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A SURVEY OF ANTIBIOTIC RESISTANCE AMONG COLIFORM BACTERIA ISOLATED FROM THE MISSOURI RIVER

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ABSTRACT

The prevalence of antibiotic resistant microorganisms in the environment is not well known but could represent a challenge to maintaining public health in the future. Fecal waste from livestock facilities, where antibiotics are routinely used, is recognized as a significant source of pollution to surface waters in the United States. We collected water from the Missouri River across a winter to spring seasonal change in order to survey the density of coliform bacteria. We tested bacterial isolates for resistance to penicillin, tetracycline, ampicillin, erythromycin, and ciprofloxacin. Coliform density in Missouri River water generally was high throughout the study (0–405 cfu 100 ml^{-1}). The highest values corresponded to a precipitation event that likely enhanced surface runoff. The lowest coliform densities corresponded to an increase in river flow volume caused by the release of reservoir water upstream. Of the isolates tested, none were sensitive to penicillin or erythromycin, both anti-microbial drugs that are normally ineffective against Gram negative bacteria such as coliforms. No isolates were resistant to ampicillin or ciprofloxacin. 12.5% percent of the isolates were resistant to tetracycline, however. All isolates were identified through biochemical testing as *Escherichia coli*. Our results demonstrate that antibiotic resistant coliforms were present in the Missouri River at the time of our study. Whether the source of these microorganisms is an environmental reservoir or livestock source needs to be determined.

† † †

Monitoring the density of coliform bacteria in surface waters is critical in order to protect public health (Alonso et al. 1999). Coliforms are Gram-negative, rod-shaped bacteria that are members of the family Enterobacteriaceae. They are normal flora of the gastrointestinal tracts of all warm-blooded and some cold-blooded animals (Harwood et al. 2000). *Escherichia coli*, a well-known resident of animal digestive tracts, is a coliform that can be shed in feces that is used as an indicator of fecal contamination in water (Cray et al. 1998). The Environmental Protection Agency (EPA) has set standards for maximum acceptable levels of coliforms and other contaminants in water. However, it is not uncommon for waterways to show heavy fecal coliform contamination. The Missouri River has been classified as an "impaired" waterway by the EPA due to the consistent presence of pathogenic bacteria (EPA's Surf Your Watershed website; www.epa.gov/surf/).

Coliform pollution of surface waters frequently is attributed to the livestock industry, due to the dispersal of animal excrement throughout the watershed (Hagedorn et al. 1999). Antibiotics have had long-term use in livestock feed to treat and prevent illness (Isaacson and Torrence 2002; Blanco et al. 2000). However, nearly half of the antibiotics used by the livestock industry today, including tetracycline and penicillin, are added to feed to promote growth—a practice that has created much controversy (Mlot 2000). As a direct consequence of heavy reliance on these drugs, antibiotic resistance has emerged in bacteria isolated from livestock (Witte 1997). Furthermore, antibiotic resistant microbes have been isolated from surface waters (Parveen et al. 1997). Tetracycine resistant bacteria were isolated from the Missouri River by students in an undergraduate microbiology lab at Creighton University, Fall 2001 (A. Treonis, unpublished).

Most of the antibiotic resistant microorganisms found in the environment to date have been determined to have a livestock source (Hagedorn et. al. 1999). While the prevalence of antibiotic resistant bacteria in surface waters has been demonstrated, there remains a critical need to further understand the sources and sinks for antibiotic resistance in the environment. Microbial contamination is known to increase following rainfall and runoff events (Kistemann et al. 2002), but it is not know whether this is correlated to an increase in the density of antibiotic resistant microbes as well.

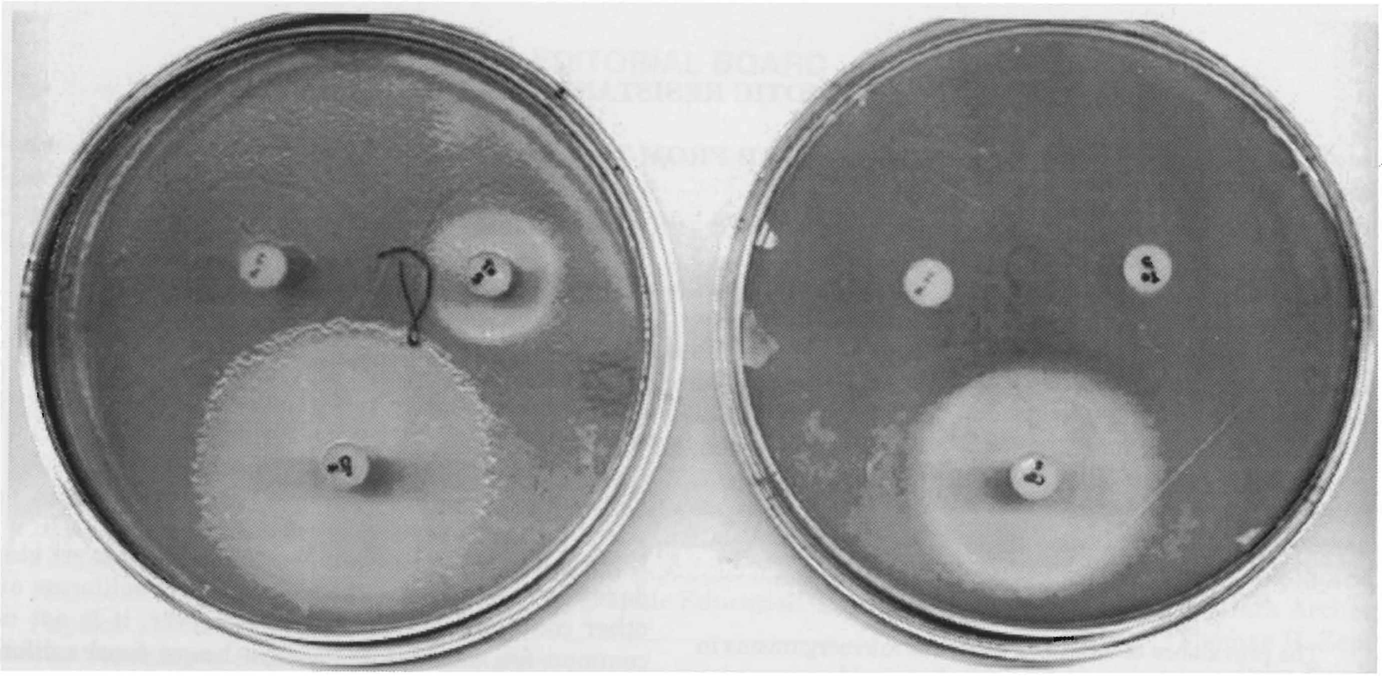

Figure 1. Petri plates containing Missouri River isolates being tested for antibiotic resistance using the disk diffusion technique. Sensi-Discs™ are shown with inhibition zones (clearing of microbial growth), which are measured to determine the level of antibiotic susceptibility.

The objectives of this study were twofold. First, we wanted to survey the density of coliforms in the Missouri River across a seasonal shift (winter to spring). Second, we wanted to determine whether these coliforms represented a reservoir of antibiotic resistance. We predicted that there would be a correlation between microbe density and rainfall events, with spring rains contributing to an increase in coliform density. We also predicted that we would isolate coliforms from river water that were antibiotic resistant.

MATERIALS AND METHODS

Water samples (100 ml) were collected over an 8 wk period (13 February–3 April 2002) from the N.P. Dodge Park (City of Omaha) boat ramp along the Missouri River. On each sampling date, four replicate samples were collected. River flow volume data was obtained from the U.S. Geological Survey web site (http://waterdata.usgs.gov/).

Tenfold serial dilutions of each sample were prepared and filtered onto membrane filters (0.45 mm, Millipore) (American Public Health Association 1995). Filters were incubated in M-Endo MF broth (Difco) at 37°C, and coliform colony forming units were counted after 24 h (American Public Health Association 1995). M-Endo MF broth is a differential media on which coliforms produce a red or pink colony with a green metallic sheen, and non-coliforms produce a clear or pink colony with no metallic sheen (Anonymous 1998).

Each week, six random coliform colonies were subcultured for further testing. The Kirby-Bauer disk diffusion technique (Bauer et al. 1959; Fig. 1) was used to assess the antibiotic sensitivity of each of the isolates on Mueller Hinton Agar (Difco). Four bacterial stock cultures (*Klebsiella pneumoniae, Salmonella typhimurium, Escherichia coli, Enterobacter aerogenes*; Carolina Biological Supply) were also included in testing to serve as controls. The zones of growth inhibition were measured for five antibiotics: tetracycline (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), ampicillin (10 μg), and penicillin (10U) (BBL™ Sensi-Disc™, Becton Dickson). Penicillin and erythromycin were selected for this study because these antibiotics are not effective against Gram negative bacteria due to resistance provided by the lipopolysaccharide outer membrane of the cell wall. These antibiotics, therefore, both served as controls. The inhibition zone diameters that indicated resistance for each of the antibiotics tested were: tetracycline: ≤ 14 mm, ciprofloxacin: ≤ 15 mm, erythromycin: ≤ 13 mm, ampicillin: ≤ 13 mm, penicillin ≤ 14 mm (Anonymous, 2001).

Isolates were identified to the species level using biochemical profiles obtained using a multi-test system (Enterotube® II; Becton Dickinson).

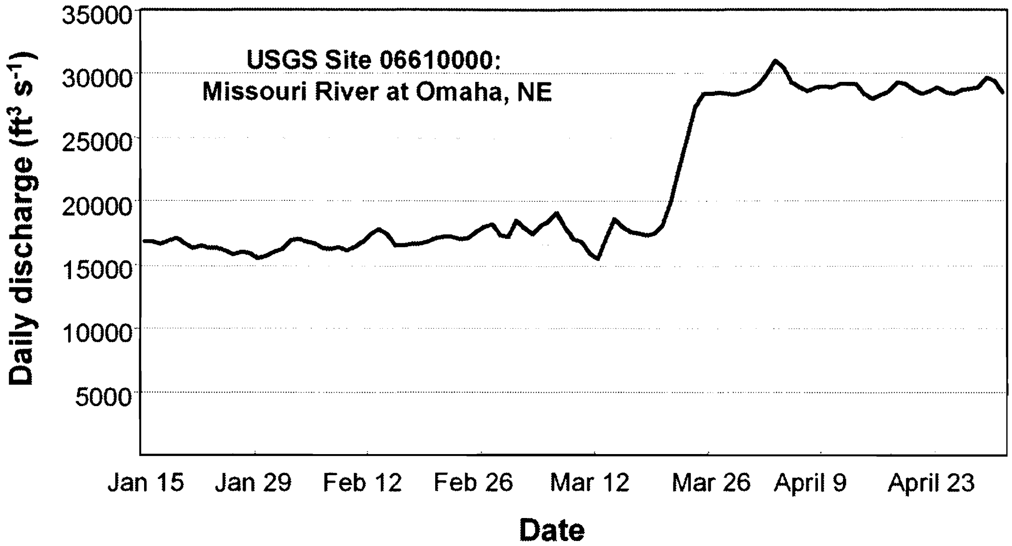

Figure 2. Missouri River discharge data at Omaha, NE (Source: USGS).

RESULTS

River flow data

The Missouri River flow rate ranged from 15000–20000 cu ft s^{-1} during the early part of our study (Fig. 2). The Army Corps of Engineers increased the river flow volume around 20 March by releasing water upstream from the reservoir behind Gavins Point Dam (west of Yankton, SD), raising the flow rate to approximately 30000 cu ft s^{-1} (Fig. 2).

Coliform density

Coliform density fluctuated little between 13 February and 20 March (Fig. 3). On 27 March, almost no coliforms were detected in the river water (Fig. 3). This sampling was followed by a dramatic increase in coliforms on 3 April (Fig. 3). Counts ranged from 0–405 cfu 100 ml^{-1}.

Disk diffusion

Out of 40 coliform isolates tested, we found that none were resistant to ciprofloxacin or ampicillin (Table 1). Forty isolates were resistant to penicillin and erythromycin (Table 1). Five isolates were resistant to tetracycline (12.5%), while 35 were sensitive (Tables 1 and 2). Tetracycline resistant bacteria were isolated from samples collected on multiple dates (Table 2). All of the stock cultures tested (all coliforms) were sensitive to tetracycline and ciprofloxacin and resistant to penicillin.

Identification

All tested isolates were identified as *Escherichia coli*.

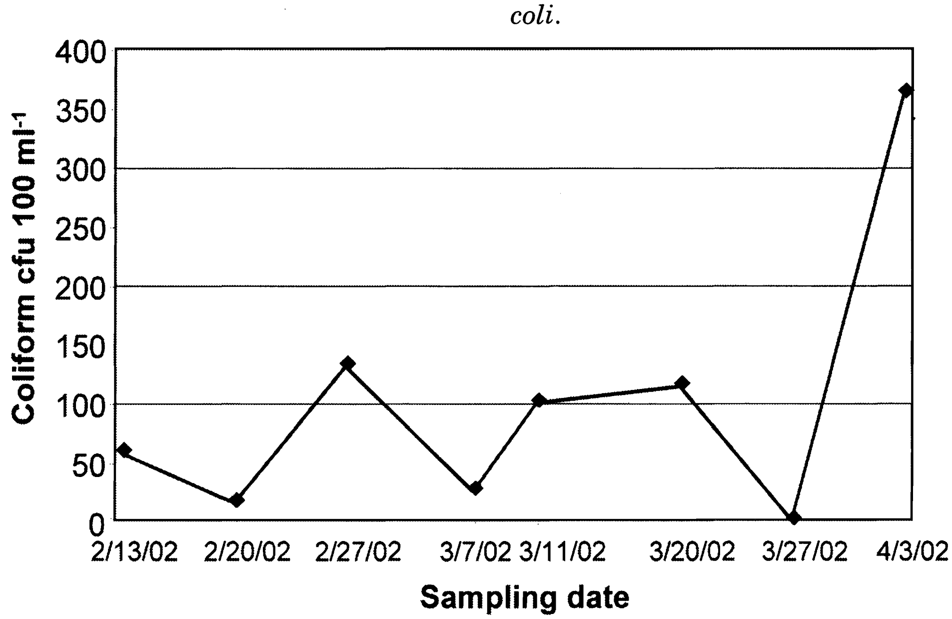

Figure 3. Coliform density in Missouri River water (Each point represents the mean of four samples).

Table 1: Antibiotic resistance among Missouri River coliforms.

	Proportion of isolates showing resistance*
Tetracycline	12.5%
Penicillin	100%
Erythromycin	100%
Ciprofloxacin	0%
Ampicillin	0%

*$n = 40$

DISCUSSION

Coliform density in Missouri River water was related to precipitation events and river flow volume. First, there was a dramatic decrease in coliform density on 27 March. This followed the release of reservoir water by the Army Corps of Engineers around 20 March, which increased river volume substantially and possibly diluted coliform inputs from tributaries. Second, there was a dramatic increase in coliform density on 3 April. This sampling date followed a heavy spring snowfall in the region. Subsequent melting may have flushed microbes from the watershed into the river water. Furthermore, increased spring temperatures may have induced a microbial "bloom". A spring increase in coliform density is consistent with similar data collected by the Omaha Metropolitan Utilities District (J. Haywood, personal communication).

Table 2. Testing details for tetracycline resistant Missouri River isolates.

Sampling date	Isolates tested for antibiotic resistance (#)*	Isolates exhibiting resistance to tetracycline (#)	Zone of inhibition for resistant isolate (mm)
2/13/02	3	0	.
2/20/02	6	1	14
2/27/02	5	1	6
3/7/02	6	1	6
3/11/02	6	0	.
3/20/02	6	2	12.5, 6
3/27/02	2	0	.
4/3/02	6	0	.

*Number varies due to an inability to obtain pure cultures or because < 6 coliforms were found in water samples on a particular date.

Of the suite of antibiotics tested, we only found resistance to tetracycline. Tetracycline is a broad spectrum antibiotic that is a common additive to cattle feed, and the cattle industry represents a significant economic activity throughout the Missouri River watershed. If the coliforms we isolated were of livestock origin, then the wide use of tetracycline in livestock feed could be leading to the creation of an environmental reservoir of bacteria resistant to this drug. Nonpoint sources of fecal coliforms to surface waters are difficult to pinpoint, however. Furthermore, the 12.5% of the isolates that were resistant could represent a natural reservoir of antibiotic resistance, which is known to exist (Isaacson and Torrence 2002). Also, antibiotics taken by humans and excreted, but not degraded during sewage treatment, may affect microbial communities in the environment. Only a more detailed strain characterization, such as DNA analyses (*sensu* Fey et al. 2000), would allow us to identify the isolates we collected as those associated with livestock. The *E. coli* we isolated may be from wild animals or even leaky septic systems in the watershed.

Of the remaining antibiotics tested, ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic frequently added to poultry feed, and ampicillin is a broad-spectrum penicillin derivative. Both of these antibiotics are effective against Gram negative bacteria, and none of the Missouri River isolates we tested showed resistance to either of these antibiotics. These results are different than those for tetracycline and suggest that there may be no natural reservoir for resistance to these drugs. Alternatively, there may have been little selection pressure for resistance to ciprofloxacin or ampicillin at the source of these isolates.

Antibiotic resistance in the clinical setting has been increasing in recent decades, creating great concern among physicians and microbiologists. While hospital-acquired, antibiotic resistant infections currently are prevalent, in the future, environmental reservoirs of antibiotic resistant bacteria may become a larger concern (McDonald et al. 1997). Our results show that coliforms are present in the Missouri River that exhibit resistance to tetracycline. We have demonstrated that there is a potential reservoir for antibiotic resistant microbes in a major U.S. waterway, although it represents a relatively small proportion of our isolates. Furthermore, we failed to isolate any multi-drug resistant coliforms in this study. It is important, however, to monitor coliform density and antibiotic resistance levels to ensure that surface waters are safe in the future.

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