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Virus Persistence in Groundwater

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More than 50% of the outbreaks of waterborne disease in the United States are due to the consumption of contaminated groundwater. An estimated 65% of the cases in these outbreaks are caused by enteric viruses. Little, however, is known about the persistence of viruses in groundwater. The purpose of this study was to determine whether measurable chemical and physical factors correlate with virus survival in groundwater. Groundwater samples were obtained from 11 sites throughout the United States. Water temperature was measured at the time of collection. Several physical and chemical characteristics, including pH, nitrates, turbidity, and hardness, were determined for each sample. Separate water samples were inoculated with each of three viruses (poliovirus 1, echovirus 1, and MS-2 coliphage) and incubated at the in situ groundwater temperature; selected samples were also incubated at other temperatures. Assays were performed at predetermined intervals over a 30-day period to determine the number of infective viruses remaining. Multiple regression analysis revealed that temperature was the only variable significantly correlated with the decay rates of all three viruses. No significant differences were found among the decay rates of the three viruses, an indication that MS-2 coliphage might be used as a model of animal virus survival in groundwater.

Almost 25% of the water used in the United States is groundwater, and the percentage is increasing. For approximately half the population, groundwater is the principal source of drinking water (5). Many small communities and some larger cities such as Tucson, Ariz., rely totally on groundwater for potable water. Traditionally, groundwater has been considered safe for human consumption without undergoing conventional drinking water treatment. But as the demand for clean water has grown, the increasingly attractive and economically favorable practice of recharging aquifers with wastewater has increased the possibility of contaminating potable waters with pathogenic bacteria and viruses present in domestic sewage (9).

Viruses are introduced into the subsurface in a variety of ways, including through septic tanks, landfills, and artificial recharge of groundwater and crop irrigation with treated sewage effluent. The burial of disposable diapers in sanitary landfills is one means by which untreated sewage is introduced into the subsurface; feces from infected individuals may contain as many as 10^6 PFU of enteroviruses g^{-1} and 10^{10} rotaviruses g^{-1} (18). When treated domestic sewage effluent is used for recharge or irrigation, between 50 and 99.999% of the viruses are inactivated, depending on the type of treatment process(es) used (7). Thus, if the concentration of viruses initially present is great enough, even extensive treatment of wastewater before injection into the soil will not remove all potentially infectious virus particles.

Several factors control the survival and migration of viruses once they have been introduced into the subsurface. In general, the climate (e.g., temperature and rainfall), the nature of the soil (e.g., clay content and moisture-holding capacity), and the virus type are the major elements in determining virus fate. Viruses can migrate considerable distances in the subsurface; virus penetration to depths as great as 67 m and horizontal migration as far as 408 m have been reported (9).

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Several investigators have studied the factors controlling virus survival in soil (17), but few have studied the survival of viruses in groundwater. Bitton et al. (4) compared the survival characteristics of three bacteria (*Salmonella typhimurium*, *Streptococcus faecalis*, and *Escherichia coli*), an enterovirus (poliovirus 1), and a coliphage (phage f2). Their experiments were done with groundwater from a 145-m-deep well in Florida; flasks were incubated at 22°C, the in situ groundwater temperature. They found that poliovirus 1 and *S. faecalis* had the lowest decay rates (although the survival pattern of *S. faecalis* was highly variable), followed by *S. typhimurium* and *E. coli*. The f2 phage had the highest decay rate, more than 10-fold that of any of the other organisms.

In a similar study, Keswick et al. (10) compared the persistence of coxsackievirus B3, poliovirus 1, rotavirus SA11, phage f2, *E. coli*, and a sewage-isolated fecal streptococcus in groundwater. The organisms were put in a McFeters survival chamber (G. McFeters, Bozeman, Mont.) and exposed to a continuous flow of groundwater from a 84-m-deep well. The temperature during the experiment ranged from 3 to 15°C. The enteroviruses poliovirus 1 and coxsackievirus B3 had decay rates lower than those of both of the bacteria. Rotavirus SA11 and the f2 phage were inactivated faster than the bacteria.

From the above discussion, it is clear that viruses are introduced into the subsurface by a variety of routes and that they migrate to potable groundwaters. It has been shown that consumption of contaminated groundwater is responsible for most of the outbreaks of waterborne disease in the United States (5) and that viruses probably are the etiologic agents in most of the cases (8). The increasingly frequent practice of land treatment of wastewater which may contain viruses, coupled with the ability of viruses to persist for long periods of time in the environment, has led to concern that viruses will become an increasingly significant cause of groundwater-related morbidity in the United States. Although the survival characteristics of viruses in surface waters and soils have been studied, little is known about virus survival in groundwater. The purpose of this study was

TABLE 1. Physical and chemical analyses of groundwater samples

Sample	pH	Ammonia (mg/liter)	Calcium hardness (mg/liter)	Total hardness (mg/liter)	Magnesium hardness (mg/liter)	Nitrate (mg/liter)	Total dissolved solids (mg/liter)	Turbidity[a] (NTU)
Wisconsin	8.0	0.122	208	424	216	11	260	0.28
Arizona	8.1	0.183	600	740	140	8.8	1,100	0.65
North Carolina 1	7.9	0.366	138	162	24	22	430	1.75
North Carolina 2	8.3	0.732	100	104	4	22	95	3.5
University of Arizona	8.2	0.061	92	102	10	8.8	190	0.65
New York 1	6.0	0.366	44	88	44	2.2	37	0.7
New York 2	7.3	0.122	138	194	56	2.2	145	0.51
Texas 1	8.0	0.427	224	448	224	2.2	850	0.8
Texas 2	7.7	0.976	354	926	572	28.6	950	1.2
California 1	8.0	0.061	216	272	70	13.2	235	0.5
California 2	8.1	0.061	216	272	56	4.4	200	0.6

[a] NTU, Nephelometric turbidity units.

to identify factors that influence virus persistence in groundwater.

MATERIALS AND METHODS

Sample collection. Groundwater (1 liter) samples were obtained from sites in Arizona, California, New York, North Carolina, Texas, and Wisconsin. All samples were collected aseptically in sterile polypropylene containers and packed on ice for transport to the laboratory.

Sample analysis. Measurements of nitrates, ammonia, sulfates, iron, and calcium, magnesium, and total hardness were made with Hach kits (Hach Co., Loveland, Colo.). Determination of total dissolved solids was made with a DS meter (Myron L Company, Encinitas, Calif.). Turbidity was measured with a turbidimeter (model 2100A; Hach Co.). An Expandomatic SS-2 pH meter (Beckman Instruments, Inc., Fullerton, Calif.) was used to measure pH. The temperatures of the water samples were measured at the time of collection. All other physical and chemical analyses were performed immediately before initiation of the experiments.

Experimental procedure. Samples (50 ml) of water were placed in sterile polypropylene centrifuge tubes (Falcon; Becton Dickinson and Co., Oxnard, Calif.). Viruses (poliovirus 1 strain LSc 2ab, echovirus 1 strain Farouk, or *E. coli* B phage MS-2) were added to achieve a final concentration of ca. 10^4 to 10^6 PFU ml^{-1}. The tubes were capped and incubated in water baths or incubators at the in situ groundwater temperature. Some samples were also incubated at two other temperatures. Subsamples (1 ml) were withdrawn on days 0, 1, 2, 3, 5, 7, 10, 15, 20, 25, and 30 and assayed in duplicate to determine the number of virus particles present.

Virus assays. The agar-overlay plaque technique of Adams (2) was used to determine the number of MS-2 phage present in a subsample. *E. coli* ATCC 15597 was used as the host bacterium.

Animal virus assays were done by the enumeration of plaques formed in infected cell monolayers (15). A continuous Buffalo Green Monkey kidney (BGM) cell line was used for all assays. The cells were grown in Nunclon six-well plates (GIBCO Laboratories, Grand Island, N.Y.) containing modified Eagle minimal essential medium and Earle salts (Flow Laboratories, Inc., McLean, Va.) supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah). After inoculation with virus-containing samples, the plates were incubated at 37°C in an atmosphere supplemented with 4% CO_2 (VWR incubator; VWR Scientific, Inc.) and observed for plaque formation.

Statistical analyses. With the $\log_{10}$ (number of viruses) as the y value and the day on which that number of viruses was present as the corresponding x value, we used linear regression to fit a line through the points. The slope of the line was called the decay rate of the virus. All decay rates are reported as positive numbers: the larger the number, the greater the decay rate.

Multiple regression analysis of the data was performed with the computer program REGRAN (19), which generated an intercorrelation matrix. REGRAN was also used to develop model equations to predict virus decay rates.

RESULTS

The physical and chemical characteristics of the water samples used are presented in Table 1.

The decay rates of MS-2, poliovirus 1, and echovirus 1 in all groundwater samples are presented in Table 2. Results of the multiple regression analysis to determine which water characteristics were correlated with decay rates show that incubation temperature was significantly correlated ($P = 0.05$) with the decay rates of all three virus types (Table 3). In addition, calcium hardness was significantly correlated with the decay rate of MS-2. By using all chemical and physical variates (temperature, pH, ammonia, nitrates, total dissolved solids, turbidity, and hardness), over 90% of the variation in decay rates among samples could be predicted.

DISCUSSION

Effect of temperature on virus persistence. The previous studies on the persistence of viruses in groundwater reported decay rates for poliovirus 1 of 0.21 $\log_{10}$ day^{-1} (9) and 0.0456 $\log_{10}$ day^{-1} (4). This investigation found 0.1615 $\log_{10}$ day^{-1} to be the mean value for the decay rate of poliovirus 1 in the 11 groundwater samples studied.

Temperature was found to be the single most important predictor of virus persistence in well water. The decay rates of MS-2 in all water samples at all incubation temperatures were analyzed as a function of temperature. Linear regression analysis gave a correlation coefficient of 0.88, which is significant at the 0.01 level. The coefficient of determination, R^2, was 0.775, indicating that 77.5% of the variation in decay rates among samples can be explained by temperature.

These results were not unexpected. Temperature had been found to be an important factor in virus persistence in all types of water, including marine, river, lake, and tap water (3, 14). However, the viruses persisted for longer periods in the well water samples than in surface waters incubated at similar temperatures. In river water at 16.5 to 27°C, inactivation rates for poliovirus 1 range from 1 day to achieve a 1 log reduction in titer (LRT) (11) to 0.25 day to achieve a 2 LRT (6). At 20°C in seawater, only 7 and 10 days were required for a 5 LRT for echovirus 1 and poliovirus 1, respectively (16). In contrast, this study found that poliovirus could persist at 26°C in well water samples for 3 to 5 days before a 1 LRT was effected. At the lower temperatures characteristic of the groundwater in most areas of the United States, both poliovirus 1 and echovirus 1 persisted for very long periods, up to 28.8 days, before a 1 LRT was achieved.

Effect of calcium on virus persistence. The concentration of calcium in the 11 groundwater samples was found to be significantly correlated with the decay rate of MS-2: as calcium concentration increased, the decay rate increased. This finding prompted further investigation to ascertain whether the increase in decay rate was due to a requirement for calcium in the assay procedure or whether it was an effect on the stability of the virus particle itself.

A few studies have investigated the role of calcium on the ability of bacteriophage to infect and replicate in host cells. Adams (1) reported on the requirement for calcium by T phages. He found that adsorption to the host cell was independent of calcium concentration. However, in the absence of calcium very few bacteria liberated viable virus progeny. Addition of calcium to T-infected bacteria in a calcium-free medium resulted in the liberation of viable virus particles. Thus, it was concluded that calcium is required during some stage of replication after penetration.

Rountree (13) found that divalent cations such as Ca^{2+} and Mg^{2+} are required for one more stage in staphylococcal bacteriophage replication in addition to adsorption to the host cell. She hypothesized that the other stage was penetration, because irreversible inactivation of phage particles adsorbed to host cells was effected by the removal of divalent cations from the medium.

Adams (2) states that adsorption to host cell surfaces can be prevented by the removal of calcium ions from the medium. It should be emphasized that these requirements for calcium and other divalent cations are virus specific, rather than host cell specific.

With MS-2 phage, this study found that the number of plaques produced by infection of susceptible host cells with similar numbers of phage particles did not vary significantly in the presence of calcium ions at concentrations ranging from 0 to 0.01 M. Interference with the assay procedure by calcium ions was therefore ruled out as the cause of the variation of decay rates among samples.

When survival experiments were performed with various concentrations of calcium in the same water sample, it was found that calcium concentration was not significantly correlated with the decay rate of MS-2 phage (M. V. Yates, Ph.D. dissertation, University of Arizona, Tucson, 1984). This suggested that some unmeasured property of the water which was correlated to the calcium concentration was involved in the observed variation in decay rates. More extensive investigation is needed to determine in what way, if any, calcium concentration affects virus persistence in groundwater.

MS-2 phage as a model of animal virus behavior. Detection and enumeration of animal viruses is an expensive, time-consuming process that requires access to a tissue culture laboratory. Bacteriophage such as MS-2 can be assayed easily and inexpensively in a short period of time using basic laboratory materials. For this reason, it is desirable to find a bacterial virus which could be used in studies as a model for animal virus behavior. Survival studies by Keswick et al. (10) and Bitton et al. (4) compared the inactivation rates of animal viruses and bacteriophage f2. The f2 phage was inactivated much more rapidly than the animal viruses in both studies; it was thus concluded that the behavior of animal viruses could not be modeled on f2.

In the present investigation, the inactivation rates of MS-2 were equal to or slower than those of the animal viruses in

TABLE 2. Decay rates[a] of viruses in groundwater samples

Sample	Temp (°C)	Decay rate[a]		
		MS-2	Poliovirus 1	Echovirus 1
Wisconsin	4	0.020	ND[b]	ND
	12	0.093	0.060	0.066
	23	0.244	ND	ND
Arizona	4	0.064	ND	ND
	12	0.162	ND	ND
	23	0.578	0.357	0.188
North Carolina 1	4	0.014	ND	ND
	12	0.030	0.138	0.186
	23	0.187	ND	ND
North Carolina 2	4	0.012	ND	ND
	12	0.095	0.114	0.174
	23	0.262	ND	ND
University of Arizona	4	0.025	ND	ND
	12	0.040	ND	ND
	23	0.325	0.676	0.628
New York 1	12	0.034	0.035	0.054
New York 2	12	0.037	0.051	0.051
Texas 1	13	0.077	0.036	0.138
Texas 2	13	0.114	0.137	0.079
California 1	18	0.082	0.185	0.151
California 2	17	0.075	0.081	0.091

[a] Decay rate = $-[(\log_{10} \text{PFU})\ \text{day}^{-1}]$.
[b] ND, Not done.

TABLE 3. Correlation between experimental conditions and virus decay rates in groundwater samples

Experimental condition	Correlation coefficient[a]		
	MS-2	Poliovirus 1	Echovirus 1
Incubation temp	0.8313	0.8251	0.7480
pH	0.3329	0.3919	0.3643
Ammonia	−0.1771	−0.2112	−0.2492
Calcium hardness	0.5689	−0.0130	−0.1539
Total hardness	0.3345	−0.1406	−0.2280
Magnesium hardness	0.0912	−0.2292	−0.2388
Nitrate	−0.0277	0.0600	0.0076
Total dissolved solids	−0.0702	−0.1866	−0.1266
Turbidity	−0.2118	−0.0487	−0.0454

[a] Numbers with an absolute value greater than 0.444 are significant at the 0.05 level (12).

most water samples. Statistical analysis of the data showed that there was no significant correlation between the decay rate of the viruses and the virus type used (MS-2, poliovirus 1, or echovirus 1). The MS-2 phage appears to behave more like the animal viruses than does the f2 phage. If the results of the survival experiments are to be used in developing criteria to predict safe distances between drinking water wells and septic tanks, the use of MS-2 as a model for animal virus behavior in groundwater would be justified in most cases. Because it is inactivated at a slower rate, its use would add an extra margin of safety in predictive models of animal virus behavior.

The criteria now used in the placement of drinking water wells relative to septic tanks and other sources of potential contamination do not take into account the possibility that viruses are present in the effluent. Because enteric viruses are the most likely etiologic agents involved in the majority of waterborne disease outbreaks in the United States, it seems important to incorporate information about their persistence in the environment in criteria being developed to determine safe distances between drinking water wells and sources of potential contamination.

There have been a few attempts with computer techniques to model the movement of viruses from sources of contamination to drinking water wells. These models assume an initial virus concentration at the contamination source and incorporate various soil characteristics, such as layering, permeability, and texture, in addition to groundwater flow variables. Until now, these models have had to use assumed values for virus decay rates. The results of this study will allow the input of more realistic values. In addition, because groundwater temperature was found to be highly correlated with the decay rate of the enteric viruses used in this study, it seems appropriate to include groundwater temperature and its relation to virus inactivation rates as one of the input variables in computer models of virus movement through the subsurface.

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