plains (041610800), and Shiawassee River at Holly (04143830). The Washington Creek reference sample also had fecal coliform isolates resistant to five antibiotics. Most of the fecal coliform isolates tested in this study were presumed to be fecal coliform bacteria and were not further identified; therefore, the clinical significance of the antibiotic resistance cannot be determined. However, because multiple antibiotic resistances are an indication of acquired resistance it is possible that this resistance could be transferred to pathogens.

Presumptive *E. coli* O157 was identified by three different methods. Sites in which all three methods (IA, GA, rfb_{0157} assay) identified potential *E. coli* O157 include 04161000 and 04161810 in August. IA, the most sensitive assay, identified four other sites in August (04166000, 04166200, 04143830, and 04148035) and two other sites in September (04166000 and 04166315) with potential for *E. coli* O157. Both the IA and rfb_{0157} assay were negative for all three reference samples. Only the reference site Au Sable River at Grayling (04135500) was positive for the GA.

Virulence genes *eae*A and *stx*2 have been reported to be associated with human disease. This gene combination was found in samples from the following sites: 04166000, 04166200, 04160900, and 04143830 in August and September; 04166100 and 04166315 in August; and 04161540, 044161580, and 04161810 in September. The *eae*A and *stx*2 gene combination was not found in any of the three tested reference samples.

One site of interest was the River Rouge at Birmingham site (04166000). Pathogenic *E. coli* tests revealed a high likelihood that *E. coli* O157 was present. This water sample also contained the *eae*A and *stx*2 genes. At this site, fecal coliform bacteria were also isolated that were resistant to cephalothin and ceftriaxone, and presumptive *E. coli* was also recovered that was resistant to cepahalothin, ceftriaxone, and cefoxitin, with intermediate resistance to gentamicin. In addition, *E. coli* carrying an integron was detected at this particular site. The combination of potential pathogens, antibiotic resistances, and transferable genetics is of clinical significance.

Very little data are available on the prevalence of clinically significant antibiotic-resistant bacteria and pathogenic *E. coli* in environmental waters. This study included two reference sites with much less urbanization than those in Oakland County. Antibiotic resistance was found at both of these sites; however, the pattern of resistance was much different. Further studies would be needed to determine whether the results seen in Oakland County are representative of what is typically seen in the environment or whether these results are typical of more urban/suburban areas. In addition, this study was unable to determine whether particular environmental factors (such as temperature and rainfall patterns, wildlife, and so forth) would influence study results. It may be possible to use DNA sequence information to determine the variants of *stx*2 genes found at these sites. Many studies have suggested that particular *stx*2 variants are more commonly associated with certain non-human host animals and that only some of the variants have been associated with human disease.

Suggestions for Future Studies

Results of this study indicate that clinically significant antibiotic resistances and pathogenic bacteria are present in the waters of Oakland County. However, indications of multiple antibiotic resistances and of pathogenic *E. coli* were found in reference samples collected outside of Oakland County. Because very few reference samples were collected for this study, additional reference samples would need to be collected from other sites to better understand how the waters in Oakland County compare to other waters. Studies identifying sources of fecal contamination, seasonality of bacteria concentrations, changes in flow, and rainfall patterns, would help in understanding the extent of the problems that do occur.

The threat that fecal indicator bacteria resistant to antibiotics poses to human health is not clearly known. However, the presence of transferable genetic elements in the environment does present a possibility that clinically-significant antibiotic resistance could be transferred to pathogens. Further studies investigating the occurrence of transferable antibiotic-resistance elements and their relation to other constituents such as antibiotics, metal, detergents, or other waste products would improve our understanding of the role the environment has on antibiotic resistances. In addition, an evaluation of particular acquired antibiotic-resistance genes, such as *van*A, could determine how closely genes found in the environment are related to those of concern in the clinical setting.

This study also has indicated potentially pathogenic *E. coli* are present in streams throughout Oakland County. The extent of the problem and threat to human health is unknown. Further studies addressing the occurrence of pathogenic *E. coli* markers (temporally and with streamflow changes) would improve our understanding.

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Glossary

Acquired antibiotic resistance – A resistance to an antibiotic as a result of a genetic mutation or acquisition of a gene(s) that causes resistance in an otherwise sensitive organism.

Clinically significant antibiotic resistances – Antibiotic resistances that include pathogens resistant to those antibiotics used to treat an infection or genes responsible for resistance that can be transferred to pathogens.

ENT cultures – Preserved stock of all isolates capable of growing on mEI media at 41.5°C from 100 mL of filtered water.

FC cultures – Preserved stock of all isolates capable of growing on mFC media at 44.5°C from 100 mL of filtered water.

GA – Assayed based on growth on sorbitol MacConkey medium containing cefixime and tellurite (CT-SMAC).

IA – Immunological assay based on the presence of an antigen specific to the *E. coli* O157group.

Integron – Transferrable genetic elements that may carry multiple genes including genes responsible for antibiotic resistance.

Intrinsic antibiotic resistance – An inherent resistance not caused by exposure to the antibiotic or acquired genes; rather, it is caused by the biology of the organism. Intrinsic resistance is not transferable to other bacteria.

Isolates – Individual colony forming unit.

Multiple-antibiotic-resistant bacteria – Bacteria resistant to two or more antibiotics.

Nosocomial – Infections acquired while in the hospital.

Pathogenic bacteria – Bacteria capable of causing disease in humans or animals.

Serotype – A serological characterization of a microorganism at the species level based on the organism's antigenic properties.

Transferable genetic elements – Plasmids, transposons, integrons, or genes that may carry genetic sequences responsible for antibiotic resistance and that can be transferred to other organisms.

Appendix A.

Fecal indicator bacteria concentrations in river-water samples collected from Oakland County, Mich., in August and September 2003.

Appendix A. Fecal indicator bacteria concentrations in river-water samples collected from Oakland County, Mich., in August and September 2003.

[Aug, August; Sept, September; *E. coli*, *Escherichia coli*; CFU, colony forming units; mL, milliliter; NT, not tested; D, quantification based on dilution]

USGS Station Number	Fecal coliforms CFU/100 mL (P31616)		*E. coli* CFU/100 mL (P50278)		Percentage of fecal coliforms that are *E.coli*		Enterococci CFU/100 mL (P90909)	
	Aug	Sept	Aug	Sept	Aug	Sept	Aug	Sept
River Rouge watershed								
04166000	2,000 D	910 D	1,000 D	910 D	50	100	852 D	420 D
04166100	5,400 D	3,300 D	1,500 D	350 D	28	11	596 D	1,380 D
04166200	126,000 D	80	25,000 D	50	20	63	1,000 D	350 D
04166315	5,400 D	1,400 D	1,500 D	1,300 D	28	93	1,120 D	1,600 D
Clinton River watershed								
04160800	430 D	1,040 D	120 D	220 D	28	21	104 D	300 D
04160900	3,700 D	450 D	1,700 D	430 D	46	96	900 D	470 D
04161000	1,620 D	1,030 D	110 D	320 D	7	31	102 D	400 D
04161540	3,500 D	480 D	1,500 D	470 D	43	98	>1,000 D	350 D
04161580	990 D	360 D	270 D	260 D	27	72	126 D	95 D
04161810	3,400 D	570 D	1,800 D	310 D	53	54	1,060 D	69 D
Other Oakland County watersheds								
04143830	980 D	290 D	140 D	100 D	14	34	210 D	390 D
04148035	2,400 D	600 D	300 D	500 D	13	83	422 D	650 D
04170000	190 D	33	20	23	11	70	70	35
04170500	6 D	3	3	3	50	100	2	13
Reference sites								
04135500	320 D	36	290 D	34	91	94	5	14
04001000	NT	380 D	NT	260 D	NT	68	NT	0

Appendix B.

Field measurements at sites in Oakland County, Mich., in August and September 2003.

Appendix B: Field measurements at sites in Oakland County, Mich., in August and September 2003.

[°C, degrees Celsius; µS/cm, microsiemens per centimeter; mg/L, milligrams per liter; ft^3/s, cubic feet per second; ND, not determined]

USGS station number	Date sampled	pH	Water temperature (°C)	Specific conductance (µS/cm)	Dissolved oxygen (mg/L)	Discharge (ft^3/s)
04143830	8/5/2003	8.06	25.79	636	7.38	9.7
04148035	8/5/2003	8.07	20.39	868	8.40	6.1
04160800	8/5/2003	7.99	23.46	707	6.60	2.2
04160900	8/5/2003	7.58	22.47	933	6.67	3.0
04161000	8/5/2003	7.73	22.32	1,164	9.01	21.0
04161540	8/5/2003	8.01	19.96	710	8.22	12.0
04161580	8/5/2003	8.34	23.46	700	8.50	3.2
04161810	8/5/2003	8.19	22.04	913	9.06	62.8
04166000	8/5/2003	8.08	22.50	1,230	7.33	9.8
04166100	8/5/2003	7.85	20.38	1,254	6.81	17.0
04166200	8/5/2003	7.67	20.65	743	4.73	3.2
04166315	8/5/2003	7.98	20.08	1,378	7.08	5.9
04170000	8/5/2003	7.66	21.86	866	6.10	31.0
04170500	8/5/2003	8.03	24.50	779	7.17	33.0
04143830	9/9/2003	7.58	19.55	611	6.17	4.5
04148035	9/9/2003	7.99	14.76	878	8.45	4.2
04160800	9/9/2003	8.13	18.52	751	8.33	0.6
04160900	9/8/2003	7.55	21.30	918	5.60	4.2
04161000	9/8/2003	7.90	21.85	1,038	9.65	21.0
04161540	9/9/2003	8.25	16.42	872	10.36	ND
04161580	9/9/2003	8.28	19.78	643	9.56	1.70
04161810	9/9/2003	8.49	19.33	1,016	10.28	33.5
04166000	9/8/2003	8.30	20.95	1,211	8.46	4.2
04166100	9/8/2003	8.01	17.51	1,394	8.20	11.0
04166200	9/8/2003	7.91	18.05	3,040	6.60	1.40
04166315	9/8/2003	8.18	17.57	1,451	9.02	3.7
04170000	9/8/2003	7.66	19.57	875	6.51	27.0
04170500	9/8/2003	8.01	22.55	774	8.49	22.0

Appendix C.

N
collected in Oakland County, Mich.

Appendix C. Number of fecal coliform and enterococci isolates resistant to tested antibiotics that were isolated in August 2003 from samples collected in Oakland County, Mich.

[CFU, colony forming unit; mL, milliliter; µg, microgram; +, positive result; >, greater than; NA, not available]

	Fecal coliform antibiotic resistance (CFU/100 mL)[1]								Enterococci antibiotic resistance (CFU/100 mL)					
USGS station number	Total fecal coliform isolates	cefoxitin 32 µg/mL	ceftriaxone 64 µg/mL	cephalothin 32 µg/mL	ampicillin 32 µg/mL	tetracycline 8 µg/mL	gentamicin 16 µg/mL	streptomycin 64 µg/mL	Total enterococci isolates	vancomycin 8 µg/mL	vancomycin 16 µg/mL	streptomycin 2000 µg/mL	gentamicin 100 µg/mL	tetracycline 16 µg/mL
Rouge River watershed														
04166200	25,000	+	1,000	+	+	+	+	5,000	>1,000	6	2	2	2	104
04166000	1,000	+	300	+	+	100	0	0	844	6	0	0	0	64
04166315	1,500	+	800	+	+	80	900	100	1,096	0	0	40	92	146
04166100	1,500	+	200	+	+	700	400	100	596	0	0	8	0	54
Clinton River watershed														
04161580	270	+	90	+	+	190	110	0	118	4	0	0	8	34
04160900	1,700	+	1,600	+	+	+	+	0	906	2	0	0	2	68
04161540	1,500	+	600	+	+	400	600	0	>1,000	0	0	14	2	90
04161810	1,800	+	1,700	+	+	600	1,600	400	1,032	0	0	2	36	56
04161000	110	+	40	+	+	+	+	90	100	2	0	2	14	6
04160800	120	+	80	+	+	+	+	+	96	0	0	16	38	58
Other Oakland County watersheds														
04148035	300	+	20	+	+	10	10	0	416	0	0	0	0	56
04143830	140	+	90	+	+	190	110	0	206	0	0	0	26	2
04170500	3	+	2	+	+	2	0	0	2	4	0	2	2	2
04170000	20	+	20	+	+	160	460	0	70	2	0	0	0	4
Reference sites														
04135500	290	+	160	+	+	70	60	0	5	4	0	0	0	0
04001000	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[1] + Indicates plates were overgrown and bacteria colonies could not be counted.

Appendix D.

samples collected in Oakland County, Mich.

Appendix D. Percentage of fecal coliform and enterococci isolates resistant to tested antibiotics that were isolated in September 2003 from samples collected in Oakland County, Mich.

[CFU, colony forming unit; µg, microgram; mL, milliliter; NA, not available]

		Percent fecal coliform antibiotic resistance							Percent enterococci antibiotic resistance				
USGS station number	Total. fecal coliform isolates	cefoxitin1 32 µg/mL	ceftriaxone 64 µg/mL	cephalothin1 32 µg/mL	ampicillin1 32 µg/mL	tetracycline 8 µg/mL	gentamicin 16 µg/mL	streptomycin 64 µg/mL	Total enterococci isolates	vancomycin 8 µg/mL	vancomycin 16 µg/mL	streptomycin 2000 µg/mL	gentamicin 100 µg/mL
Rouge River watershed													
04166000	50	6	4	10	50	NA	0	2	42	7	0	0	0
04166100	50	4	4	100	12	NA	2	2	50	0	0	0	4
04166200	8	88	63	63	38	0	0	13	35	0	0	0	0
04166315	14	57	29	100	33	NA	0	50	50	6	0	2	0
Clinton River watershed													
04160800	50	46	30	100	1	44	30	12	30	33	7	0	3
04160900	45	44	16	98	16	56	4	11	47	9	0	4	0
04161000	50	64	0	98	100	NA	0	0	39	3	0	8	0
04161540	48	29	54	100	2596	15	23	13	35	20	9	6	0
04161580	36	86	100	97	100	72	39	69	50	2	0	2	0
04161810	50	56	14	88	76	24	32	6	50	8	0	0	0
Other Oakland County watersheds													
04143830	29	83	3	100	97	21	17	10	39	15	8	0	0
04148035	50	24	8	84	56	NA	12	2	50	22	8	0	4
04170000	33	45	6	73	73	0	0	24	35	6	0	0	0
04170500	3	100	100	0	0	0	0	0	13	38	15	0	0
Reference sites													
04001000	30	23	0	97	23	27	20	27	0	NA	NA	NA	NA
04135500	5	20	0	40	0	60	0	20	14	7	0	0	0

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