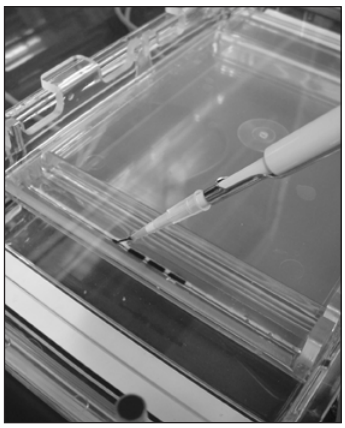
US groundwater may be subjected to fecal contamination from a variety of sources. This study sought to develop a preliminary assessment database on virus occurrence in groundwater systems at the national level. Information on physical and geological characteristics of groundwater wells, along with various microbial and physicochemical water quality parameters, was collected, and possible correlation with the presence of human viruses was investigated. Groundwater samples from 448 sites in 35 states were collected and assayed for microorganisms and chemical contaminants. Infective viruses, viral nucleic acid, bacteriophages, and bacteria were present in 4.8, 31.5, 20.7, and 15.1% of samples, respectively. Statistical analysis showed that one-time sampling is not sufficient for proper risk characterization. No significant direct correlations existed between the presence of virus and microbial indicators. However, when only the sites with repeat sampling were examined for correlations between indicators and pathogens, it was observed that if a site tested positive for a microbial indicator, it also tested positive at some point in time for pathogens.



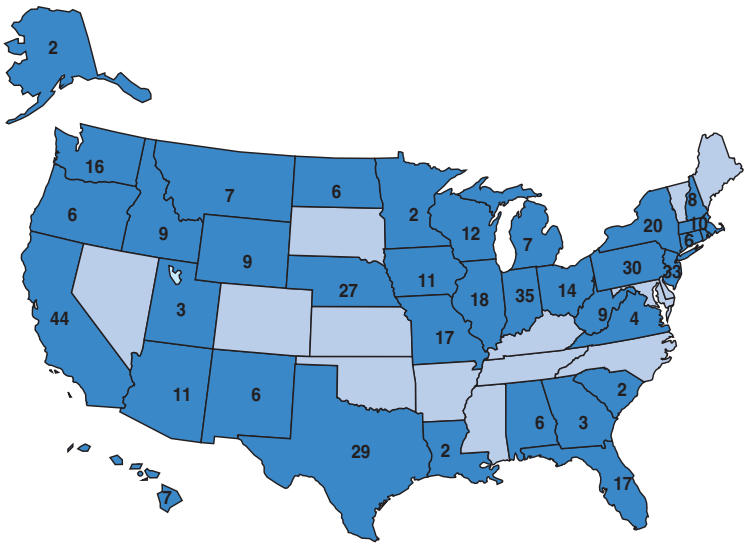
Occurrence of Viruses IN US GROUNDWATERS

BY MORTEZA ABBASZADEGAN, MARK LECHEVALLIER, AND CHARLES GERBA

More than 140 enteric viruses are known to infect humans. Enteric viruses are excreted in the feces of infected individuals and may directly or indirectly contaminate water intended for drinking. US groundwater may be subjected to fecal contamination from a variety of sources, including sewage treatment plant effluents, onsite septic waste treatment discharges, land runoff from urban, agricultural, and natural areas, and leachates from sanitary landfills. Under proper conditions, viruses have been observed to travel more than 328 ft (100 m) through the subsurface (Keswick et al, 1982a). Because of their smaller size, viruses (23–80 nm) are transported farther in groundwater than bacteria (0.5–3 µm) or protozoan parasites (4–15 µm). Thus, the occurrence of viruses in groundwater in the absence of coliforms may not be surprising. Information on the occurrence of viruses in groundwater is largely limited to studies in which land application of domestic wastewater has been practiced (Goyal et al, 1984), to outbreak investigations (Hejkal et al, 1982), or to when viruses have been purposely added (Keswick et al, 1982b). Surveys on viruses in groundwater have been limited because of the need to concentrate large volumes and the expense of using animal cell culture. However, the development of molecular-based methods for virus detection in groundwater allows for application of less expensive methods to detect a wide range of viral contamination (Abbaszadegan et al, 1999a; Abbaszadegan et al, 1993).

Evidence for a possible route of fecal contamination of surface water and groundwater is provided by the detection of enteric viruses in either surface water or groundwater and the continued occurrence of viral waterborne disease.

FIGURE 1 Location of wells sampled (shaded areas) and number of sites sampled in each state



Wells sampled are indicated in the shaded areas; also included is the number of wells sampled in each state.

The US Environmental Protection Agency (USEPA) and the Centers for Disease Control and Prevention (CDC) have maintained a database of disease outbreaks in the United States since 1971 (Craun & Calderon, 1996). The database is formulated on the responses to a voluntary reporting by state and local public health officials, but a large number of waterborne outbreaks may not be recognized or reported (Craun, 1986). Between 1971 and 1994, 650 outbreaks of waterborne disease and 569,754 cases of illness were reported in the United States, with 58% associated with groundwater sources and 33% associated with surface water sources. Eight percent of all reported outbreaks were due to enteric viruses (hepatitis A virus [HAV], Norwalk virus, and rotaviruses) (Craun & Calderon, 1996). It is suspected that many waterborne disease outbreaks for which no etiological agent was identified (about 47% of all reported outbreaks) may be caused by viruses. Analysis showed that 10% of the groundwater outbreaks were caused by viruses, whereas 4% of the surface water outbreaks were viral (Craun, 1999).

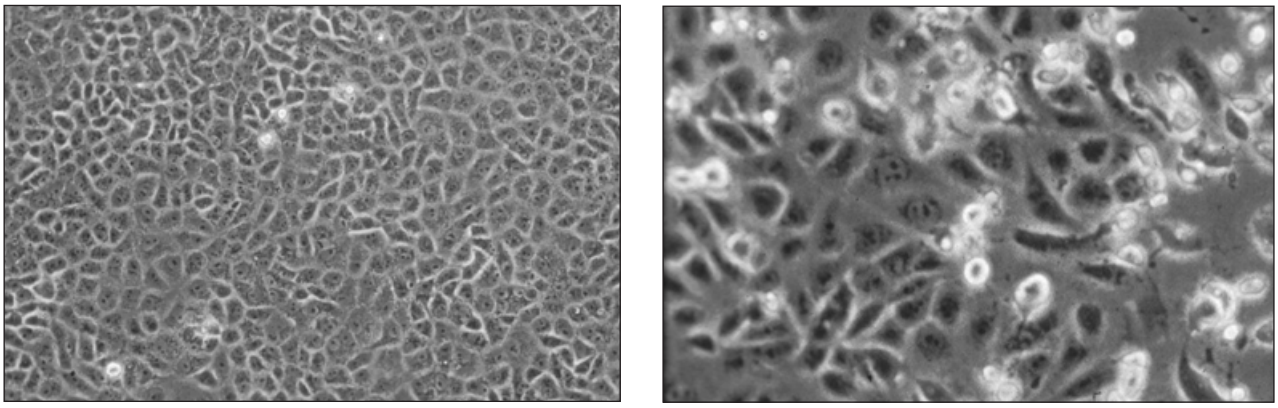
Examination of waterborne outbreak data for public groundwater systems (community and noncommunity) for 1971–94 showed that three types of source water contamination were involved: untreated water, disinfected water, and filtered systems. Eighty-one percent of these 356 groundwater outbreaks were related to source water contamination, with 45% attributed to untreated water and 35% to improperly disinfected water. Community systems using groundwater experienced 113 outbreaks, whereas noncommunity systems using groundwater experienced 243 outbreaks. Thirty-one percent and 62% of outbreaks in community systems were traced to distribution system and source water contamination, respectively. The majority (89%) of outbreaks in noncommunity systems were associated with source water contamination (7% were related to the distribution system). Among recognized causes of groundwater viral outbreaks in public water systems during 1971–94, HAV was associated with eight community and nine noncommunity systems. Norwalk virus was identified as the etiologic agent in 4 community and 12 noncommunity outbreaks (Craun, 1999).

THE GROUND WATER RULE

The Safe Drinking Water Act (SDWA) of 1974 established mandates for protecting the nation's public drinking water supply. The SDWA was amended in 1986, which required USEPA to establish National Primary Drinking Water Regulations requiring disinfection as treatment for the inactivation of microbiological contaminants for all public water supply systems, including systems supplied by groundwater sources. This mandate was again amended in 1996 to require disinfection for groundwater sources "as necessary." This amendment required USEPA to develop the Ground Water Rule (GWR), which specifies the appropriate use of disinfection and also addresses other components of groundwater systems to ensure public health protection. A revised version of the GWR was issued in May 2000 (USEPA, 2000).

When the rule was developed, the following items were unknown: the percentage of water supplies at risk from viruses, the level of viruses in contaminated groundwater supplies, the criteria necessary to identify contaminated wells, and the level of treatment necessary to ensure safe drinking water, which is critical in the development of such a rule. Therefore, a database of virus occurrence and virus concentrations in public groundwater systems needs to be developed. Advanced molecular techniques, such as polymerase chain reaction (PCR), may allow for the routine monitoring of source water or finished water for the presence or absence of viruses in groundwaters.

The purpose of this project was to apply recent advances for the detection of enteric viruses in groundwater using traditional cultural methods and recent advances in the molecular methods. It was of interest to determine how water quality or hydrogeological conditions at a site might determine the susceptibility of a well to viral contamination. However, the primary objective of



Twenty-one sites and 22 samples tested positive for viral infectivity by exhibiting cytopathic effect in Buffalo green monkey (BGM) kidney cells (right). A normal uninfected BGM cell culture monolayer is shown on the left for comparison.

this research was to develop a preliminary assessment database on virus occurrence in groundwater systems at the national level. The database will be used to develop criteria for determining the vulnerability of groundwater to viral contamination. The specific objectives are as follows: (1) to determine the occurrence of virus contamination in the untreated source water of public groundwater systems, (2) to investigate water quality parameters and the occurrence of microbial indicators in groundwater to determine correlations with human viruses, and (3) to analyze data (microbial, physical, and chemical measurements) to develop a quantitative approach that can be used to screen groundwater systems to identify wells that are at risk for fecal contamination.

TABLE 1 Microbial occurrence results of sites sampled*

Microorganisms Tested	*n*†	Positive	Negative	Unknown
Viruses cell culture				
Infectious enteroviruses	442	21 (4.8%)	427 (96.6%)	0 (0 %)
Viruses reverse transcriptase–polymerase chain reaction				
Enterovirus	448	68 (15.2%)	360 (80.3%)	20 (4.5%)
Rotavirus	448	62 (13.8%)	363 (81.0%)	23 (5.1%)
Hepatitis A virus	448	31 (6.9%)	399 (89.1%)	18 (4.0%)
Norwalk virus	317	3 (0.9%)	309 (97.5%)	5 (1.6%)
Combined (any virus)‡	448	141 (31.5%)	302 (67.4%)	5 (1.1%)§
Bacteria				
Total coliform	445	44 (9.9%)	401 (90.1%)	0
Enterococci	355	31 (8.7%)	324 (91.3%)	0
Clostridium	57	1 (1.8%)	56 (98.2%)	0
Combined (any bacteria)**	445	67 (15.1%)	378 (84.9%)	0
All (three bacteria)††	57	0 (0%)	100%	0
Bacteriophages				
Escherichia coli C	444	18 (4.1%)	426 (95.9%)	0
E. coli C 3000	444	48 (10.8%)	396 (89.2%)	0
Salmonella WG-49	440	42 (9.5%)	398 (90.5%)	0
Combined (any host)‡‡	444	92 (20.7%)	352 (79.3%)	0
All (three hosts)§§	440	1 (0.2%)	99.8%	0

*Norwalk virus, enterococci, and *Clostridium* assays were begun later in the course of the study.
†*n* = number of sites/wells
‡Sample tested positive for any of the viruses using reverse transcriptase–polymerase chain reaction.
§For some samples, the reverse transcriptase–polymerase chain reaction assay was inhibited for all of the four viruses.
*Site tested positive or negative for one or more of the bacteria listed.
††Site tested positive or negative for all of the bacteria listed.
‡‡Site tested positive or negative for one or more of the bacterial hosts listed.
§§Site tested positive or negative for all of the three microorganisms at the same time.

TABLE 2 Comparison between cell culture versus RT–PCR* results for all viruses†

Status of Cell Culture	RT–PCR Positive (Positive for Enterovirus, Rotavirus, Hepatitis A, or Norwalk Virus)	RT–PCR Negative	RT–PCR Unknown
Cell culture positive	12	8	1
Cell culture negative	129	289	3
Cell culture unknown	0	5	1

*RT–PCR—reverse transcriptase–polymerase chain reaction
†The numbers in this table represent sites. The total number of sites tested and compared in this table is 448. The cell culture and the reverse transcriptase–polymerase chain reaction results using enterovirus primers were compared because the Buffalo green monkey cell line used is known to be sensitive to enteroviruses. The primer set used detected three groups of enteroviruses.

TABLE 3 Comparison among total coliform, enterococci, and bacteriophage versus RT–PCR* results

	RT–PCR Positive (Positive for Any Virus†)	RT–PCR Negative	RT–PCR Unknown
Total‡ coliform positive	19	25	0
Total coliform negative	120	276	5
Enterococci§ positive	9	22	0
Enterococci negative	86	236	2
Bacteriophage** positive	34	55	3
Bacteriophage negative	105	245	2
Total†† cultural positive	55	99	3
Total cultural negative	86	203	2

*RT–PCR—reverse transcriptase–polymerase chain reaction
†Enterovirus, rotavirus, hepatitis A, and Norwalk virus
‡For coliform, a total of 445 sites were compared in this table. Three samples were excluded for which one assay result was undetermined.
§For enterococci, a total of 355 sites were compared in this table; samples excluded for which one assay result was undetermined.
**For bacteriophage, a total of 444 sites were compared in this table; samples excluded for which one assay result was undetermined.
††For total cultural, all 448 sites were compared with either one of cell culture, enterococci, total coliform, *Clostridium*, and bacteriophage.

MATERIAL AND METHODS

Water sampling program. ***Site selection.*** The sample collection strategy allowed sampling from different geographical locations with a variety of physical and chemical characteristics. Some of the samples used in this study were initially collected for a project evaluating the efficacy of the PCR method for the detection of viruses in groundwater (Abbaszadegan et al, 1999a). Sites selected for that project were chosen based on their widely varying characteristics, such as very high or low mineral or metal content, pH, or temperature. Also included were sites known to be under the influence of surface water. Only 12 samples were collected from such wells.

Initially, groundwater sources were selected at random from sites volunteered by water utility personnel agreeing to participate in the project. Seven hundred fifty wells were available for selection; however, any of the following criteria resulted in exclusion of sites from the sampling campaign: sites known to be under the influence of surface water, wells with no log records available, and poorly constructed wells. The remaining sites were selected based on their geological characteristics to match the actual national profile for groundwater sources. The objective of these selection criteria was to ensure that the samples closely represented the various geological formations throughout the United States used for groundwater production. All of the wells were actual drinking water production wells and not monitoring wells.

Sampling kit. To provide consistent sampling procedures, 30 identical sample kits were assembled. Each kit contained all the equipment needed to collect a sample, including all hoses and connectors, a filter and a filter housing, protective gloves, reusable ice packs, sample bottles, a sample data sheet, and a detailed written protocol. The kits also included a flowmeter and an inline flow-restricting device to limit the filtration rate to 4 gpm (0.25 L/s). In addition, to help ensure that the sampling procedure was consistent, a 10-min professionally produced VHS video was included to demonstrate all of the details of the procedure.

Sample collection and cell culture assay. Sample collection, filter elution, virus flocculation and reconcentration, cell culture assay, and positive and negative controls were performed as described earlier (Abbaszadegan et al, 1999a).

Bacteriophage assay. The following phage hosts were used for the coliphage assays: *Escherichia coli* C,[1] *E. coli* C-3000,[2], *Salmonella typhimurium* WG49, for male-specific (FRNA) bacteriophage (Havelaar et al, 1993). MS-2[3] and φ X174[4] were used as positive control. Bacteriophage assays were performed as described earlier (Abbaszadegan et al, 1999b).

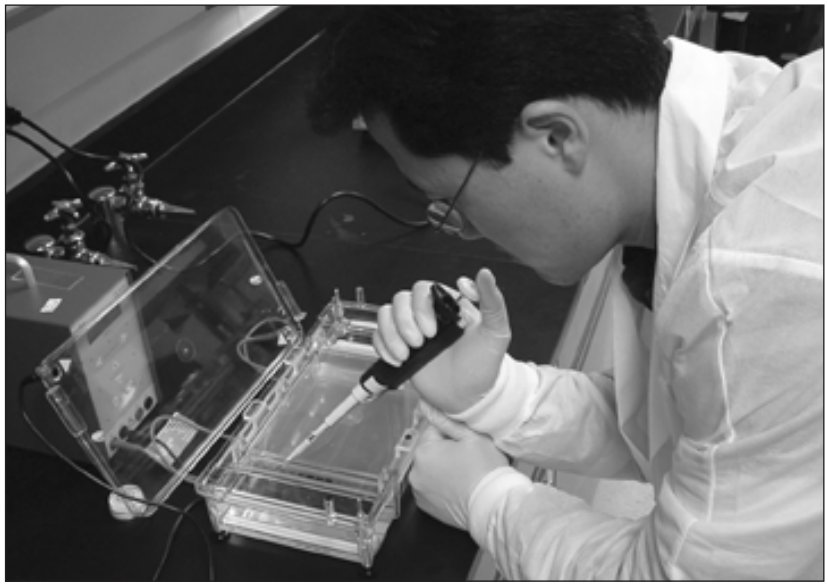
Test for bacteria. A 1-L sample of raw water was collected in a sterile polypropylene container and returned with the sampling kit. Within 24 h of receipt, three 100-mL portions of the sample were assayed for total coliforms by membrane filtration method and incubated on mEndo Agar LES[5] for 24 h at 37°C. The volume of sample assayed for enterococci and *Clostridium* was 300 and 400 mL, respectively (Abbaszadegan et al, 1999b).

Reverse transcriptase–PCR method and primers used for virus detection. The sample preparation, primers, and large

volume PCR conditions were followed as described earlier (Abbaszadegan et al, 1999a). The Norwalk virus primers NV3-5'- GCA CCA TCT GAG ATG GAT GT-3' and NV51-5'-GTT GAC ACA ATC TCA TCA TC-3' produced a 205-bp product and targeted the 58-kD capsid protein (Moe et al, 1994).

Geological and well construction information. The participating utilities completed a detailed questionnaire for each well, providing information on the geology, construction, location, and physical characteristics.

Statistical analysis. Statistical analysis was performed by an independent firm.[6]



Gel electrophoresis is the most common method of identifying positive samples after completion of a polymerase chain reaction.

RESULTS

Groundwater samples were collected from 448 sites (wells), from 117 utilities, or from state agencies in 35 states (Figure 1). Out of 448 sites, 25 sites were sampled more than once. A total of 539 samples was collected from all the sites. Each sample was assayed for virus infectivity using cell culture assay, the presence of viral nucleic acid using reverse transcriptase–PCR (RT–PCR), bacteriophage using three hosts, total coliforms, enterococci, *Clostridium perfringens,* total organic carbon, and various metals and minerals.

Virus cell culture assays. Twenty-one sites (4.8% of 442) and 22 samples (4.1% of 529) tested positive for viral infectivity by exhibiting cytopathic effect (CPE) in Buffalo green monkey (BGM) kidney cells. One of the sites, which was sampled more than once, tested positive twice for viruses using cell culture.

The most probable number/100 L ranged from 0.09 to 1.86 for the samples that were virus-positive. All of the samples tested positive when seeded with poliovirus type 1 (LSc strain) as a positive control.

Virus RT–PCR assays. Sample concentrates were assayed for viral ribonucleic acid (RNA) by the RT–PCR method. Four pairs of primers, specific for enterovirus, rotavirus, HAV, and Norwalk virus, were used. Each sample was assayed twice with each primer pair—once after having been seeded with a positive control virus and once unseeded. The seeded reactions were performed to determine whether the sample would inhibit the RT–PCR. If no PCR product was found in the seeded reaction, the sample was deemed inhibitory. These samples were recorded as "unknown" with respect to the RT–PCR assay results (Table 1).

Bacterial and bacteriophage assay results. The first three sites were not tested for total coliform, and not all samples were tested for all three bacteria. The enterococci and *Clostridium* assays were begun later in the course of the study. Four hundred forty-four sites were tested for all bacteriophage except for *Salmonella* WG-49 host, which was tested at 440 sites. Four sites were chlorinated and not tested for any bacteriophage (Table 1).

Comparative results. Seven microorganisms (three bacteria, three bacteriophages, and one virus) were tested by using cultural assays, and four viruses were tested using RT–PCR. Cultural methods included cell culture assays for infectious enteroviruses, double-layer agar assay for bacteriophage, and membrane filtration method for bacterial indicator assays. The target microorganisms for this project are indicative of fecal contamination of source water. A total of 40.6% of sites were positive for one or more of the seven microorganisms using cultural assay, and 31.5% (141/448) of the sites were positive for at least one of the four viruses by RT–PCR (Table 1).

Comparison of virus assay methodologies. Both the cell culture assay and the RT–PCR assay attempt to detect viruses in the sample (Table 2). However, they are not directly comparable. The cell culture assay can only detect infectious viruses, whereas the RT–PCR assay can potentially detect both infectious and noninfectious viruses. Additionally, the RT–PCR assay is designed to detect specific viruses, based on their RNA sequence. Conversely, the cell culture assay can potentially detect any infectious virus capable of causing infection in the cultured cells. For instance, BGM cells used in the cell culture assay could possibly show infection from mainly enteroviruses and possibly rotaviruses or other viruses, whereas the RT–PCR assay does not result in this kind of ambiguity. Additionally, each assay differs in level of sensitivity and the effect of inhibitory or toxic substances in the sample and the equivalent sample volume examined. Viral

TABLE 4 Classification efficiency* for low- and high-risk wells for the calibration data set using all the microbial results to identify risk type

Parameter	Scoring Based on 25th and 95th Percentiles			Scoring Based on 25th and 90th Percentiles			Scoring Based on 25th and 50th Percentiles			Scoring Based on 25th and 75th Percentiles		
	Low Risk n = 32	High Risk n = 82	Overall n = 114	Low Risk n = 32	High Risk n = 82	Overall n = 114	Low Risk n = 32	High Risk n = 82	Overall n = 114	Low Risk n = 32	High Risk n = 82	Overall n = 114
Unsaturated soil	8.33	96.43	52.38	33.33	91.07	62.20	79.17	50.00	64.59	66.67†	78.57†	72.62†
Maximum grout	20.00	95.45	57.73	30.00	90.91	60.45	65.00	54.55	59.78	45.00	75.00	60.00
Minimum screen	12.00	95.83	53.92	12.00	91.67	51.84	76.00	50.00	63.00	28.00	75.00	51.50
Nitrate	6.25	88.31	47.28	6.25	88.31	47.28	65.63	50.65	58.14	56.30	75.32	65.81
Index A‡	12.50	97.53	55.02	21.88	93.83	57.86	75.00	59.26	67.13	53.13	80.25	66.69
Index B	17.86	97.01	57.44	28.57	92.54	60.56	71.43	49.25	60.34	39.29	79.10	59.20
Index C	6.25	96.25	51.25	12.50	93.75	53.13	75.00	56.25	65.63	53.13	80.00	66.57
Index D	15.63	92.59	54.11	28.13	88.89	58.51	68.75	59.26	64.01	62.50†	77.78†	70.14†
Index E	18.75	91.25	55.00	18.75	88.75	53.75	68.75	51.25	60.00	46.88	75.00	60.94
Index F	14.29	95.31	54.80	32.14	87.50	59.82	82.14	46.88	64.51	60.71	78.13	69.42
Index G	22.22	93.94	58.08	40.74	87.88	64.31	77.78	48.48	63.13	70.37†	74.24†	72.31†
Index H	12.50	91.25	51.88	28.13	87.50	57.82	78.13	52.50	65.32	71.88†	72.50†	72.19†
Index I	15.38	94.92	55.15	23.08	91.53	57.31	73.08	45.75	59.42	42.31	71.19	56.75
Index J	15.63	88.61	52.12	15.63	86.08	50.86	81.25	49.37	65.31	59.38	72.15	65.77
Index K	18.75	88.75	53.75	25.00	86.25	55.63	65.63	50.00	57.82	56.25	71.25	63.75

*Percentage of individual parameters and indexes
†Indicates overall classification efficiency >70%
‡Meaning of indexes: A—unsaturated soil, minimum screen distance, maximum grout depth, nitrate; B—unsaturated soil, minimum screen distance, maximum grout depth; C—unsaturated soil, minimum screen distance, nitrate; D—unsaturated soil, maximum grout depth, nitrate; E—minimum screen distance, maximum grout depth, nitrate; F—unsaturated soil, minimum screen distance; G—unsaturated soil, maximum grout depth; H—unsaturated soil, nitrate; I—minimum screen distance, maximum grout depth; J—minimum screen distance, nitrate; K—maximum grout depth, nitrate

TABLE 5 Sewage source distances for cell culture and RT–PCR* positive sites

Distance Range *ft (m)*	Sewage Source Distance *ft (m)*	Sites Sampled	Cell Culture Sites	RT–PCR Positive Sites (Enterovirus Only)	RT–PCR Positive Sites (All Primers)
≤150 (46)	≤150 (46)	174	10 (47.6%)	29 (42.6%)	59 (41.8%)
	151–250 (46–76)	46	2 (9.5%)	7 (10.3%)	12 (8.5%)
	251–350 (77–107)	32	2 (9.5%)	7 (10.3%)	11 (7.8%)
	351–450 (107–137)	21	1 (4.8%)	2 (2.9%)	8 (5.7%)
	451–550 (137–168)	28	2 (9.5%)	3 (4.4%)	8 (5.7%)
151–550 (46–168)		127	33.3%	27.9%	27.7%
Total for <550 (168)		301	80.9%	70.5%	69.5%
	551–650 (168–198)	8	0	2 (2.9%)	2 (1.4%)
	651–999 (198–304)	11	0	2 (2.9%)	3 (2.1%)
	1,000–1,999 (304–609)	44	1 (4.8%)	7 (10.3%)	11(7.8%)
	2,000–2,999 (610–914)	23	0	1 (1.5%)	5 (3.5%)
	3,000–3,999 (914–1,219)	7	1 (4.8%)	1 (1.5%)	2 (1.4%)
≥5,000 (1,524)		21	0	1 (1.5%)	4 (2.8%)
None†		33	2 (9.5%)	6 (8.8%)	16 (11.3%)
Total sites		448	21	68	141

*RT–PCR—reverse transcriptase–polymerase chain reaction
†No source of contamination was reported by the utility.

pathogens were detected in 141 sites using RT–PCR and 21 sites using cell culture assay. However, only 12 sites showed viral contamination using both cell culture and RT–PCR, and 289 sites were negative for all viruses using both techniques (Table 2).

To ensure consistent sampling procedures, 30 identical sample kits were assembled. Each contained all of the equipment needed to collect a sample, along with a flowmeter and an inline flow-restricting device. A 10-min video was also included to demonstrate all of the details of the procedure to further provide for consistency.

Virus assays compared with other potential indicator assays. One objective of this study was to evaluate other biological and physical attributes of groundwater as potential indicators of the presence of viruses. These assays are often simpler to perform and are less expensive and more familiar to water utility personnel. To this end, the authors examined the relationship between virus assay results and the results of assays for total coliforms, enterococci, and bacteriophage. In addition, total cultural assays (cell culture, bacterial, and bacteriophage assays) were examined with the RT–PCR results. Fifty-five sites were positive for microbial indicators and pathogens using cultural and RT–PCR assays, whereas 203 sites were negative by both assays (Table 3).

Comparing individual nonviral assay results with both RT–PCR and cell culture viral assay results showed no significant correlations ($p = 0.05$) with any individual indicator. However, a more extensive statistical analysis was performed in which wells were first classified as high or low risk based on one or more positive fecal indicator assays.

The ability of abiotic parameters to correctly predict the susceptibility of wells to fecal contamination was examined by developing a screening tool. This process included grouping the wells according to the physical parameters, establishing a quantitative threshold for selected parameters, and analyzing the quantitative threshold to obtain a score. Wells were identified as being at high or low risk of vulnerability to fecal contamination using microbial occurrence data. The data were randomly divided into a calibration subset and a validation subset. Half of the wells identified as high risk and half of the wells identified as low risk were randomly selected and combined into the calibration set. The remainder of the wells was combined into a validation set. Summary statistics (sample size, mean, and standard deviation) were calculated for the calibration data set in which wells were classified as low or high risk based on microbial data. Mann-Whitney *U*-tests were performed on the quantitative parameters of the calibration data set to determine which parameter can be used to distinguish wells based on the level of risk of microbial contamination. Many of the parameters in this study were qualitative such as screened, confined aquifer over bedrock, casing, groundwater under the influence of surface water, chlorination, geology, and deposit type. Therefore, a numeric value was assigned to each parameter for statistical evaluation. Parameters were subcategorized if necessary. Spearman's correlation was performed on both quantitative and qualitative parameters to distinguish them based on level of risk. Eleven indexes—A–K—(Table 4) were created using calibration data. The scores of each critical parameter were scaled (score of 1, 3, or 5 for each well) so that each parameter had a similar effect on the index. Percentiles for the high-risk wells were calculated for each of the selected parameters. The classification efficiency of each parameter and index was calculated as the percent of wells that were correctly classified. Classification efficiencies for each parameter and each index were calculated for both the calibration and validation data set. Parameter or indexes that correctly classified at least 70% of the wells were considered to be the acceptable indicator of the vulnerability of groundwater wells to fecal contamination. When wells were classified using all the microbial results, the parameter unsaturated soil and indexes D (unsaturated soil, maximum grout depth, nitrate), G (unsaturated soil, maximum grout depth), and H (unsaturated soil, nitrate) correctly classified more than 70% of the wells using the 25th and 75th percentiles to score the parameter values (Table 4).

Physical characteristics. Various physical characteristics of each well were gathered through a questionnaire for the study. The depth of the wells and the percent samples collected in each depth category are summarized in Figure 2. Turbidity ranged between 0.0 and 85 ntu, temperature ranged between <1 and 35°C, TOC ranged between 0.123 and 85.37 mg/L, and pH ranged between

TABLE 6 Comparison of samples collected at each geology deposit with the percent production at national level

Geology/Deposit	Number of Sites Collected	Sites Collected %	Production at National Level %*
Unconsolidated	287	64.1	69.5
Alluvial sand and gravel	180	62.7	32.7
Coastal plain	31	10.8	17.1
Fluvial/eolian	0	NA†	2.4
Glacial valley	19	6.6	2.3
Glacial outwash	44	15.3	13.8
Glacial valley and/or outwash	0	NA	1.2
Other	8	2.8	NA
Unknown	5	1.7	NA
Bedrock	122	27.2	30.5
Carbonates	46	37.7	18.1
Sandstone and conglomerate	40	32.8	8.3
Siltstone	11	9.0	0.2
Plutonic igneous and metamorphic	10	8.2	0.6
Limestone	4	3.8	
Volcanic	8	6.6	3.4
Unknown	3	2.5	NA
Unknown	39	8.7	NA
Total	448	100	100

*Based on US Geological Survey (1990)
†NA—not applicable

4 and 9.6. The samples that tested positive with cell culture and RT–PCR were analyzed for the distance to a source of contamination (Table 5). The sources were identified by the participating utilities and included septic tanks, sewer lines, and wastewater plants. Samples were tested in three categories: 147 samples in <150 ft (46 m) distance, 154 samples in 151 to <550 ft (46 to <168 m) distance, and 147 samples in >550 ft (<168 m) distance. Examination of cell culture and PCR positive results showed that the majority of positive results from all three categories had a sewage source fewer than 150 ft (46 m) away (all three in the 41–47.6% range) and 80.9% of sites that tested positive had a sewage source closer than 550 ft (167 m) (Table 5).

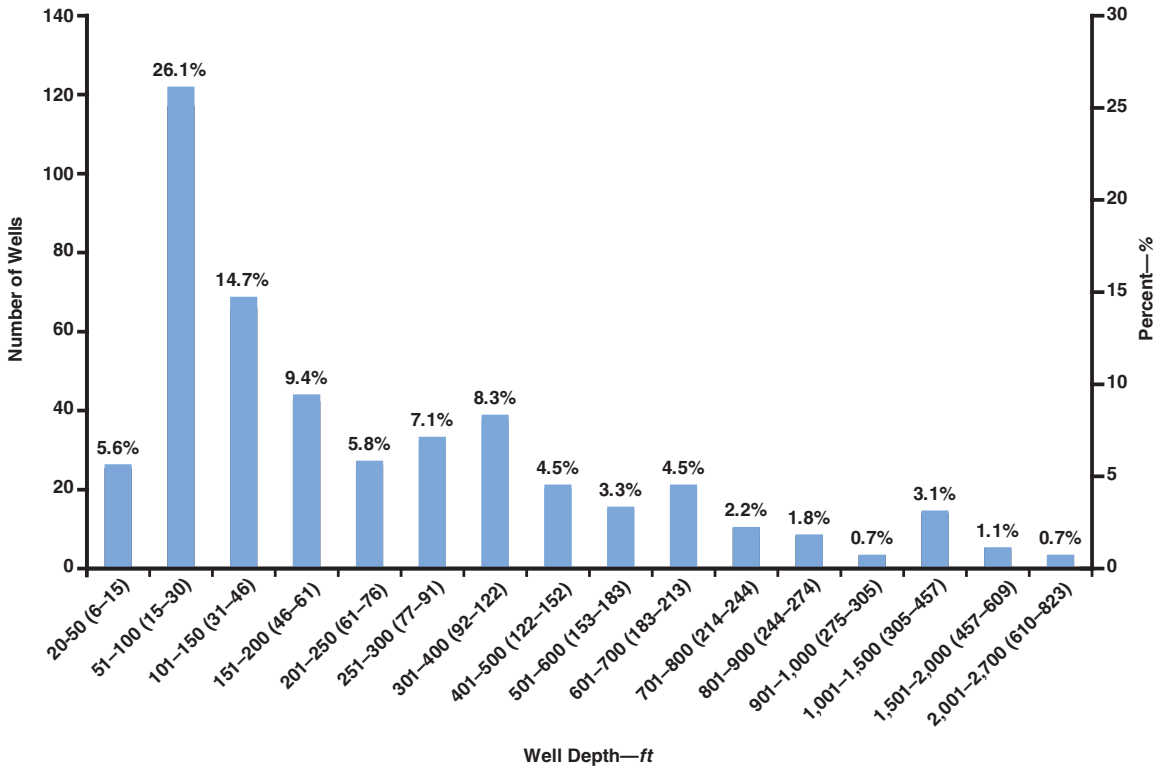
Analysis of results by geological formation. Geological formations are classified into two basic groups: bedrock or unconsolidated. Table 6 categorizes the 448 wells by classification and by subgroups. The positive viral and bacterial assay results within those classifications are summarized in Table 7. The bedrock-situated wells represented 27.2% of total wells sampled, and 25.9% of these wells were positive by biological assays, whereas the unconsolidated wells represented 64.1% of total wells sampled, and 60.9% of these wells were positive for biological assays. Within the bedrock and within the unconsolidated formation, the sedimentary/carbonate formation and the alluvial subgroup had the highest positive sites by biological assays (Table 7). This would seem to indicate that these two particular formations—unconsolidated/alluvial and bedrock/sedimentary-carbonate—are more likely to test positive for biological indicators and thus may represent a higher risk of fecal contamination.

Repeat sampling. Twenty-five sites were sampled more than once. Thirteen sites were negative for all the assays. The remaining 12 sites tested positive for one or more of the assays. When the results were analyzed considering all of the samples collected from the 448 sites to determine whether a correlation existed between virus occurrence and any of the indicators in groundwater, no such correlation was observed. However, when only the sites with repeat sampling were examined for correlations between indicators and pathogens, it was observed that if a site tested positive for a microbial indicator, it also tested positive, at some point in time, for pathogens (Table 8).

Seasonality. Seasonal trends can be seen for some of the viruses tested by RT–PCR. Enteroviruses have seasonal peaks in summer and early fall (CDC, 1989; Strikas et al, 1986). Rotavirus infections peak from late autumn to early spring and start in the southwest regions of the country moving to the northeast states by spring (Torok et al, 1997; Gouvea et al, 1990; Matson et al, 1990). HAV infections do not show a seasonal pattern (CDC, 1996). In this study, a seasonal trend was observed by the detection of a higher number of virus-positive samples during the summer months and in the early fall, except for HAV, in which case a peak was observed during February and March (Figure 3). No clear trend was observed for the cultural methods (Figure 4). Figure 5 shows the groundwater temperature trend for cell culture and RT–PCR positive samples.

The objective of this analysis was to investigate the relationship between RT–PCR and cell culture methods of virus detection in certain groundwater temperature groups. The number of samples in all temperature groups was not the same. Within all temperature groups, a similar trend was observed between the RT–PCR and cell culture–positive samples. At the 11–15°C temperature range, the number of virus-positive samples by both RT–PCR and cell culture methods was the great-

FIGURE 2 Depth of the wells and percent samples collected in each depth category



More than 75% of the wells sampled ranged from 51 to 500 ft (15 to 152 m) in depth.

est, suggesting longer survival of viruses in this temperature range.

DISCUSSION

The primary objective of the study was to develop a preliminary assessment database on virus occurrence in the untreated source water of public groundwater systems at the national level. In addition, the authors investigated various water quality parameters and the occurrence of microbial indicators in groundwater and their possible correlation with the presence of human viruses. The analysis of the data (microbial, physical, and chemical measurements) was used to develop a quantitative approach that can be utilized to screen groundwater systems to identify wells that are at risk for fecal contamination.

In order to determine whether this study is truly representative of US groundwater sites as a whole, USEPA conducted a comparative study between this study and the national database for nitrate data (USEPA, 1997). Groundwater data are typically not compiled into national databases with the exception of nitrate. Therefore, nitrate data were used in the comparative study. Nitrate data for 216 sites from this study were compared with those of 216 sites in the US Geological Survey (USGS) database (Lanfear, 1992). Analysis of variance statistical tests conducted to compare the mean log nitrate concentrations in this study versus the USGS data indicated that at a 95% confidence level, there was no significant difference (USEPA, 1999). The USEPA report concluded that the samples appropriately represented wells in the United States with low nitrate concentration.

PCR assay compared with cell culture. Groundwater samples analyzed for the occurrence of viruses using RT–PCR indicate higher viral contamination compared with cell culture methods (Table 1 and Figure 5). PCR assay is indicative of the presence of viral nucleic acid and not necessarily the infectious viral particle. Therefore, the PCR results should be interpreted as indicative of the possibility of virus transport within the aquifer and a potential risk of disease rather than an absolute public health problem. The total of the cultural indicators (coliforms, enterococci, *Clostridium*, bacteriophage) were positive in 40.6% of the samples tested, whereas 31.5% of the total sites were positive for RT–PCR, suggesting that the general overall indication of fecal contamination of groundwater sources did not substantially differ between the molecular technique (PCR) and the cultural methods.

The greater number of virus-positive samples could be due to several reasons including (1) greater sensitivity

TABLE 7 Biological assays by geologic formation*

Formation	All Sites (wells)		Cell Culture Positive Sites		Enterovirus RT–PCR† Positive Sites		Positive Sites for Any Bacteria		Positive Sites for Bacteriophage	
	n‡	%	*n*	%	*n*	%	*n*	%	*n*	%
Bedrock	122	27.2	4	19.0	22	32.4	18	26.9	23	25.0
Igneous/metamorphic	10	8.2	0	NA§	0	NA	0	NA	0	NA
Sedimentary/carbonates	46	37.7	3	14.3	11	16.2	7	38.9	14	15.2
Sedimentary/ sandstone	40	32.8	1	4.8	6	8.8	7	38.9	5	5.4
Siltstone	11	9.0	0	NA	2	2.9	2	11.1	3	3.3
Volcanic	8	6.6	0	NA	3	4.4	1	5.6	1	1.1
Limestone	4	3.8	0	NA	0	NA	1	5.6	0	NA
Unknown	3	2.5	0	NA	0	NA	0	NA	0	NA
Unconsolidated	287	64.1	15	71.4	38	55.9	41	61.2	54	58.7
Alluvial	180	62.7	9	42.9	24	35.3	24	58.5	34	37.0
Coastal plain	31	10.8	2	9.5	2	2.9	5	12.2	6	6.5
Glacial outwash	44	15.3	1	4.8	7	10.3	7	17.1	8	8.7
Glacial valley	19	6.6	1	4.8	2	2.9	4	9.8	2	2.2
Abandoned mine	3	1.0	0	NA	0	NA	1	2.4	0	NA
Other	5	1.7	0	NA	0	NA	0	NA	1	1.1
Unknown	39	8.7	2	9.5	8	11.8	8	11.9	15	16.3
Totals	448		21		68		67		92	

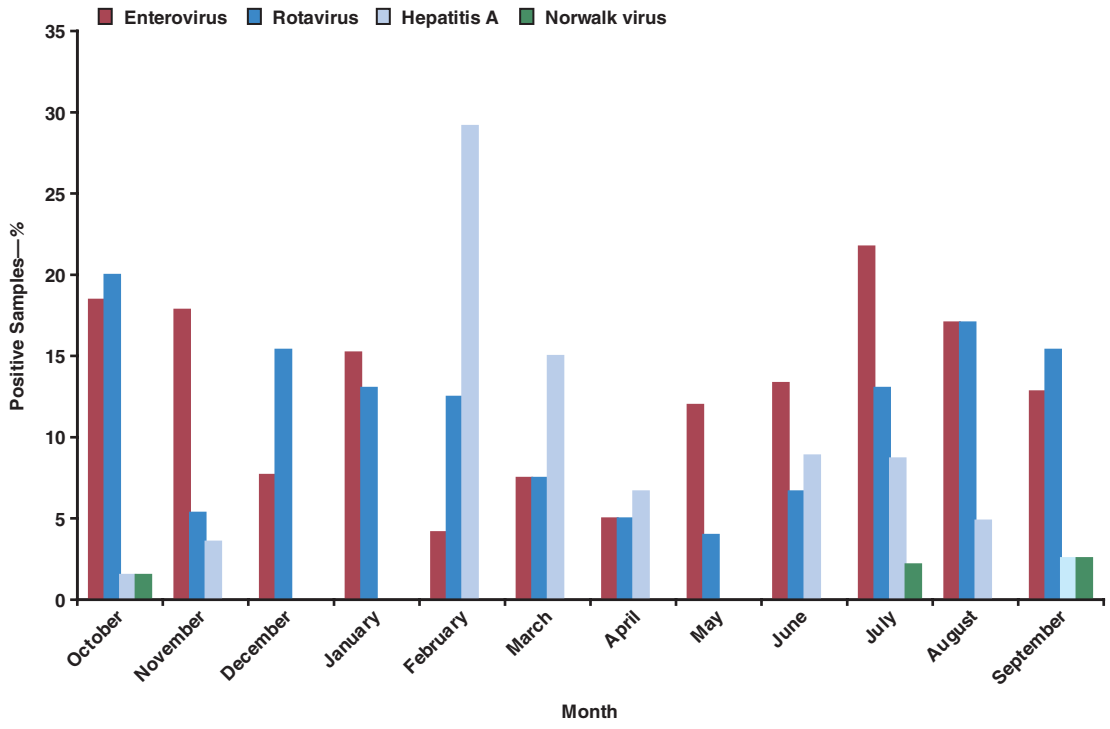
*The percentages refer to the portion of the total for the assay category column. For example, in the column "Cell culture positive," four positive samples were from bedrock formations, which represents 19.0% of the 21 positive cell culture assays. None of the assays—cell culture, RT–PCR, bacterial, or bacteriophage—showed any significant trends when the percentages of positive assays are compared with the percentages of the actual samples within each geological formation category.
†RT–PCR—reverse transcriptase–polymerase chain reaction
‡*n* = number
§NA—not applicable

TABLE 8 Multiple hits for sites with repeated sampling*

States With Repeat Sampling Sites	Number Sampled	Number of Sites That Tested Positive	Viruses				Indicators				
			Cell Culture	Reverse Transcriptase–Polymerase Chain Reaction			WG49	*Escherichia coli* C-3000	*E. coli* C	Coliform	*Enterococci*
				Enterovirus	Rotavirus	Hepatitis A Virus					
Illinois	2	2	1†	1†	0	0	1†	1†	0	2†	ND‡
Iowa	2	2	1†	2†	0	0	0	2†	0	1†	1†
Indiana	2	2	0	1†	0	0	0	0	0	1†	0§
New Jersey	2	2	0	0§	1†,§	0	0	0	0	2†	0
Ohio	2	2	1†	1†	0	0	0	0	1†	0	0§
Arizona	4	2	2†	1†	0	0	1†	1†	0	0	ND
Pennsylvania	17	5	0	2†	1†	0	1†	0	1†	3†	0§
Massachusetts	2	2	0	0§	0	1†	0	1†	1†	0	0§
MO site 1	2	2	0	1†	0	0	0	1†	0	0	0§
Idaho	2	2	1†	2†	0	0	0	0	0	0	ND
Virginia	2	2	0	1†	1†	0	0	1†	0	0	1§
MO site 2	10	2	1†	0	1†	0	0	0	0	2†	1†

*Only 25 sites were sampled more than once. Thirteen sites were negative, and 12 sites tested positive for one or more of the assays.
†Indicates more than one microbial test was positive for a site; the number in the table denotes the number of replicates that tested positive for a particular assay. WG49, *E. coli* 3000, and *E. coli* C are three hosts used for the enumeration of bacteriophage. Enterovirus, rotavirus, and hepatitis A virus were assayed by polymerase chain reaction.
‡ND—no data for all replicates
§Denotes no data for one replicate

FIGURE 3 Seasonal trends for polymerase chain reaction–positive samples



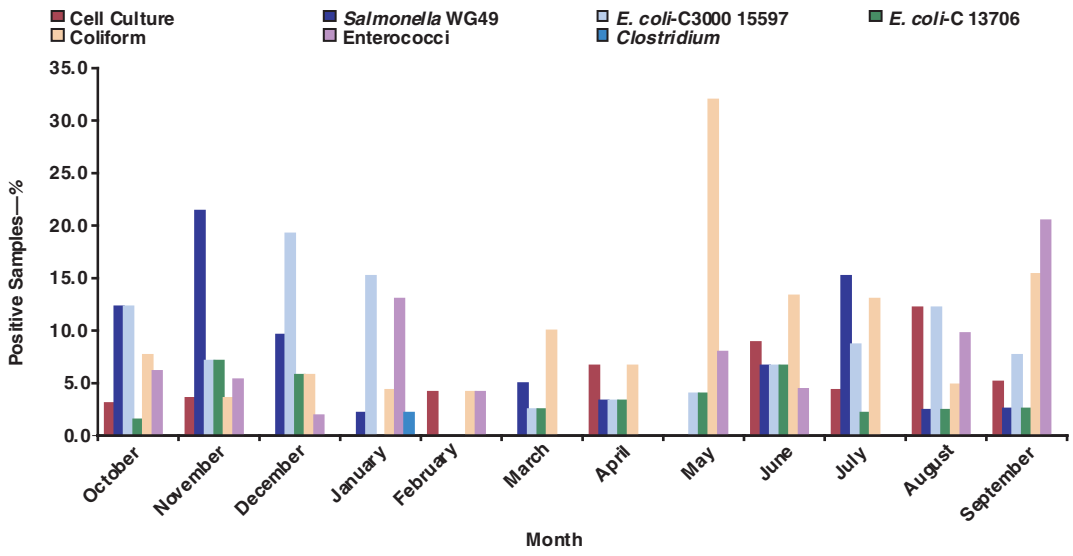
of the PCR method for the detection of viruses in water samples; (2) not all enteric viruses cause CPE in the BGM cell line; thus, non–CPE-forming viruses were not detected by cell culture assay; (3) the ability of PCR to detect a wider variety of viruses than the cell culture method; and (4) the possibility of detecting noninfectious viral nucleic acids by PCR.

RT–PCR and cell culture methods assayed differed in their equivalent volumes of the original sample (5 versus 600 L, respectively); the difference in the assays' sensitivities must be considered when the test results are compared in order to understand the practical application for water analysis. The minimum detection level of viruses using a cell culture assay is 1 pfu per tested sample volume. Because hundreds of virus particles may be required to produce a single plaque-forming unit, assay methods such as PCR that detect virus particles directly will result in significantly greater sensitivity. The minimum detection limit of viruses using RT–PCR is one virus particle, and it has been shown that PCR can consistently detect 10^{-2} pfu of virus (Abbaszadegan et al, 1993). Because the RT–PCR assay is approximately 100 times more sensitive, assaying 100 times less sample by RT–PCR approximately equates to the volume assayed by cell culture.

Distance to source of contamination. The sites that were positive for cell culture and RT–PCR were analyzed for the distance to a source of contamination (Table 5). Transport of virus through soil to groundwater has been reported in a number of studies (Gross & Mitchell, 1990; Bales et al, 1989; Moore et al, 1981; Gerba et al, 1975), and a similar trend was observed in this study in which almost 81% of the sites that tested positive had a sewage source <550 ft (168 m) away. It is plausible that the observation of higher numbers of positive wells close to the sewage source is probably a result of virus transport from the sewage source to the groundwater wells (Wellings et al, 1975; Wellings et al, 1974).

Repeat sampling. Research suggests that male-specific bacteriophages are similar to human enteric viruses (Sobsey, 1990) in size, shape, survival, and transport behavior in the environment. In addition, their removal during coagulation is similar to that of enteroviruses (Abbaszadegan et al, 1997). Correlations between the presence of F-specific RNA bacteriophages and enteric viruses in freshwater have been reported (Havelaar et al, 1993). Occurrence of indicators in water samples and possible correlations with both RT–PCR and cell culture results were investigated. Out of 448 sites, 25 were sampled more than once. It appeared that often when a well was positive once for any of the biological assays tested, it was likely to be positive in future sampling for at least one of the assays. The 12 sites that tested positive for repeat sampling at different occasions were positive for pathogens by cell culture and/or PCR. Only two sites tested positive for pathogens but were negative for indicators. The other 10 sites that tested positive for pathogens also tested pos-

FIGURE 4 Seasonal trends for positive cultural methods

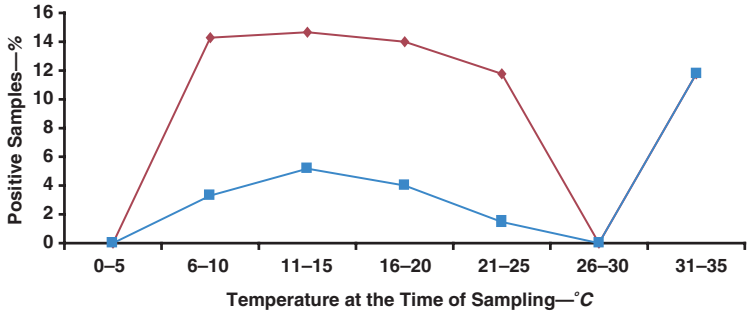


itive for bacteriophage and/or bacteria. Even though no direct correlation between the assays existed, this indicated the vulnerability of the wells to fecal contamination. These limited number of repeat sampling results suggest that microbial indicators such as coliforms, enterococci, or male-specific bacteriophage may be useful for monitoring those groundwater sources. The lack of a 1:1 correspondence in all the monitoring data between viral occurrence and microbial indicators such as coliforms, enterococci, or male-specific bacteriophage indicates the limitations of the indirect assessment of viral contamination of groundwater; however, these indicators may be considered for a site-specific monitoring strategy.

Seasonality. Although an equal number of samples were not collected in all seasons, an increased rate of PCR-positive results was observed in the summer months and in the early fall. No clear trend was observed for the culturable methods (Figure 4). Enteroviruses have seasonal peaks in summer and early fall (CDC, 1989; Strikas et al, 1986). Rotavirus infections, which peak from late autumn to early spring, start in the southwest region of the country and move to the northwest states by spring (Torok et al, 1997; Gouvea et al, 1990; Matson et al, 1990). A similar pattern was observed for enteroviruses using RT–PCR; however, in the case of rotavirus, a minor shift toward fall was observed. PCR confirms the presence of virus genome regardless of its status of infectivity. A plausible explanation of seasonal trend observed by PCR but not by culturable methods could be that a low number (n = 21 sites) of samples tested positive by cell culture and that the method is not a sensitive assay for the detection of infectious viruses. The groundwater temperature-grouped data for the occurrence of viruses showed a similar trend between PCR and culturable methods (Figure 5). These patterns suggest the validity of PCR assay as a tool for virus monitoring.

Although no waterborne outbreaks were documented during this study, greater detection of HAV in February was observed. It has been reported that annually 3 to 6% of community water systems in a six-state area exceeded total coliform levels during a four-year period (GAO, 1997). Between 0.3 and 1.3% of the systems con-

FIGURE 5 Sample temperature trends for cell culture and enterovirus polymerase chain reaction–positive samples



tained *E. coli* or fecal coliform bacteria. However, because the Total Coliform Rule requires samples to be collected from the distribution system, these rates may not reflect the occurrence of bacterial indicators in the source water. Total coliform occurrence rates in private water systems (which are typically not disinfected) ranged between 15 and 59% (GAO, 1997) and are consistent with the findings of this study.

All of the sites that tested positive (by cell culture, PCR, or any of the indicators) practiced disinfection, typically with injection of chlorine at the well head and maintenance of a chlorine residual in the distribution system. There was some difficulty in recruiting nondisinfected systems to participate in the study because the possibility of finding fecal indicators would require corrective (possibly disinfection) action. Only seven sites in this study were not using any disinfection procedure, but all of these sites tested negative for viruses. It is possible that the level of microbial contamination of groundwater observed in this study was skewed because of the large representation of disinfected groundwaters. It was the objective of this study to develop a sampling scheme that would represent the type of geological formations used for production of drinking water in the United States. It was reasoned that the soil type would be indicative of the possibility of viral transport within the aquifer. However, there is some evidence that the placement (siting) of certain wells was made with regard to the level of treatment provided. For example, the majority of wells positive for culturable viruses were followed by full, conventional (coagulation, sedimentation, filtration, disinfection) treatment. It can thus be argued that the placement of the well near a surface water source (subject to contamination) was permitted because the treatment provided was adequate to handle the type of microbial contaminant that might be present. A similar line of reasoning could be applied to the disinfected wells examined in this study. The high occurrence rate of microbial indicators may be related to the fact that disinfection was provided so that large setback distances were not required by the governmental agencies that permitted the placement of the well. However, the mean setback distance to surface water for sites in this study (3,400 ft [1,036 m]) far exceeded the mean distance (462 ft [141 m]) for the average community groundwater system (USEPA, 1997). Therefore, without additional research focused on exclusively nondisinfected systems, it cannot be determined whether the level of microbial occurrence found in this study is generally representative of both disinfected and nondisinfected wells. However, it may be prudent to provide protective barriers (including disinfection) until the possibility of contamination can be eliminated.

When water samples were positive for enteroviruses by cell culture analysis, the participating utility was notified of the need to maintain a disinfectant residual for the source. However, because the samples were taken before disinfection, the positive results do not necessarily indicate a health risk for the communities served by the water provider. Repeat testing at selected sites failed to recover culturable viruses following disinfection. A drawback of the PCR method is the lack of a viability or infectivity determination, and the health significance of

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viruses detected by this method is unknown. It is recommended that epidemiology studies and quantitative microbial risk assessment be conducted to assess the health effects of viruses detected by PCR methods in groundwater.

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FOOTNOTES

[1]ATCC 13706, American Type Culture Collection, Rockville, Md.
[2]ATCC 15597, for MS-2 phage, American Type Culture Colleciton, Rockville, Md.
[3]ATCC 15597-B1, American Type Culture Collection, Rockville, Md.
[4]ATCC 13706-B1, American Type Culture Collection, Rockville, Md.
[5]Difco Laboratories, Detroit, Mich.
[6]Technology Planning and Management Corp., Scituate, Mass.

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