# Viral contamination of household wells near disposal sites for human excreta: final report prepared for the Wisconsin Department of Natural Resources. [DNR-144] 2002

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# Viral Contamination of Household Wells Near Disposal Sites for Human Excreta

**Final Report Prepared for the Wisconsin Department of Natural Resources**

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**Summary**

Recent studies monitoring ground water for enteric viruses have focused on municipal wells, whereas the incidence of viruses in private household wells is unknown. However, household wells may be more susceptible to viral contamination than municipal wells because they are shallower, may be less carefully maintained, and can be located in close proximity to areas with high loading rates of human excreta. For regions of the US that rely heavily on ground water, like the northern Midwest, data on virus incidence in private wells would complement the municipal well data to more fully characterize the extent and conditions of groundwater contamination with enteric viruses.

The objective of this study was to estimate the incidence of viruses in private household wells by sampling fifty wells in seven distinct Wisconsin hydrogeologic districts. Wells were divided into two sets of 25, one set sampled for one year and a new set sampled the second year. Each well was sampled four times, once each season. All wells were privately owned and served a single household. Wells were selected based on their proximity to land-spreading sites of human septage or rural subdivisions with high densities of conventional septic systems. The rationale for this selection scheme was that viruses only would be detected if there is a fecal source and that these wells then would likely represent the upper limit of virus contamination. Viruses were concentrated using a 1 MDS cartridge filter, detected by reverse-transcriptase polymerase chain reaction (RT-PCR), and confirmed by Southern hybridization. In addition, all samples were tested for enteroviruses by cell culture. Virus groups investigated included enteroviruses, rotavirus, hepatitis A, and the human caliciviruses (genogroups 1 and 2). Companion water samples were collected for water quality indicators including total coliforms, *E.coli*, fecal enterococci, male-specific coliphages, nitrate, and chloride.

Among the fifty wells, four (8%) were positive for viruses. Three wells were positive for hepatitis A and the fourth well was positive for both rotavirus and G1 calicivirus in one sample and an enterovirus in another sample. Contamination was transient, as none of the wells was virus-positive for two sequential samples. Enteric virus incidence among samples was 3% (5/194). None of the samples were positive for

enteroviruses by cell culture. RT-PCR inhibition occurred in 8% (16/194) of the ground water samples, primarily those collected during the winter. Male-specific coliphages were found in two samples, however, this indicator and the others were not statistically associated with virus occurrence on either a per-sample or per-well basis. The sensitivity and positive predictive value of the indicators were generally low, except for chloride.

This study is the first in the US to systematically monitor private household wells for virus contamination, and combined with data for municipal wells, provides further insight on the extent of ground water contamination with enteric viruses.

## Introduction

Groundwater is a common transmission route for waterborne infectious disease. Forty-three percent of all waterborne disease outbreaks (125 out of 291) in the USA between 1981 and 1990 were due to contaminated groundwater (Craun 1992). In 1991 and 1992 there were 34 waterborne disease outbreaks recognized in the USA and 76% were attributed to well water (Moore et al 1993). Outbreaks from groundwater consumption have been due to Norwalk virus (Lawson et al 1991, Kukkula et al 1997), Norwalk-like virus (Beller et al 1997), and hepatitis A (Bowen and McCarthy 1983, Bloch et al 1990). For one-half of all waterborne disease outbreaks reported in the USA the causative pathogen could not be identified and was assumed to be viral (Craun et al 1997). Public health officials suspect that contaminated groundwater may be responsible for many cases of endemic enteric disease that are too sporadic to easily identify the infection source.

Enteric viruses are the most likely human pathogens to contaminate groundwater. Their extremely small size (around 30 nm) and slow die-off rates allow them to infiltrate and survive in soils, eventually reaching aquifers. Depending on such factors as soil chemistry and permeability, soil organic carbon content, virus type, pH, ionic strength, and rainfall, viruses can move considerable distances in the subsurface (Gerba and Bitton 1984, Sobsey et al 1986, Yates and Yates 1988, Gerba and Rose 1990). Penetration to depths as great as 67 m and horizontal migration as far as 408 m in glacial till and 1600 m

in fractured limestone have been reported (Keswick and Gerba 1980, Robertson and Edberg, 1997). Viruses can survive for several months in soils and groundwater when temperatures are low and soils are moist (Yates et al 1985, Jansons et al 1989, Straub et al 1993, Robertson and Edberg 1997). Groundwaters beneath wastewater irrigation sites (Keswick and Gerba 1980, Goyal et al 1984) and septic drain fields (Vaughn et al 1983, Alhajjar et al 1988) have become contaminated with enteric viruses. Viruses are shed in enormous quantities in feces, ($10^9 - 10^{10}$/g, Melnick and Gerba, 1980) so that even an 8 log attrition rate during transport through the subsurface environment can still result in virus concentrations in groundwater that are infectious.

The question confronting public health and regulatory agencies 10 years ago was, do viruses survive and travel through soils? We now know that they do. The question now is, what is the incidence of viral contamination in groundwater? Just as with other groundwater contaminates, like $NO_3$ or herbicides, basic monitoring data are necessary to assess the extent of the problem. Recent studies monitoring ground water for enteric viruses have focused on community and non-community wells, but there are no studies on the incidence of viruses in private household wells. However, these wells may be more susceptible to viral contamination than community wells because they may be less carefully maintained, and can be located in close proximity to areas with high loading rates of human excreta. To accurately assess the extent of groundwater contamination with viruses, private wells need to be examined. The objective of this study was to estimate the occurrence of human enteric viruses in private household wells located near sites with high loading rates of human excreta, specifically sepatage landspreading sites and subdivisions with high densities of septic systems. The rationale for this approach was that if wells near sources of enteric viruses were not contaminated, then other wells in regions with lower excreta loading rates would not likely be contaminated. Secondary objectives included comparing the occurrence of enteric viruses among wells in different hydrogeologic settings, and assessing the predictive value of indicators of water sanitary quality for virus contamination.

## Methods

Well selection and homeowner enrollment

Well selection was stratified by major hydrogeologic district so that several wells were sampled for viruses in 7 of the 9 major hydrogeologic districts in Wisconsin (Zaporozec and Cotter 1985) (Figure). These included: District 2) Northern Drift – Precambrian deposits, specifically the eastern portion with permeable sand and gravel deposits; District 3) Central Precambrian, fractured Precambrian igneous rock overlain by thin, low permeability till; District 4) Eastern Drift – Paleozoic sandstone and dolomite, the Silurian dolomite near Sturgeon Bay is fractured and soils are thin; District 5) South Central Drift – Paleozoic, specifically the southern portion with permeable soils and high water production; District 6) Central Sand Plain, sand-gravel aquifer that is highly productive and permeable; District 7) West Central Drift – Paleozoic, sandstone aquifer overlain with Pleistocene deposits, specifically the high yielding western portion; and District 8) Southwestern Paleozoic (Driftless region), sandstone or dolomite aquifers overlain by thin soil. District 1, Lake Superior, and District 9, alluvial valleys, were not included in the study.

Wells were eligible for virus sampling if they were located near sites with high volumes of land-applied septage or sites with high densities of septic systems. Land-application sites were identified from Wisconsin Department of Natural Resources (WI DNR) records indicating site location and volume of septage disposed, and sites were ranked in each hydrogeologic district by the total volume of septage. These sites were considered potential sources of viruses because of the large volume of human septage applied to the land (13,000 gallons/acre/week or 1/2 inch/week is the maximum allowable application rate (Wisconsin Administrative Code, Chapter NR 113, 1996)), and while liming is often required before application, its effectiveness in inactivating some viruses in septage, like Norwalk-like virus or rotavirus, has not been investigated. Sites that did not have septage applied the year previous to well selection or did not have at least one home within 500 meters of the site were excluded. Septic system sites were identified from recommendations from county sanitarians and state hydrogeologists or WI DNR well construction reports. Confirmation that a household well was located in subdivision with septic systems

was obtained from the homeowner at the time of enrollment. Homeowners with wells meeting the eligibility criteria were sent a letter describing the study and a few days later they were telephoned to confirm eligibility and request their participation. Only one well was selected per septic system or sepatage land-application site.

Sampling schedule.

Two sets of 25 wells (50 unique wells) were sampled, one set sampled from January 1999 to November 1999 and a new set sampled from November 1999 to June 2000. Wells were sampled four times, once per season: spring (April); summer (June, July, August); autumn (October, November); and winter (January, February). All samples in a given sample collection period were collected in 3 to 6 weeks.

Virus concentration, elution, and flocculation

A laboratory technician collected all samples from a household tap, usually located at the well pressure tank before the water passed through a water softener or home filtration unit. Samples were collected aseptically; gloves were worn, taps were disinfected with 70% ethyl alcohol, and the entire virus filtration apparatus was sterile. Enteric viruses were concentrated by passing approximately 1500 l of well water through a positively charged, cartridge filter (1MDS, CUNO Inc., Meriden CT). If the well water pH was > 8.0, it was adjusted to neutrality by injecting 0.1M HCl during sample collection. Filters were transported on ice and stored < 2 days at 4°C.

Viruses were eluted by passing 1 l of 1.5% beef extract with 0.05 M glycine, (pH 9.5) through the filter twice. The eluate pH was adjusted to 3.5 and the solution was stirred slowly for 30 minutes at room temperature followed by centrifugation at 4°C. The resulting pellet was resuspended in 15-30 mL of sterile 0.15 M $Na_2PO_4$ (pH 9.0-9.5), centrifuged at 6000 x g for 10 minutes at 4°C, and the supernatant was decanted and adjusted to pH 7.0-7.5. Final sample concentrates were stored at –80°C.

Virus recovery controls were performed approximately quarterly. Forty liters dechlorinated tap water containing 200 plaque forming units (PFU) attenuated poliovirus was filtered using a 1 MDS cartridge filter using the same collection apparatus as used in the field. Viruses were eluted and

flocculated as described for the well water samples. Viruses were enumerated using BGMK cells and the most probable number total culturable virus assay. The recovery efficiency during the study period was 102% ± 43% (mean ± 1 SD, n=5).

Viral RNA extraction and purification

Five hundred µl final sample concentrate was added to 500 µl 4M guanidine isothioocyanate solution, vortexed for 3 min, combined with 1 ml buffered acidic phenol-chloroform (5:1), vortexed again, and after centrifugation the aqueous portion was removed and combined with an equal volume of chloroform/isoamyl alcohol. The vortexing and centrifugation steps were repeated and 750 µl of the aqueous layer was removed and applied to a sterile column of a DNA-grade Sephadex G-100 (Sigma). The first 750 µl column eluant (fraction 1) was discarded. Three successive 750 µL aliquots of Tris-EDTA buffer were applied and these fractions (fractions 2, 3, and 4) were collected in separate microcentrifuge tubes with 50 µL of Chelex 100 resin and stored at –80°C. Preliminary studies in our laboratory demonstrated that the majority of viral RNA was eluted in fraction 3, lesser amounts were found in fractions 2 and 4, and no RNA was detected in fractions 1, 5 or 6.

RT-PCR

The reverse transcription polymerase chain reaction (RT-PCR) was performed to detect five groups of enteric viruses: enteroviruses, rotavirus, hepatitis A, and human caliciviruses genogroups 1 and 2. Separate RT and PCR reactions were performed for each virus group. Fifty µL chromatography column eluent, 50 µL nuclease-free water, and 4 µL (2µg) random hexamers (ProMega, Madison, WI) were mixed, heated for 4 minutes at 99°C, and then supplemented with 186 µL reverse transcription reaction mixture. This mixture contained 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 70 µM of each dNTP (Applied Biosystems, Foster City, CA), 200 U RNasin (ProMega) and 500 U of SuperScript II reverse transcriptase (Life Technologies, Rockville, MD). Reaction tubes were heated in a thermal cycler (Stratagene, LaJolla, CA) set at 25°C for 15 min, 42°C for 60 min, 99°C for 5 min and then held at 4°C until PCR amplification. After the RT reaction, an 8.6 µl PCR cocktail was added containing 10 U Taq DNA polymerase (Applied Biosystems) and 0.4 µM of each primer

(Integrated DNA Technologies, Coralville IA). Primer pairs and target genes for each virus group are listed in Table 1. Amplification conditions for enteroviruses, rotavirus and hepatitis A were an initial denaturation step for 4 min at 96°C followed by 35 cycles of denaturation (94°C, 75 s), annealing (55°C, 75 s), and extension (72°C, 75 s). The amplification conditions for human caliciviruses G1 and G2 were similar to the other virus groups except there were 40 cycles and the annealing and extension temperatures were 50°C and 60°C, respectively. All amplifications ended with a final extension period of 72°C for 7 min. Reaction products were electrophoresed using a 1.6% agarose gel containing ethidium bromide, and amplicon of the size expected for the virus group tested (Table 1) was detected by UV light illumination (Gel-Doc system, Bio-Rad Laboratories, Hercules, CA).

RT-PCR controls included a beef extract negative control, a master mix negative control, and an RNA extraction and PCR positive control for each virus group tested. RT-PCR reactions were batched, using one positive control per batch to minimize the possibility of amplicon contamination. Standard quality assurance measures for PCR were followed.

RT-PCR Inhibition Control

Inhibition of the RT-PCR reaction was checked for every well water sample by seeding 50 µL of the chromatography column eluent (fraction 3) with an RNA control constructed from an amplicon of the Norwalk virus polymerase gene. The amplicon included a 123 base pair deletion so that it could be distinguished from wildtype amplicon (Schwab 1997). Instead of running the control internally as described previously (Schwab 1997) it was amplified in separate reactions using Norwalk-specific primers NVp35 and NVp36 (Table 1). Master mix composition and thermal cycling conditions were the same as for human caliciviruses G1 and G2. A sample was classified as inhibited if the expected RT-PCR product was not evident after gel electrophoresis.

Southern hybridization

Amplicon was transferred from the gel to an uncharged nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in 10x SSC solution (1.5 M sodium chloride, 0.15 M sodium citrate) using a vacuum blotter (Bio-Rad Model 785 Vacuum Blotter, Hercules, CA) with 5 in of Hg of vacuum applied

for 90 min. Gels were depurinated (0.4 M HCl for 15 min) and denatured (0.4 M NaOH for 15 min) before blotting. DNA was crosslinked to the membrane with 1.2 kJ/$m^2$ of UV light (UV Stratalinker 2400, Stratagene). Membranes were pre-hybridized with hybridization buffer (ExpressHyb™ ClonTech, Palo Alto, CA) in a roller tube rotated in a rotisserie hybridization incubator for 60 min at 42°C. The buffer was replaced with hybridization buffer containing 500 ng of oligonucleotide probe labeled with digoxigenin (DIG). Oligoprobes used in this study are listed in Table 1. DIG-labeling was performed with the DIG oligonucleotide 3'-end labeling kit (Boehringer Mannheim, Germany), following the manufacturer's instructions and incorporating the manufacturer's controls. Hybridization was conducted overnight at 42°C. The membrane was then washed twice at room temperature with 50-100 mL 2x SSC/0.1% sodium dodecyl sulfate.

Successfully hybridized probes were detected using the DIG nucleic acid detection kit (Boehringer Mannheim), an enzyme-linked immunoassay using anti-digoxigenin alkaline phosphatase conjugate and the colorimetric substrates 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium salt. The color reaction was allowed to proceed for 16 hours. The assay detection limit, as reported by the manufacturer, is 0.1 pg of homologous DNA.

Indicator assays

Samples for water quality indicators were taken at the same time as the virus samples. Microbial indicators included total coliform, *E. coli*, fecal enterococci, and male-specific coliphage, and chemical indicators included chloride anion and nitrate. Two chromogenic substrate assays, Colisure (IDEXX Laboratories, Wesbrook, ME) and Colilert (IDEXX), both that test total coliform and E. coli simultaneously, were performed in parallel and if either test was positive a sample was classified as positive. Fecal enterococci were tested by Enterolert (IDEXX), another chromogenic substrate assay.

Male-specific coliphages ($F^+$ coliphage) were enumerated by the double-agar-layer plaque assay (Sinton et al 1996). One liter well water, pH adjusted to neutrality if necessary, was filtered through a sterile 47 mm 1 MDS filter disk (CUNO, Inc.) at a rate < 300 ml/min. Coliphages were eluted from the filter with two 5 mL aliquots of 3% beef extract/1.5% Tween 80 (pH 9.0). The host bacterium *E. coli*

$F_{amp}$ in log-phase growth was added to the top agar layer (55°C, 0.7% agar), this was combined with 2 ml of the beef extract eluent, and the mixture was poured over the bottom nutrient agar layer (1.1% agar). The entire eluent was assayed by pouring five plates per sample, 2 ml eluent per plate. Plates were incubated overnight at 37°C. Summing the number of plaques in the five plates estimated the number of coliphages per liter. The positive control was beef extract seeded with MS2 coliphage (ATCC#15597-B1) and the negative control was unseeded beef extract. Recovery efficiency of the filter-elution method was checked each time a batch of coliphage samples was analyzed; mean recovery efficiency was 83.7% ± 46.7% (mean ± 1 SD, n = 42).

Chloride was measured with a chloride ion selective combination electrode (Orion Model 96-17B, Thermo Orion, Beverly, MA) and ISE meter, (Orion Model 720A). Chloride ionic strength adjustor (Thermo Orion) was added to all standards and samples to minimize interferences. Nitrate was measured with a nitrate ion selective combination electrode (Orion Model 9707 *ionplus*) and Orion ISE meter. Nitrate interference suppressor solution (Thermo Orion) was added to all standards and samples.

Culturable enteroviruses

Buffalo green monkey kidney (BGMK), Rhabdomyosarcoma (RD), and Caco-2 cell lines were grown to confluent monolayers in tissue culture flasks with Eagle's minimum essential medium (MEM) with Earle's salts, HEPES buffer, penicillin-streptomycin-fungizone solution, and 10% fetal bovine serum (FBS). A 1.0-2.5 ml aliquot of final sample concentrate was filter-sterilized (Millex-GV, 0.22 μm pore size, Millipore, Bedford, MA) and inoculated into each of six 175 $cm^2$ flasks containing cell monolayers and 2.0-2.5 ml sterile saline. There were two flasks per cell line and thus 6.0-15.0 ml of the final sample concentrate was assayed per sample. Two flasks from each cell line served as controls; the negative control was inoculated with 0.15 M $Na_2HPO_4$, the diluent of the final sample concentrate, and the positive control was inoculated with 20 PFU/ml poliovirus Type 3. All flasks were rocked for 90 min at room temperature. Cell monolayers were washed with pre-warmed saline with 2% FBS and then 50 ml of Eagle's MEM with 2% FBS was added to each flask. Flasks were incubated at 37°C for 14 days and

examined on an inverted microscope (Nikon Eclipse TS100) for viral cytopathic effect (CPE). Positive cultures were frozen at -70°C when 75% of the monolayer showed CPE. Negative cultures were frozen at -70°C after removing 40 ml of media and then thawed to release any unobserved virus present in the cells. One ml of freeze-thaw supernatant was passed into a 25 $cm^2$ flask containing the same cell line. Flasks were incubated at 37°C and observed for another for 14 days to confirm the first passage results.

Statistical Analysis

For each water quality indicator its sensitivity, specificity, positive predictive value, and negative predictive value were calculated relative to the virus detection results. These parameters were calculated on a per sample basis, comparing indicator and virus tests for the same sample, and on a per well basis, comparing indicator and virus tests for the same well with the well classified as indicator or virus positive if any of its samples were positive. Sensitivity is defined as the percent of virus positive samples (or wells) that were also positive by the indicator (i.e. true positive rate), whereas specificity is the percent of virus negative samples (or wells) that were also negative by the indicator (i.e. true negative rate). Positive predictive value is the percent of indicator positive samples (or wells) that were positive for virus, whereas negative predictive value is the percent of indicator negative samples (or wells) that were negative for virus. In addition, kappa statistics (Cohen 1960) were calculated together with 95% confidence limits as a measure of agreement between the water quality indicators and the virus detection results on a per-sample basis (without adjustment for multiple samples per well) and on a per-well basis.

## Results

Well site and construction characteristics

To achieve the target enrollment of 50 wells, 82 households with private wells had to be contacted and requested to participate in the study (i.e. participation rate = 61%). Five to eleven wells were located in each of 7 hydrogeologic districts of Wisconsin (Figure 1). Twenty-four wells were near septage land application sites (median application rate, 909,000 gal; range 0.2 – 3.8 mil gal/yr), and 26 wells were located in subdivisions with septic systems (median septic system density, 38; range, 16 – 186

systems/square mile). The majority of wells were drilled (47) and a small number were driven point (2) or hydro-jetted (1). Household wells with a wide range of depths, casing depths, and construction ages were included in the study (Table 2).

Sample characteristics

Forty-six wells were sampled 4 times, once each season as originally planned, 2 wells were sampled 3 times and 2 wells were sampled twice for a total of 194 samples. Six samples were missed due to homeowners withdrawing from the study or leaving their residence for the winter. Sample volume, pH, and temperature data are summarized in Table 2. Only two samples had an initial pH > 8.0, requiring the pH to be lowered during sample collection to ensure virus adsorption. In homes with water softeners or plumbed filtration units, all samples except one were taken prior to softening and all samples except 7 were taken prior to filtration. Filter types included cellulose heavy sediment filters (6) and activated charcoal (1). None of these seven samples were identified later as virus positive.

RT-PCR Inhibition

Inhibition of RT-PCR, as evidenced by the Norwalk virus internal standard failing to amplify, was observed in 8% (16/194) of the samples. The 16 inhibitory samples were from 14 different wells; two wells had two inhibitory samples. Sample inhibition appeared seasonal as one-half (8/16) of the inhibitory samples were collected during the winter, one-quarter (4/16) were collected in the fall, and 3 out of 16 and 1 out of 16 were collected in the summer and spring, respectively. RT-PCR was eventually performed successfully on all 16 samples by diluting the target fraction of the chromatography column 1:10 (13 samples), analyzing the chromatography fraction previous to the target fraction (2 samples), or by both using the earlier fraction and diluting it 1:10 (1 sample). One of the inhibited samples was later determined to be virus positive.

Virus prevalence by RT-PCR

Of the 194 samples tested by RT-PCR for enteric viruses, 5 samples (3%) were virus positive (Table 3), as evidenced by a positive Southern hybridization blot. Four samples were positive for one virus and one sample was positive for two viruses, rotavirus and human calicivirus genogroup 2.

Hepatitis A was the most commonly identified virus and each of the other virus groups tested was detected at least once, except human calicivirus genogroup 1. The amplicon from the enterovirus positive sample was sequenced and found to have 98% identity with poliovirus Type 3 (GenBank BLAST search, e-score 2 x 10**-96).

Expressing virus prevalence on a per well basis, 4 out of the 50 wells sampled (8%) were virus positive (Table 3). Virus positive wells were found in three of the seven hydrogeologic districts included in this study (Figure). Two wells were located in the Door County Peninsula near Sturgeon Bay, one well was in the northern part of the state near Eagle River, and the other well was in the south near Brodhead.

The construction and site characteristics of the four virus positive wells were similar (Table 4). All were drilled and had a casing depth complying with state code, and they were located at sites classified in this study as having a high density of septic systems. The surface geology was available for three of the wells and reported to include coarse textured components, namely sand and gravel.

Virus occurrence in wells was intermittent (Table 5). Three of the four positive wells had only one positive sample out of four collected. The other well was positive for enteroviruses in the summer, negative in the autumn, and then positive for rotavirus and Norwalk-like-G2 virus in the winter. Viruses were detected in wells only during the summer and winter sampling periods.

Enterovirus prevalence by cell culture

Of 194 samples tested by cell culture, all were negative for enteroviruses; CPE was not observed in any of the three cell lines.

Water quality indicators

The proportions of positive water sanitary quality indicators are reported on both a per sample and per well basis (Table 6). Among microbial indicators, total coliform bacteria were the most common, whereas *E. coli* was detected in only one sample. Male-specific coliphage was detected in 2 wells. Four wells exceeded the10 ppm Maximum Contaminant Level (MCL) for $NO_3$ and 20 wells had a chloride level > 28 ppm, suggesting fecal contamination.

The diagnostic accuracy of the water quality indicators is reported in Table 7. On a per-well basis, sensitivities of the indicators ranged from 0% for *E. coli*, fecal enterococci, $F^+$ coliphage, and high $NO_3$, to 75% for high Cl, while specificities ranged from 63% for high Cl to 97.8% for $F^+$ coliphage. Given the small number of virus positive results, estimates of sensitivity were imprecise (e.g. the estimate of 75% has 95% confidence limits which range from 19.4% to 99.4%). The highest positive predictive value was 15%, for the Cl indicator on a per well basis. The maximum Kappa statistic was 0.135 for Cl, suggesting slight agreement between this indicator and virus detection results (Landis and Koch 1977), but all Kappa statistics were non-significant.

## Discussion

*Virus prevalence in household wells.* We believe this is the first study in the United States to have systematically sampled private household wells for human enteric viruses. Of the 50 wells sampled throughout the state of Wisconsin, 4 (8%) were positive for viruses, including hepatitis A, rotavirus, poliovirus, or Norwalk-like virus genogroup 2. Expressing virus prevalence on a per sample basis, 5 out of 194 samples (3%) were positive. By comparison, in a study of 30 municipal wells in 17 states and two US territories, seven wells (23%) were positive for enteroviruses by cell culture (Federal Register, 2000). Using RT-PCR virus detection methods, Abbaszadegan et al (1999) analyzed 150 samples from municipal wells in 35 states and found 30.1%, 13.8%, and 8.6% positive for enteroviruses, rotavirus, and hepatitis A, respectively. Enteroviruses have been the most frequently detected virus in municipal wells (Abbaszadegan et al 1999; Federal Register, 2000), whereas in the present study hepatitis A was more common. Although private household wells may be located closer to fecal sources and maintained less adequately than municipal wells, the household wells in this study had a lower virus contamination rate. Because the household wells were purposely selected to be near human fecal sources, the actual statewide virus contamination rate in Wisconsin may be lower than 8%. On the other hand, the selected wells appear to have been representative of the level of groundwater quality generally found throughout the state. The Wisconsin State Laboratory of Hygiene tested approximately 15,000 household well water

samples in calendar year 2000 and found 20% positive for total coliforms and 2% positive for *E. coli* (J. Standridge, personal communication), similar to the 28% and 2% prevalence rates, respectively, reported in the present study. Likewise, the proportion of wells exceeding the USEPA maximum contaminant level for $NO_3$ (10 ppm) was 8%, similar to the 6.6% statewide rate estimated from a 1994 survey of 534 wells in Wisconsin (CDC 1998).

*Enterovirus prevalence by cell culture.* The RT-PCR methods for detecting RNA viruses in environmental samples are sensitive and specific, but one limitation is that successfully amplifying the target viral nucleic acid does not necessarily mean that infectious virons are present. This limitation is moot if the virus is nonculturable, as are human caliciviruses, and RT-PCR is the only method of detection available. Enteroviruses, however, are easily cultured and we used a standard procedure to detect infectious virons, intending to complement the RT-PCR data. Of the 194 samples from 50 wells, none were positive for enteroviruses by cell culture. The one sample positive for poliovirus by RT-PCR was negative by cell culture, suggesting that at the time of sampling the virus was inactivated in the water.

*Virus occurrence by hydrogeologic district.* It appeared that well susceptibility to viral contamination did not correspond to specific major hydrogeologic districts of the state as 3 of the 7 districts included in the study had contaminated wells. On a smaller geographic scale, the two contaminated wells in the Door County peninsula, near Sturgeon Bay, were consistent with the local hydrogeology of this region. The peninsula has shallow topsoil underlain with extensively fractured dolomite allowing contaminants to easily reach groundwater and travel far distances (Rayne et al 2001). Both wells were modern, conventionally drilled, and their casings were 52 meters deep. Assuming the casings were intact and the well covers properly sealed, the viruses had to be transported at least 52 meters deep before they could enter the household water supplies. It is likely that the wells in the Door County peninsula are among the most vulnerable in the state for virus contamination.

*Characteristics of virus-positive wells.* This study was designed to estimate the prevalence of viruses in household wells. The design was not appropriate to identify the types of wells, soil factors, or fecal sources associated with virus occurrence, but several observations are noteworthy. First, all four

virus-positive wells were relatively new and complied with Wisconsin state code (e.g. minimum casing depth of 40 ft), suggesting that current well construction practices do not prevent virus contamination. Non-compliant wells may have a greater frequency of virus contamination. Second, consistent with previous reports that virus transport is greatest in sand and gravel (Yates and Yates 1988), three of the four virus-positive wells in this study were located in coarse textured soils. Third, even though half of the 50 wells enrolled in the study were located near septage land application sites, all the virus-positive wells were in subdivisions with high densities of septic systems, suggesting that septic systems were more likely to be a contamination source than land application sites. Enteric viruses can pass through a septic drain field and reach ground water (Vaughn et al 1983; Scandura et al 1997). In one study conducted in south-central Wisconsin, after dosing a properly functioning conventional septic system with poliovirus vaccine strain, the virus could be detected over 3 months in a monitoring well located 6 m from the drain field edge (Alhajjar et al 1988). The findings of the present study, however, do not preclude septage land application sites as a potential source of viral contamination. Additional research is needed to identify sources of virus contamination and the conditions under which household wells are most vulnerable.

*Intermittent virus contamination.* Only the summer and winter samples were virus positive, however, whether virus occurrence in Wisconsin household wells is truly seasonal could not be inferred from just four positive wells. What can be concluded is that virus occurrence in a particular well can be intermittent, and the vulnerability of a well to viral contamination cannot be characterized from a single sample. *A priori*, sampling on a seasonal basis would appear justified given that hydrogeological parameters that affect virus transport and survival vary seasonally, for example recharge rate and groundwater temperature.

*RT-PCR inhibition.* Naturally-occurring water contains dissolved compounds, such as humic and fulvic acids, that inhibit the polymerase chain reaction (Tsai and Olson 1992; Ijzerman et al. 1997). In this study of well water the presence of inhibitory compounds was sample-specific; often times when one sample was inhibited to RT-PCR the other three samples from the same well were uninhibited. The majority of inhibited samples were collected during the winter months, the period in Wisconsin with the

lowest rate of groundwater recharge (Steuer and Hunt 2001). The concentration of inhibitory compounds in well water may peak at this time due to the absence of recharge dilution, or perhaps due to an influx of dissolved organic matter leached from deciduous leaves dropped several months previously. These findings underscore the importance of checking each well water sample for RT-PCR inhibition.

Inhibition may also depend on the specific set of primers and nucleic acid target of the PCR so, ideally, inhibition should be checked for each virus tested. The Norwalk virus inhibition control used in this study included a 123 base pair deletion so that if amplicon contamination occurred, the smaller DNA product could be discerned as a false positive. (Amplicon from the inhibition control was never detected in any of the samples.) Similarly modified controls were not available for the other viruses. Instead we chose to rely on this single control to assess inhibition and avoid seeding all 194 samples with wildtype strains from each virus group, which would have increased the risk of false positives. The tradeoff was that virus-specific inhibition of some samples may have gone undetected, and the study results include some false negatives.

*Association of indicators with virus occurrence.* Ideally, virus presence could be accurately predicted from a microbial or chemical indicator, thereby avoiding the expense, time, and training involved with virus testing. In previous studies, standard microbial indicators, such as total coliform bacteria, fecal coliforms, and *E. coli*, have not been associated with virus occurrence (Gerba et al 1979, Griffin et al 1999, Jiang et al 2001). Fecal enterococci appear to be a good predictor of gastrointestinal disease risk, (Cabelli 1982, Moe et al 1991, Barrel et al 2000, Borchardt et al in review), which would include viral etiologies, but thus far an association between fecal enterococci and the presence of specific viruses in water has not been demonstrated (Jiang et al 2001). Recently, the focus has been on male-specific and somatic coliphages as potential indicators because they are shed in human feces, and being viruses, they presumably possess survival and transport characteristics similar to the pathogenic virus groups (Armon and Kott 1996, Leclerc et al 2000). At the time this study was designed male-specific coliphages appeared to be the more promising indicator of the two types and we opted to omit somatic coliphages from the panel of indicator tests. Somatic coliphages have the limitation of low fecal

specificity because they can infect non-fecal coliform bacteria, but on the other hand, they are shed by a greater number of people (Leclerc et al 2000).

We assessed the predictive accuracy of four microbial indicators and two chemical indicators for virus contamination. The sensitivity and positive predictive values of all six indicators were low or zero, although the small number of virus-positive samples and wells limited this assessment. Likewise, while the specificity and negative predictive values were generally > 90%, these parameters may be biased upward just by virtue of the large number of virus and indicator negative samples and wells. Taking a biological instead of statistical perspective, it is possible for a fecal indicator to be present and pathogenic viruses absent simply due to the lack of infections and virus shedding (i.e. indicator has low positive predictive value). In contrast, if an enteric virus were present, recently shed in feces, one would expect the fecal indicator to be present as well (i.e. indicator has high sensitivity). That was not the case in this study. Of the virus-positive samples or wells, none were positive for *E. coli*, fecal enterococci, or male-specific coliphages, the three indicators most specific to fecal contamination. Total coliform, which is less fecal specific than the other three indicators, nevertheless, had higher sensitivities and positive predictive values.

Of the six indicators tested, chloride showed the most promise with a per-well basis sensitivity of 75%. Chloride is excreted in elevated concentrations in human feces with the specific concentration varying with dietary intake. As a conservative anion, when released to groundwater it is attenuated only by dilution, which makes it an excellent marker for locating the subsurface fecal plume emanating from a septic system (Alhajjar et al 1990). The plume Cl concentration is determined by the original effluent concentration, the distance from the septic system, and the background Cl level. Alhajjar and colleagues (1990) measured Cl concentrations in wells down gradient from septic systems at 17 sites in Wisconsin and found the median concentration ranged from 12 to 35 mg/L, depending on the distance from the septic system. The median Cl concentration in the effluents (241 samples) was 64 mg/L. Based on these numbers we selected Cl concentrations greater than 28 mg/L in the household wells to indicate fecal contamination. Even with this crude approximation of an appropriate concentration threshold, chloride

was a surprisingly good indicator of virus occurrence, much better than the microbial indicators. Microbial indicators may have different die-off, shedding, and transport rates that the pathogens they are intended to indicate. A conservative chemical species, universally present in feces, would perhaps make a better indicator. One shortcoming of chloride is that in some regions there are other anthropogenic sources, such as de-icing salt and fertilizers. Still, its value as a virus indicator should be explored further.

*Epidemiologic implications.* In the United States it is estimated there are 267 million episodes of acute diarrhea each year (Glass et al 2001). The majority of diarrheal illnesses are endemic (i.e. non-outbreak). How many of these are attributable to drinking water is unknown, let alone the fraction attributable to drinking from contaminated household wells. Borchardt et al (in review) provided an initial estimate, reporting that in a defined population of children in central Wisconsin, 11% of acute diarrhea without an identifiable etiology and thus presumably due to a viral agent was attributable to drinking from household wells positive for fecal enterococci. Alternatively, the relative importance of household wells as a disease transmission route can be gauged from the potential number of people exposed. Fifteen million households in the US use a private well as their primary drinking water source (US Census 1990). Assuming that the 8% virus contamination rate reported in the present study is a reasonable estimate of the national rate, then 1.2 million households are exposed to enteric viruses through their private wells. However, the generalizability of the 8% rate is uncertain. It may be underestimated given that the sanitary quality of Wisconsin groundwater is relatively high compared to other Midwest states (CDC 1998). It may be overestimated because the wells in this study were located near fecal sources. What is certain is that some household wells are contaminated with human enteric viruses, presenting a risk for disease transmission that should be investigated further.

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**Figure Legend**

Figure. Location of household well sampling sites and virus-positive wells.