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ABSTRACT—Karst topography of soils in southeastern Minnesota is responsible for the formation of sinkholes, subsurface cracks, and underground rivers which may enhance the transportation of surface contaminants into groundwater. The present study was conducted to determine the presence of human pathogenic viruses, coliforms, fecal coliforms and coliphages in private rural wells of this area. The occurrence of drug resistance in bacteria isolated from groundwater also was studied. Coliform bacteria were detected at least once from 22 of the 26 sites sampled over 34 months. Water from 10 sites yielded drug-resistant indicator bacteria; 25 of 38 (65.8 percent) total coliforms and 9 of 27 (33.3 percent) fecal coliforms tested were found to carry drug resistance. Human enteric viruses were detected by DNA hybridization and/or virus isolation techniques in nine samples from seven different sites, some in the absence of fecal coliforms. Of the 161 samples tested for coliphages, 13 samples from seven sites were found positive. On two occasions, coliphages were isolated from samples in which coliforms were absent. These findings indicate that potential public health problems exist in this region.

Introduction

Several well-documented outbreaks of hepatitis A and viral gastroenteritis traced to groundwater contamination have destroyed the widely held misconception that groundwater is safe from pollution. At least 673 water-related outbreaks of disease occurred in the U.S. between 1946 and 1980, of which 295 (44%) were attributed to the contamination of groundwater (1). Septic tanks, animal feedlots, land application of wastewater, and leakage from municipal sewer systems and treatment lagoons contribute to pollution of subsurface waters. Although the fate of pathogenic bacteria in soil has been studied since the early part of this century, only during the last decade have serious efforts been made to understand the fate and migration of human enteric viruses in the subsurface environment (2). Viruses are considered more resistant than bacteria to natural inactivation and sewage treatment processes, including disinfection, and they have been shown to travel long distances in soil, both vertically and horizontally (1). Application of domestic sewage and/or animal manure to land may, therefore, result in groundwater contamination. Most of the research on pollution of drinking water has focused on municipal water systems and semipublic water systems, which number 40,000 and 200,000, respectively. Much less attention has been paid, however, to the approximately 10,000,000 individual water systems that account for 13 to 14 percent of the waterborne disease outbreaks reported annually (3).

Groundwater serves as a water source for 65 to 70 percent of the Minnesota population. The southeastern part of the state is underlain by carbonate rocks with subsurface cracks, sinkholes, and underground rivers which allow rapid percolation of surface water and runoff to underground aquifers (4). The rapid transport of water may short circuit potential soil filtration of pathogens and may result in groundwater contamination. Although groundwater is disinfected before distribution via public water supplies, water from private rural wells is usually used for human and animal consumption without any treatment or disinfection. The present study was undertaken to determine the extent to which rural groundwater supplies in southeastern Minnesota are contaminated with human pathogenic viruses. The presence of fecal indicator bacteria and coliphages in groundwater and their relationships, if any, to the presence of viruses also was determined, and the presence of drug resistance in indicator bacteria was studied.

Materials And Methods

Sampling sites

Sampling wells were selected from a list of owners who had requested the Olmstead County Health Department to test their wells for nitrate and coliform contamination (5, 6). A total of 24 wells and two springs was included in this study. Locations of the wells are shown in Figures 1-3. The sampling was done between September 1984 and June 1987.

Indicator bacteria and drug resistance

Standard methods were used for the enumeration of total and fecal coliform bacteria in water (5, 7). Drug resistance in selected isolates of bacteria was determined by the previously described procedures (5, 6).

Virus Concentration

For the concentration of human and animal enteric viruses, 400 L of water were passed through an electropositive filter (1 MDS; AMF-Cuno, Meriden, CT.) The adsorbed viruses were eluted with 1500 mL of 3 percent beef extract (pH 10.5). To reduce the volume of the eluate, the pH was adjusted to 3.5

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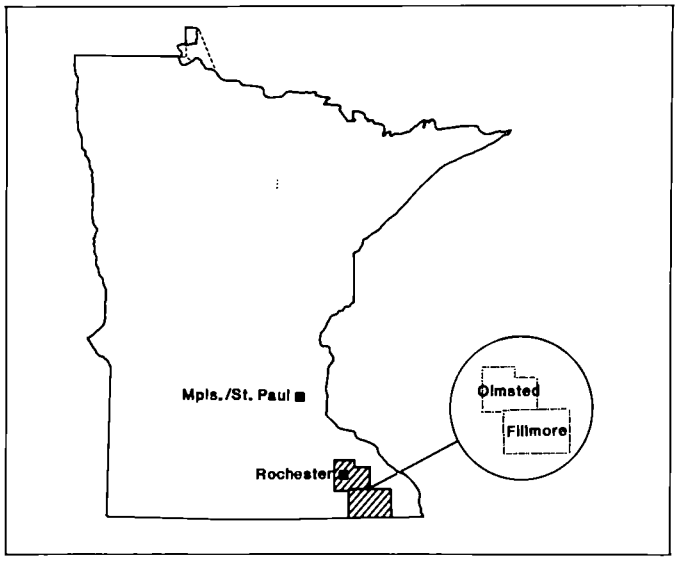

Figure 1. Map of the State of Minnesota showing the two counties where this study was carried out.

with 2N HCl, which resulted in the formation of a virus-containing floc (5-9). At pH 3.5, the eluate was allowed to flocculate for 10-30 minutes and then was centrifuged at 1500 xg for 10 minutes at 4°C. The supernatant was discarded and the floc was resuspended in 10-20 mL of 0.05 *M* glycine (pH 11.5). After neutralizing the concentrate to pH 7.5, we removed 0.5 mL and stored it in cryotubes at -20°C for phage assay. The remaining eluate was treated with antibiotics and assayed for human and animal viruses.

Virus Isolation

Isolation of human enteric viruses from concentrated water samples was achieved by inoculation of Buffalo Green Monkey (BGM) kidney cells. After virus adsorption, the inoculated cultures were incubated at 37°C for 7-10 days. Two blind passages were given to each sample before considering it negative for viruses. Progeny viruses were identified by using pools of enterovirus antisera (10). Porcine kidney (PK15) and bovine turbinate (BT) cells under liquid overlay were used for the isolation of porcine and bovine enteroviruses, respectively (11).

Virus Detection by DNA Hybridization

The method of Margolin *et al.* (12) was used for hybridization. Poliovirus type 1 (Mahoney) cDNA probe (115-7440 bp) inserted into a pBR322 plasmid and labelled with both 32p dCTP and 32p dATP was used. Briefly, the samples were extracted with phenol:chloroform followed by two extractions with chloroform and three extractions with water-saturated ether. The latter was removed by bubbling air through the sample. The samples then were spotted onto Gene Screen Plus (DuPont NEN, Wilmington, DE) using a BioRad dot-blotter. The hybridization membrane was baked at 80°C in a vacuum oven for 2 hrs. Prehybridization was done at 42°C for 6 hours with constant agitation, and hybridization was carried out for 36-48 hours. The membrane was washed and results visualized by autoradiography.

Coliphage Assay

Coliphages were detected by using *Escherichia Coli* B (ATCC 11303-1) as a host (6, 13-14).

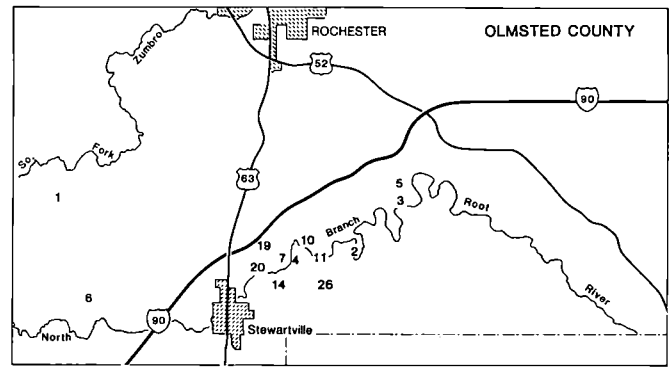

Figure 2. Location of wells sampled - Olmstead County

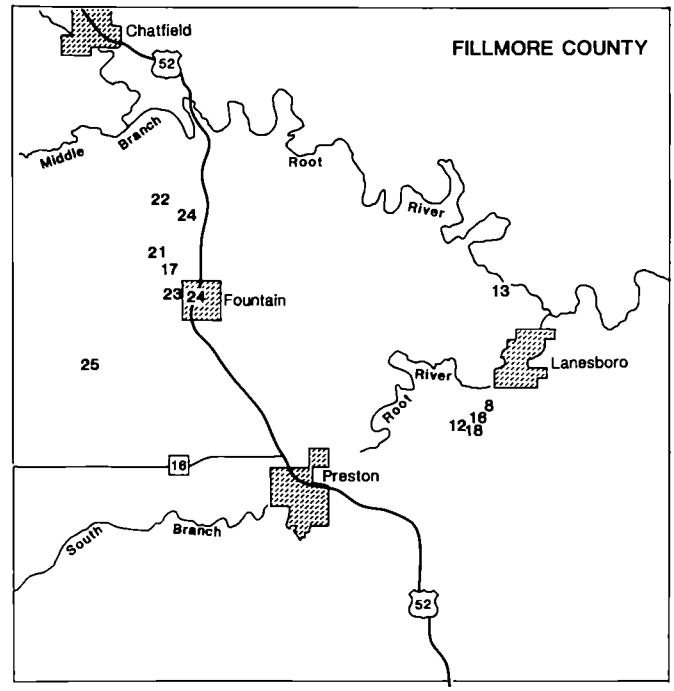

Figure 3. Location of wells sampled - Fillmore County

Results

A total of 268 samples were collected from 26 wells during the 34 month period. All samples were examined for the presence of human pathogenic viruses and fecal indicator bacteria. As shown in Table 1, 145 (54%) samples were positive for total coliforms and 67 (25%) had fecal coliforms. Coliforms were isolated at least once from 22 of the 26 sites and fecal coliforms were present in 18 of them. At 21 sites, total coliforms (Table 1) occurred in levels exceeding the limit of one total coliform per 100 mL of potable drinking water suggested by the National Technical Advisory Committee (15). Of the 38 isolates tested for drug resistance, 25 (66%) showed resistance to at least one antibiotic, and 17 (45%) showed multiple resistance. The most frequent drug resistances among total coliforms were to ampicillin, cephalothin, nitrofurantoin, and tetracycline.

Fecal coliforms were found in 18 of the 26 sites sampled (Table 1). According to current standards (15), drinking

Table 1. Occurrence of indicator bacteria in well water[a]

Site No.	Number of Samples	Number Positive for TC/FC	Range per 100mL of: TC	FC
1	8	2/0	0-1	0
2	8	4/0	0-97	0
3	8	5/1	0-16	20
4	15	5/2	0-103	0-2
5	7	1/1	0-2	0-2
6	7	0/0	0	0
7	15	2/1	0-30	0-1
8	24	10/4	0-89	0-23
9	6	5/0	0-44	0
10	25	13/4	0-100	0-60
11	26	20/20	0-TNTC	0-TNTC
12	24	17/9	0-198	0-120
13	15	10/4	0-85	0-9
14	12	5/0	0-3	0
15	2	1/1	0-9	2
16	13	10/6	0-210	0-14
17	14	14/12	34-TNTC	33-350
18	2	2/2	16-21	2-6
19	5	0/0	0	0
20	4	0/0	0	0
21	7	6/1	0-3	0-1
22	2	0/0	0	0
23	4	3/3	0-70	0-3
24	5	3/1	0-400	0-50
25	5	5/4	10-232	0-126
26	5	2/1	0-2	0-1
TOTAL	268	145/67		

[a] TC = total coliforms; FC = fecal coliforms; - not applicable, TNTC too numerous to count

water should be free of fecal coliform contamination in a 100 mL sample. Of the 22 sites showing total and/or fecal coliform contamination, 18 are used actively as sources of drinking water. We tested 27 fecal coliform isolates for drug resistance, and nine showed resistance (33%) to one or more of the 12 antibiotics. Four (16%) showed multiple resistance. Resistance to tetracycline and cephalothin was found to be the most prevalent in fecal coliforms. Highest levels of indicator bacteria were detected immediately after rainfalls of 0.6 cm (0.25 inches) or greater, and levels dropped off rapidly within two days after the rain stopped. Several wells that were negative for indicator bacteria on repeated sampling were positive after a rainfall event, and were negative again upon subsequent sampling. As mentioned earlier, eight wells included in this study were found to contain no coliforms in a single sample examined by the County Health Department. Of these, only three were found to be consistently negative for coliforms on repeated sampling in this study.

Of the 161 samples tested for coliphages, 13 (17%) were found to be positive, with numbers ranging from 60-2820 coliphages per 400 L of groundwater (Table 2). Coliphages appeared in seven of the 18 sites sampled, with multiple occurrences at four sites (6). Viruses were isolated from only two sites: site 11 yielded echovirus type 25 and site 17 yielded coxsackie virus type B-2 (Table 2). The former site is a private well, and the latter is a spring near the city of Fountain, Minnesota. Three samples from each site were tested by the cDNA dot-blot hybridization technique (12). Eight samples from six sites were positive. Of the samples positive for viruses by either method, all nine had total coliforms, but only five had fecal coliforms. None of the samples yielded viruses of veterinary importance.

Discussion

Detection of bacterial contamination of groundwater in southeastern Minnesota is not surprising because this region is characterized by active karst topography and sinkhole development over large areas. Highest and most frequent levels of fecal and total coliforms occurred in four of the shallowest wells (≤30 feet deep) e.g., sites 11, 12, 24 and 25. Deeper wells (70 feet and above) and cased wells showed little or no contamination, indicating that well construction plays an important role in preventing or encouraging contaminant invasion. On examining water supply records in a rural county in Ohio, Brewer and Lucas (3) found that 342 of 2,005 wells (17%) were positive for coliforms. They found a high correlation ($r = 0.86$) between well depth and log concentration of coliforms (based on data from 21 wells). Another highly contaminated site in this study (site 17) is a spring; Cave Spring, MN 23: A0037, northwest of Fountain, MN. This spring is directly connected to raw sewage and septic tank effluents (16) formerly dumped into sinkholes. Dye tracing studies have demonstrated a transit time of approximately 20 hrs from sewage outfall (16).

Viruses were isolated from only two sites in this study, but seven additional sites were positive by DNA hybridization. Nucleic acid probes are considered to be much more sensitive than routine virus detection procedures. The cDNA probe used in this study has been shown to be capable of detecting as few as 1 PFU of poliovirus (12). The samples that were positive by gene probe assay but negative for virus isolation by tissue culture may have contained inactive virus that could not replicate in cell cultures. The DNA probe also has been shown to cross-hybridize with other enteric viruses but with a 2-log reduction in sensitivity. Non-detection of coxsackievirus type B-2 from site 17 (Table 2) may be explained on this basis.

Farm animals are known to excrete a variety of viruses in their feces (17). Many of these viruses are pathogenic to farm

Table 2. Human viruses and coliphages in groundwater[a]

Site	Number of samples positive/tested for: Human viruses: Detected by gene probe	Human viruses: Virus isolation and identification in cell culture	Coliphages
7	1/3	0/15	0/8
8	1/3	0/24	1/13
10	1/3	0/24	2/14
11	3/3	1/26[b]	4/14
12	1/3	0/24	07
13	0/3	0/15	1/13
14	0/3	0/15	2/10
16	1/3	0/13	0/5
17	0/3	1/14[c]	2/9
18	0/3	0/2	1/2
	8/30	2/170	13/95

[a] Sites not shown in this table were negative for human viruses and coliphages
[b] Echovirus type 25
[c] Coxsackievirus type B-2

livestock, and some may be transmissible to humans. The non-detection of animal viruses in this study may indicate a real absence of such viruses, or it may reflect the fact that the methods used in this study were designed to concentrate human enteric viruses (6, 8) and may not have been suitable to concentrate and detect animal enteric viruses.

A major concern regarding the exposure of humans and animals to fecally contaminated water is the potential introduction of foreign drug-resistant bacteria (18, 19). If these bacteria colonize the gastrointestinal tracts of humans and animals, the potential exists for diseases to develop that are resistant to therapeutic levels of commonly employed antibiotics. One-third of the fecal coliforms tested in this study showed some level of drug resistance. The source of these drug resistant bacteria is considered to be animal or human carriers that have had repeated or long-term exposure to these drugs (18, 19). These bacteria may by themselves constitute a threat by colonizing the human gastrointestinal tract or may transfer their drug resistance to other human bacterial pathogens (19).

Although a high correlation has been shown between the presence of coliforms and the occurrence of bacterial pathogens in surface waters and water distribution systems, several studies indicate that the lack of demonstrable coliform levels in water does not preclude the presence of viruses (20-22). Such was the case in the present study. Some investigators have shown that coliphages are better indicators of viruses than total and fecal coliforms (23,24). In this study, however, no such correlation was found. Thus, two samples positive for coliphages were from water free of both total and fecal coliforms, and six coliphage-positive samples had total coliforms but not fecal coliforms. It should be realized, however, that the sample volume for coliphages was much more than that for indicator bacteria. Further studies are needed to determine if an increase in sample volume may provide better correlation between indicator bacteria and coliphages.

Groundwater contamination in southeastern Minnesota may be related to the karst topography which is characterized by the presence of sinkholes, subsurface cracks, and disappearing streams. These structures can act as pathways by which contaminants can reach groundwater. Surface solids generally act as a natural filter for water as it passes downward to the aquifer. However, in a karst area, the direct connection between surface water and groundwater reduces the filtration function of the soil. The occurrence of karst features in this region may account for the transient and recurring nature of detectable levels of indicator bacteria observed in this study.

Hallberg *et al.* (25) assessed regional groundwater quality problems in a karst carbonate aquifer in the Big Spring Basin, Clayton County, Iowa. They found that bacterial contamination of the aquifer was related to peak runoff periods. Persistent bacterial problems were suspected to be associated with faulty domestic water systems rather than the karst-groundwater system. They separated groundwater discharge into two principal components i.e., a base flood or infiltration component and a peak conduit flow related to surface water run-in to sink holes. The run-in component was believed to contribute to the peak bacterial problems in groundwater.

The infiltration component should be better defined. According to Gupta (Department of Soil Science, Univ. of Minneosta, 1989) one to two inches of water may not be enough to saturate soil so that water drips out over the restricting layer (or shale) and flows to the conduit. However, if the same one or two inches of water from a watershed concentrates downslope in small area, it will saturate the soil and will carry contaminants with the infiltered water. For the purposes of the present report, however, it is sufficient to say that if the infiltering water does not come into contact with adequate depth and right type of soil, viruses may not be removed (1, 2).

This study has demonstrated bacterial and viral contamination of groundwater in parts of southeastern Minnesota. When people continually consume contaminated water they increase their risk of exposure to hazardous substances. It is essential, therefore, to increase public awareness of the health risks associated with groundwater contamination. Plans for better watershed management and improved well construction are essential if the purity of groundwater in southeastern Minnesota is to be improved. Attempts should be made to decrease the interaction of surface waters with groundwater. Proper sinkhole management is also important. To protect wells from contamination, deeper wells should be dug and they should be cased by two or more linings. The variable nature of contamination at several sites in this study indicates the importance of repeated sampling to determine the sanitary quality of well water. It should be realized that this study was conducted in a small portion of southeastern Minnesota. Further studies are necessary to determine whether these results reflect the situation in the whole region.

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