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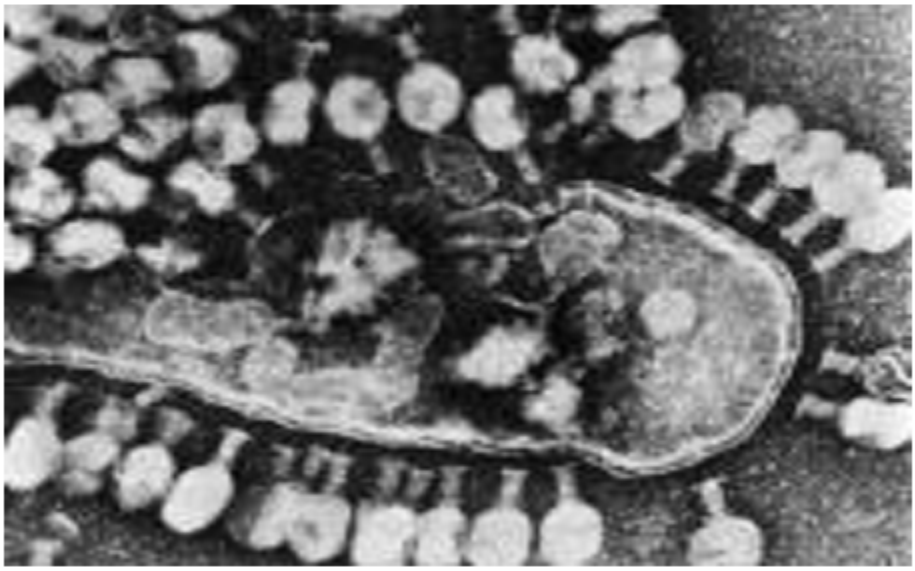
National Field Study for Coliphage Detection in Groundwater: Method 1601 and 1602 evaluation in regional aquifers



FINAL REPORT

EPA/822/R/06/002

National Field Study for Coliphage Detection in Groundwater: Method 1601 and 1602 Evaluation in Regional Aquifers

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Executive Summary

The United States Environmental Protection Agency (EPA) office of water in compliance with the Safe Drinking Water Act (SDWA) is responsible for developing regulations to protect the nation's drinking water supply from drinking water contaminants.

EPA has proposed a groundwater rule which will require states to determine groundwater systems that are vulnerable to fecal contamination. Studies were conducted by EPA on virus fecal indicator occurrence across the U.S. A round robin testing for proposed coliphage indicator has also been conducted.

A three year field study was conducted by Office of Science and Technology under the overall supervision of Dr. mark Sobsey to determine the performance of method 1601 and 1602 in detecting somatic and male-specific Coliphages in groundwater. In addition, Method 1601 and 1602 were tested using the confirmation procedure, as proposed by EPA, for all methods to detect microbes in groundwater.

The investigation was conducted in four different regional aquifers across the united States. These aquifers were in the Southeast region, the Northeast region, the south west region, and the upper Midwest region.

Results obtained, show that coliphages can be used as a tool for screening groundwater samples for the presence of fecal contamination. However the results show that there was no direct correlation of the presence of human enteric viruses and the presence of viral indicators.

The inclusion of coliphages along with conventional bacterial indicator analysis increases the likelihood for detection of fecally contaminated samples. The absence of detection of human enteric viruses in the presence of viral indicator suggest that the presence of pathogens may not routinely be detected unless under heavily contaminated conditions.

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INTRODUCTION AND BACKGROUND

Background

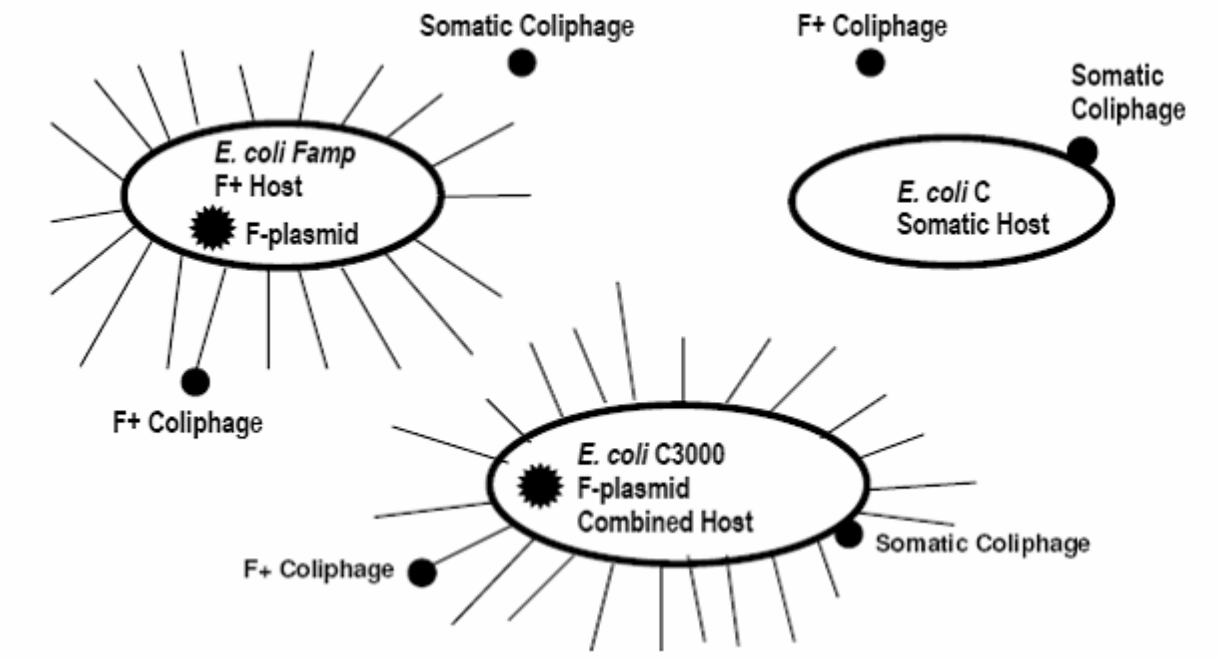
This report summarizes the results of Phase I and phase studies on a project to evaluate and, if necessary further improve, EPA Methods 1601 and 1602 to detect coliphages in groundwater. In the first phase of the study samples of groundwater were seeded with known quantities of naturally occurring coliphages from sewage and the recovery efficiency of the methods in detecting these added coliphages was determined in a series of controlled experiments performed concurrently by four participating laboratories located in different regions of the country. The data from the seeded sample recovery experiments were used to further establish and quantify the performance characteristics of the methods.

In the second phase of the study EPA Methods 1601 and 1602 were applied to geographically representative samples of groundwater potentially vulnerable to fecal contamination in order to compare the performance of the different coliphage methods and to compare their ability to detect fecally contaminated groundwater relative to the detection of fecal indicator bacteria and the detection of culturable enteric viruses. Each of the four geographically representative laboratories (southeast, northeast, upper Midwest and southwest) was to analyze at least 16 groundwater samples for coliphages, indicator bacteria and enteric viruses, for a total target number of 64 samples to analyzed for second and final phase of the project. Several other tasks were linked to this effort to further validate and improve the coliphage methods and their ability to detect and characterize coliphages in groundwater

Experimental Approach

Coliphages and their detection methods. Coliphages are viruses infecting *Escherichia coli* bacteria. Coliphages are present at high concentrations in sewage and other fecal wastes and they are indicators of fecal contamination of groundwater, other waters and other environmental media. There are two main groups of coliphages: somatic and male-specific. The relationships between these coliphages and their host bacteria, showing specific bacterial strains as examples, are summarized in Figure 1. The conventional method to detect coliphages is by their ability to infect host cells in which they replicate (proliferate), producing large numbers of progeny viruses and lysing (killing) the host cells in the process. It is this killing and lysis of host cells that forms the basis of most coliphage infectivity assay methods, including those employed for coliphage analysis by the EPA methods.

Figure 1. Somatic and Male-specific (F+) Coliphages and their Relationship to Host Bacteria



Somatic coliphages infect host bacteria by attaching directly to the outer cell wall (outer cell membrane). The male-specific coliphages infect only male F+ strains of bacteria by attaching to the hair-like appendages projecting from the cell surface, called F-pili or fimbrae, that are the characteristic male trait. Somatic coliphage hosts lack the F-pili and cannot be infected by F+ coliphages. F+ coliphage hosts differ in their ability to be infected by somatic coliphages. Some F+ coliphage hosts are very resistant to somatic coliphage infection because they have an outer cell membrane that differs from those of *E. coli* (such as the Salmonella typhimurium strain WG49) and *E. coli* Famp (which was experimentally selected as a somatic-coliphage resistant mutant). Other F+ coliphage hosts such as *E. coli* C3000 have not been subjected to selection for resistance to somatic coliphages and are susceptible to F+ coliphage infection as well as somatic coliphage infection. Therefore, some host bacteria are infected only by somatic coliphages (*E. coli* C and CN13), others only by male-specific coliphages (*E. coli* Famp and *Salmonella typhimurium* WG49) and yet others by both groups of coliphages (*E. coli* C3000).

There are still questions about which groups of coliphages, somatic, male-specific or both groups together, are the appropriate and preferred indicators of fecal contamination. There is evidence in support of both somatic and male-specific coliphages as being effective and useful virus indicators of fecal contamination. Some have suggested that both somatic and male-specific coliphages should be detected as fecal indicator viruses of contamination of groundwater and other waters. It is the understanding of the authors that EPA has so far not made any final decisions about which of the coliphage groups to target for detection in future guidelines or regulations. It also been suggested that both groups of coliphages, somatic and male-specific,

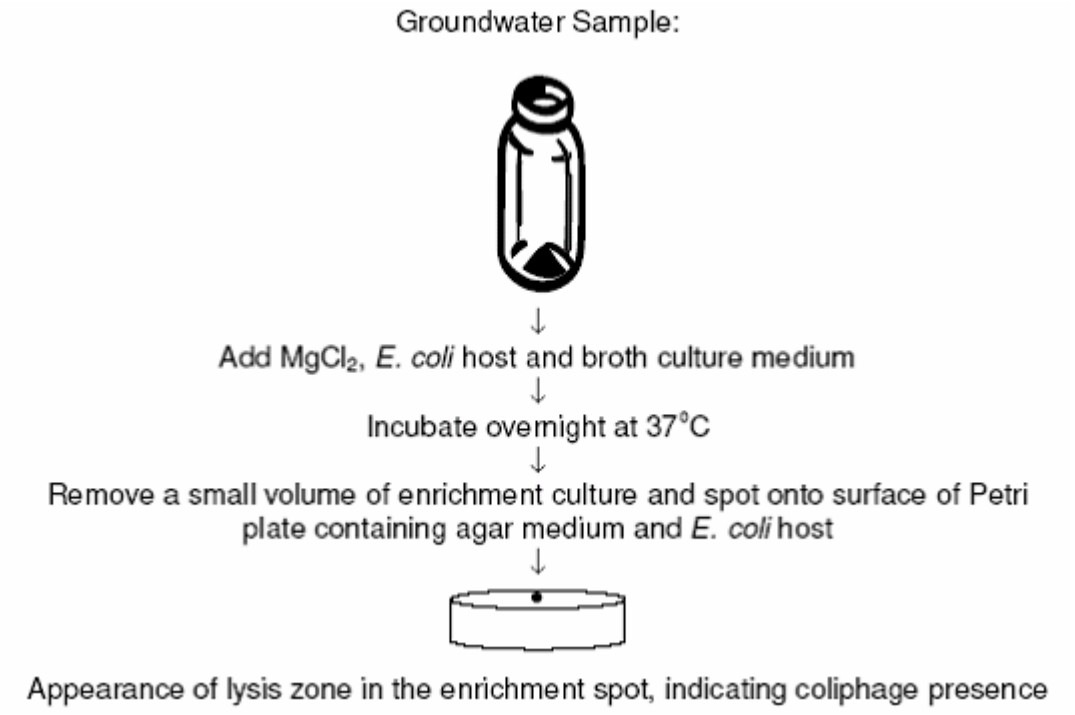
could be simultaneously detected on a single coliphage host, thereby giving the greatest probability and highest sensitivity in detecting any coliphage indicative of fecal contamination.

EPA Methods for Coliphage Detection in Ground Water. EPA Methods 1601 and 1602 were developed to detect somatic and male specific coliphages in large volumes of groundwater, with target sample volumes of up to 1000 mL in Method 1601 (an enrichment method) and 100 mL in method 1602 (a Single Agar Layer plaque assay method), respectively. The methods are based upon the ability of the coliphages to infect host bacteria, which results in the lysis of the host bacteria. This a widely used approach to detect coliphages. In plaque assays or other assays on solid media, such as those containing agar, the lysis of the host bacteria is visualized as zones of lysis or clearing of the bacteria as discrete, circular areas (called a lysis zones or plaques) in a confluent layer (or "lawn") of host bacteria in a solid nutrient medium. In liquid enrichment cultures in broth media, the lysis of host bacteria can in principle be observed as the clearing of turbidity from the culture as the bacteria are lysed and their resulting cell debris settles out of suspension. Because such clearing of broth cultures as evidence of host cell lysis can be hard to observe due to interference from other bacteria that may grow in the broth culture, other ways to confirm the presence of phages are often used. One of the most common ways is to take some of the enrichment culture containing phages, apply it to a lawn of bacteria in an agar medium, and allow the phages to infect and lyse the host cells in the lawn to produce a clear zone of lysis that can be readily observed.

Method 1601. Method 1601 is a so-called two-step "enrichment" method and the steps of the method are outlines in Figure 2. In the first step of this method, liquid bacterial media, magnesium chloride (to promote coliphage attachment to the host bacteria), and the *E. coli* host are added to the water sample, making a liquid (broth) culture for coliphage infection of the *E. coli* host bacteria. After allowing for coliphage infection and lysis of the host bacteria during overnight incubation, a small volume (several microliters) of the enrichment culture is placed on the surface of a Petri dish of agar medium containing *E. coli* host bacteria (a spot). This is the second step of the method. If the applied sample contains coliphages able to infect the host bacteria, a circular zone of host cell lysis (clearing) develops after several hours of incubation in the spot where the sample was applied. Such a lysis zone in the spot indicates coliphage presence in the enrichment broth and is a positive result. If no such lysis zone develops in the sample spot on the plate, the enrichment culture of the sample is considered negative for coliphages.

Figure 2.

Method 1601 – Two-Step Enrichment-Spot Plate Method for Coliphage Presence-Absence



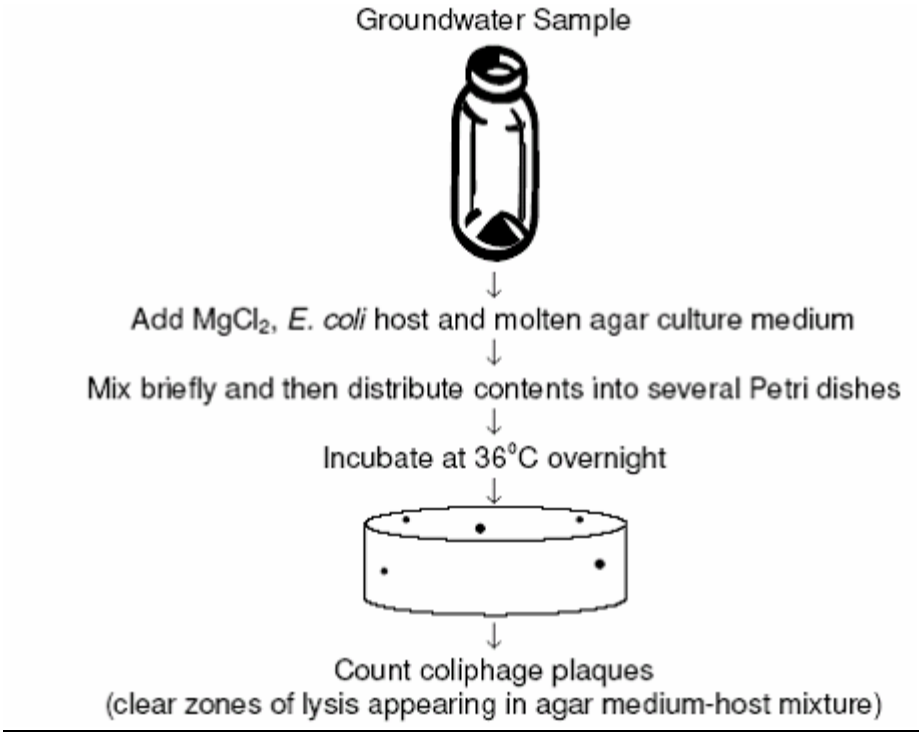
When Method 1601 is applied to a single sample volume, the analysis provides a determination of the presence or absence of coliphages in the sample volume analyzed. If the method is applied to multiple sample volumes, each in separate enrichment cultures, the method is capable of giving an estimation of the concentration of coliphages in the water sample, based on which sample enrichment volumes become positive and negative for coliphages.

Method 1602. EPA Method 1602 is a so-called single agar layer method for the enumeration of coliphage plaques (discrete clear zones of lysis of host bacteria) developing in a culture of host bacteria in an agar medium in a Petri dish. As shown in Figure 3, a 100-mL sample of

groundwater is supplemented with magnesium chloride, host bacteria and then combined with molten agar medium. The mixture is then distributed into Petri plates, the agar medium is allowed to solidify and the plates are incubated overnight for the development of coliphage plaques, which are clear, circular zones of lysis, each produced by a separate or individual coliphage. The plaques are then counted to determine the total number of number of coliphages in the sample, assuming each plaque arose from an individual infectious coliphage.

Figure 3.

Method 1602 – Single Agar Layer (SAL) Plaque Assay Method for Coliphage Enumeration



Confirmation of Positive Results by Methods 1601 and 1602. For both Methods 1601 and 1602, EPA has proposed a method to confirm positive results. For confirmation of positive results, material is removed (picked or aspirated with a capillary pipette or a micropipettor) from the lysis zones of enrichment spots on agar medium-host cell plates (Method 1601) or from the plaques that develop in agar medium-host cell plates of Method 1602. The recovered material is transferred to a small volume of buffered water, mixed briefly, and then a small volume (several microliters) of the material is placed ("spotted") on the surface of a Petri dish of agar medium containing *E. coli* host bacteria. If the applied material contains coliphages capable of infecting the host bacteria, a circular zone of host cell lysis develops after incubation (for several hours or overnight) in the spot where the sample was applied. Such a lysis zone in the spot is indicative of coliphage presence in the material recovered from either a lysis zone on the spot plate of an enrichment broth (Method 1601) or from the plaque of a Single Agar Layer plate (Method 1602). If no such lysis zone develops in the sample spot on the confirmation plate, the sample (presumptive lysis zone from an enrichment culture or presumptive plaque from an SAL plate) is considered negative for coliphages.

Simultaneous Detection of both Somatic and Male-specific Coliphages on a Single Host. EPA Methods 1601 and 1602 were originally developed to separately detect somatic and male-specific coliphages using separate *E. coli* hosts able to support the growth of only one or the other coliphage group (somatic or male-specific, respectively). *E. coli* CN13 is used to detect somatic coliphages and *E. coli* Famp is used to detect male-specific coliphages (Figure 1). It was later suggested that perhaps a single *E. coli* host could be used to simultaneously detect both

somatic and male-specific coliphages present in a groundwater samples rather than having to use two separate *E. coli* hosts to separately detect each coliphage group (Figure 1). If the presence of either or both groups of coliphages indicates fecal contamination, simultaneous detection of both on one host would reduce time, effort, materials and cost and provide appropriate data about coliphage presence in a sample. As previously noted, *E. coli* C3000 is such a host. However use of a single *E. coli* host bacterium capable of detecting both somatic and male-specific coliphages had not been adequately tested for its performance characteristics in previous studies on the development and evaluation of Methods 1601 and 1602 and their application to either seeded samples or field samples of groundwater.

Survival of Coliphages in Groundwater. In the development and evaluation of methods for coliphage detection in groundwater, the question has been raised as to how long samples can be held before being subjected to analysis. It has been suggested that samples may have to be collected and sent to a distant lab capable of coliphage analyses, but that the time between sample collection and analysis may be more than 1 or 2 days. If the sample holding time is 2 or more days will the coliphages still be present and be detectable? To address this question additional experiments were done as an added task in **Phase II** of this study at the request of Dr. Nena Nwachuku, the EPA project manager. Groundwater was seeded with known, low level amounts of mixed populations of sewage-derived coliphages and aliquots of these samples were subjected to coliphage analysis by Methods 1601 and 1602 on days 0, 2, 3 and 6. These assay days were chosen to model those that might be used if samples were shipped to a lab for coliphage analysis and even held overnight before analysis once received by the lab. The

resulting data on coliphage concentrations were analyzed to determine if the coliphages were stable and still detectable for periods ranging from 1 to 6 days.

Field Application of Methods 1601 and 1602 to Detection of Coliphages as Indicators of Fecal Contamination in Vulnerable Groundwater. An important test of the newly developed EPA methods to detect coliphages in groundwater, Methods 1601 and 1602, would be to validate their performance for coliphage detection in vulnerable groundwater, in comparison with the detection of fecal indicator bacteria and human enteric viruses in the same samples. Preferably such studies would apply the methods to different, geographically representative groundwater in order to make sure that the methods were not adversely affected by interfering constituents in the groundwater, or so-called "matrix effects". Furthermore, the concurrent detection of coliphages by Methods 1601 and 1602 in the same groundwater samples would provide an opportunity to compare their relative detection sensitivities and lower limits of coliphage detection. In addition, the concurrent detection of coliphages as well as fecal indicator bacteria and enteric viruses in the same groundwater samples would make it possible to determine if coliphages were as good or better than fecal indicator bacteria or enteric viruses in identify fecally contaminated ground water. Such analysis would make it possible to determine if one of these microbe groups was a superior indicator of fecal contamination because it was detected more frequently and/or at higher concentrations. Such analyses were done in **Phase II** of this study.

PHASE I STUDIES

PURPOSES, GOALS AND TASKS OF PHASE I STUDIES

The overall purposes and goals of Phase I studies were to determine the performance characteristics of Methods 1601 and 1602 in detecting and quantifying somatic and male-specific coliphages in ground water samples seeded with known quantities of natural, mixed populations of coliphages obtained from municipal sewage. These studies were done using certain modifications and additions to Methods 1601 and 1602 in order to address recommendations suggested for the methods after their original development, evaluation and multi-laboratory testing. Specifically, host *E. coli* C3000 was tested for simultaneous detection and quantification of both somatic and male-specific coliphages in addition to testing the methods with the individual hosts previously specified for separate detection of somatic (*E. coli* C3000) and male-specific (*E. coli* Famp) coliphages. In addition, Methods 1601 and 1602 were tested using the confirmation procedure, as proposed by EPA for all methods to detect microbes in ground water.

The key tasks and activities of the Phase I studies are listed below.

1. Recruit a total of 4 experienced laboratories, each from a different region of the country, to test Methods 1601 and 1602 using the standard protocols with the modifications indicated: a) include host *E. coli* 3000 for simultaneous detection of both somatic and male-specific coliphages, and b) include confirmation of presumptive positive results obtained from samples.

The 4 laboratories are:

University of North Carolina (UNC), under the direction of Mark D. Sobsey (southeast)

University of New Hampshire (UNH), under the direction of Aaron Margolin (northeast)

Texas A&M University (TAMU), under the direction of Suresh Pillai (southwest)

Wisconsin State Hygiene Lab (WSHL), under the direction of David Battigelli (upper Midwest)

2. Develop bench sheets (bench laboratory aids or protocols in easy-to-follow format) to be used by analysts performing the methods in these repeated, weekly experimental trials.

3. Perform weekly experimental tests (trials) of the methods using the developed bench sheets.

4. Test each method (1601 and 1602) simultaneously by the 4 laboratories on a weekly basis, using locally collected ground waters seeded with the same stock of sewage-derived coliphages prepared and distributed weekly by the lead or reference laboratory (UNC) and all three *E. coli* hosts (CN13 for somatic, Famp for male-specifics and C3000 for both).

5. Perform repeated trials of each method and submit the results to the lead (UNC) laboratory for compilation and data analysis in order to develop and evaluate a sufficient database to characterize the performance of the methods.

6. Identify any deficiencies or limitations encountered with the methods. If possible within a short time period (no more than a few weeks), devise and implement modifications or corrective measures to improve the performance characteristics of the methods.

7. Based on the compiled data from the 4 laboratories, determine if the performance characteristics of the methods are of sufficient quality to recommend the use of the methods to detect coliphages in ground water samples.

8. Save (archive) representative coliphages detected by each method on each *E. coli* host for further characterization by the UNC laboratory to determine if the coliphage isolates have properties consistent with a fecal origin. These properties include bacterial host range, growth temperatures and taxonomic group (sub-set of representative isolates only).

PHASE I METHODS AND MATERIALS

The methods and materials used in this project are those specified in the documents for US EPA Methods 1601 and 1602. Stepwise procedural steps in the application of these methods for the specific purpose of this study are also given in the laboratory bench sheets (laboratory bench protocols) presented in the Appendix to this report. The only departures or modifications to Methods 1601 and 1602 employed in this study are: (a) the addition of *E. coli* C3000 as a host bacterium for the simultaneous detection of both somatic and male-specific coliphages, and (b) the addition of the newly proposed confirmation procedure for plaques from plates of Method 1602 and from lysis zones of plates from Method 1601.

Method 1601

For Method 1601, the two-step enrichment method, the goal was for each of the 4 participating laboratories to seed 30+ liters of ground water with a quantity of coliphage stock (filtered sewage) to achieve between 1 and 2 infectious units of coliphages per liter of water. The seeded water was then aliquotted into 30 1-liter volumes. Groups of 10 1-liter volumes were subjected to the enrichment assay method using one of the three host bacteria, thereby testing each host bacterium for coliphage detection using 10 replicate 1-liter volumes per host bacterium per weekly experiment. As negative control samples, three additional 1-liter volumes of unseeded ground water were also subjected to coliphage analysis by the two-step enrichment method using each of the three different *E. coli* host bacteria. As negative controls, these samples were intended to demonstrate no background level of coliphages were present in the ground water

prior to seeding with sewage-derived coliphages. A total of 8 replicate experiments were conducted, one experiment per week, between May and July, 2001.

Method 1602

For Method 1602, the single agar layer (SAL) method, the goal was for each of the 4 participating laboratories to seed replicate 300-mL volumes of water with a quantity of coliphage stock (filtered sewage) to give about 100 infectious units of coliphages per 100 mL of ground water. The seeded water was aliquotted as 3 100-mL volumes and each of these volumes was assayed by the single agar layer method using one of the three different *E. coli* host bacteria. As negative controls, 3 100-mL volumes of unseeded ground water were subjected to coliphage analysis by the SAL method using each of the three different host bacteria. As negative controls, these samples were intended to demonstrate no background level of coliphages were present in the ground water prior to seeding with sewage-derived coliphages. A total of 10 replicate experiments were performed, once experiment per week, during February and April, 2001.

PHASE I RESULTS

Coliphage Recovery by Method 1602

Table 1 shows the recovery of seeded coliphages by method 1602 (single agar layer assay) for the total of 10 successive trials performed weekly. In some initial weekly trials no data were available from the WSLH laboratory. This was due to other obligations that precluded their participation. In the interest of time, the initial three experiments were performed among the other three laboratories in order to initiate the project and to begin addressing potential logistical issues of coordination among laboratories. No serious logistical problems arose among the three labs participating initially. This indicated a reliable system for concurrent method performance among the labs using the same coliphage stocks prepared by UNC lab to seed test groundwater. The WSLH also was unable to participate another week due to a state-mandated holiday.

Table 1: Recovery of Seeded Coliphages in 100-mL Groundwater Samples by Method 1602 (Single Agar Layer Assay)

Date	Host	UNC	TAMU	WSLH	UNH
21-Feb-01	C3000	2%	14%	no data	22%
	CN13	80%	90%	no data	65%
	Famp	246%	160%	no data	24%
27-Feb-01	C3000	9%	79%	no data	131%
	CN13	43%	43%	no data	127%
	Famp	22%	15%	no data	53%
6-Mar-01	C3000	9%	6%	no data	45%
	CN13	37%	19%	no data	50%
	Famp	101%	18%	no data	35%
13-Mar-01	C3000	9%	0%	20%	26%
	CN13	19%	0%	35%	23%
	Famp	72%	0%	28%	33%
20-Mar-01	C3000	12%	8%	56%	37%
	CN13	20%	9%	94%	39%
	Famp	91%	68%	92%	45%
27-Mar-01	C3000	4%	8%	39%	81%
	CN13	73%	30%	120%	118%
	Famp	48%	20%	40%	70%
3-Apr-01	C3000	10%	16%	28%	88%
	CN13	30%	36%	70%	77%
	Famp	49%	47%	67%	72%
10-Apr-01	C3000	5%	17%	34%	93%
	CN13	21%	63%	64%	96%
	Famp	26%	10%	32%	77%
17-Apr-01	C3000	44%	no data	49%	86%
	CN13	139%	no data	77%	84%
	Famp	72%	no data	94%	98%
24-Apr-01	C3000	33%	55%	32%	77%
	CN13	117%	88%	76%	89%
	Famp	36%	33%	26%	84%

The percent coliphage recovery data were subjected to Analysis of Variance (ANOVA) to discover if there were significant recovery differences among the hosts and/or the laboratories (Table 2) . As shown in Table 2, there were significant differences in recovery among the 3 hosts ($p=0.00008$), and significant differences in recovery among the four labs ($p=0.000002$). The highest recovery (73%) was obtained using *E coli* CN13, the lowest (39%) was obtained using *E coli* C3000 and an intermediate recovery of 46% was obtained with *E coli* Famp. The differences among host bacteria were consistent (not significantly different) within each

laboratory (p=0.42). This latter result suggests that recoveries among the three host bacteria are generally similar within a lab and therefore, the hosts are equivalent on a within-lab basis. In other words, the three different *E. coli* hosts will give similar recovery efficiencies when used by an individual lab to analyze mixed populations of coliphages of sewage (fecal) origin in a groundwater matrix.

Table 2: Descriptive Statistics for Seeded Coliphage Recovery by Method 1602

	OVERALL				C3000			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**49%**	**35%**	**56%**	**68%**	**14%**	**23%**	**37%**	**69%**
Median	35%	19%	49%	75%	9%	14%	34%	79%
Mode	NONE	0%	NONE	NONE	NONE	NONE	NONE	NONE
Std. Dev.	52%	37%	28%	31%	14%	26%	12%	35%
Var.	27%	14%	8%	10%	2%	7%	2%	12%
Minimum	2%	0%	20%	21%	2%	0%	20%	22%
Maximum	246%	160%	120%	131%	44%	79%	56%	131%
Count	30	27	21	30	10	9	7	10
95% CI	19%	15%	13%	12%	10%	20%	11%	25%
	CN13				Famp			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**58%**	**42%**	**77%**	**77%**	**76%**	**41%**	**54%**	**59%**
Median	40%	36%	76%	81%	61%	20%	40%	62%
Mode	NONE	NONE	NONE	NONE	NONE	NONE	NONE	NONE
Std. Dev.	43%	33%	26%	33%	65%	49%	30%	25%
Var.	18%	11%	7%	11%	43%	24%	9%	6%
Minimum	19%	0%	35%	23%	22%	0%	26%	24%
Maximum	139%	91%	120%	127%	246%	160%	94%	98%
Count	10	9	7	10	10	9	7	10
95% CI	31%	25%	24%	24%	47%	38%	28%	18%

The differences in coliphage recovery efficiency among the laboratory groups led us to question whether there were differences in the groundwater of each region (i.e., a "matrix" effect) which might account for those observed differences in recovery. Further experiments were conducted using regional groundwater and additionally reagent water (as a control measure) in an attempt to answer this question. Four replicate experiments were conducted and these data are summarized

in Table 3, with descriptive statistics in Tables 4a, 4b and 4c for hosts *E. coli* C3000, CN13 and Famp, respectively..

Table 3: Coliphages Recovery Efficiency of Method 1602 Concurrently Applied to Seeded Groundwater and Reagent Water

Date	Matrix	Host	UNC	TAMU	WSLH	UNH
3-Apr-01	ground	C3000	10%	16%	28%	88%
	reagent	C3000	51%	22%	26%	87%
	ground	CN13	30%	36%	70%	77%
	reagent	CN13	61%	49%	57%	74%
	ground	Famp	49%	47%	67%	72%
	reagent	Famp	64%	88%	48%	72%
10-Apr-01	ground	C3000	5%	17%	34%	93%
	reagent	C3000	52%	13%	37%	92%
	ground	CN13	21%	63%	64%	96%
	reagent	CN13	76%	66%	63%	78%
	ground	Famp	26%	10%	32%	77%
	reagent	Famp	66%	18%	47%	75%
17-Apr-01	ground	C3000	44%	no data	49%	86%
	reagent	C3000	73%	no data	67%	76%
	ground	CN13	139%	no data	76%	84%
	reagent	CN13	135%	no data	68%	82%
	ground	Famp	72%	no data	94%	98%
	reagent	Famp	87%	no data	138%	97%
24-Apr-01	ground	C3000	33%	55%	32%	77%
	reagent	C3000	31%	13%	51%	79%
	ground	CN13	117%	88%	76%	89%
	reagent	CN13	103%	48%	65%	83%
	ground	Famp	36%	33%	26%	84%
	reagent	Famp	59%	49%	78%	79%

Table 4a: Descriptive Statistics: Coliphage Recovery by Method 1602 for Seeded Groundwater and Reagent Water on Host *E. coli* C3000

	GROUNDWATER				REAGENT WATER			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**23%**	**29%**	**36%**	**86%**	**52%**	**16%**	**45%**	**84%**
Median	21%	17%	33%	87%	52%	13%	44%	83%
Std. Dev.	18%	22%	9%	7%	17%	5%	18%	7%
Var.	3%	5%	1%	0%	3%	0%	3%	1%
Minimum	5%	16%	28%	77%	31%	13%	26%	76%
Maximum	44%	55%	49%	93%	73%	22%	67%	92%
Count	4	3	4	4	4	3	4	4
95% CI	29%	55%	14%	11%	27%	13%	28%	12%

Table 4b: Descriptive Statistics: Coliphage Recovery by Method 1602 for Seeded Groundwater and Reagent Water on Host *E. coli* CN-13

	GROUNDWATER				REAGENT WATER			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**77%**	**63%**	**72%**	**86%**	**94%**	**54%**	**63%**	**79%**
Median	73%	63%	73%	86%	90%	49%	64%	80%
Std. Dev.	60%	26%	6%	8%	32%	10%	5%	4%
Var.	36%	7%	0%	1%	11%	1%	0%	0%
Minimum	21%	36%	64%	77%	61%	48%	57%	74%
Maximum	139%	88%	77%	96%	135%	66%	68%	83%
Count	4	3	4	4	4	3	4	4
95% CI	95%	65%	9%	12%	52%	25%	8%	6%

Table 4c: Descriptive Statistics: Coliphage Recovery by Method 1602 for Seeded Groundwater and Reagent Water on Host *E. coli* Famp

	GROUND				REAGENT			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**46%**	**30%**	**55%**	**83%**	**69%**	**52%**	**78%**	**81%**
Median	43%	33%	49%	81%	65%	49%	63%	77%
Std. Dev.	20%	19%	32%	11%	12%	35%	42%	11%
Var.	4%	4%	10%	1%	2%	12%	18%	1%
Minimum	26%	10%	26%	72%	59%	18%	47%	72%
Maximum	72%	47%	94%	98%	87%	88%	138%	97%
Count	4	3	4	4	4	3	4	4
95% CI	32%	47%	51%	18%	20%	87%	67%	18%

These coliphage recovery data were subjected to ANOVA. No interaction effects were indicated ($p>0.09$ in all cases), implying that any observed factor differences were consistent throughout the experiment. As expected, there were significant differences in recovery among the host bacteria ($p= 0.00007$), with recovery for *E. coli* C3000 (45%) being lower than recoveries for *E. coli* CN13 (73%) and *E. coli Famp* (63%). There were also significant differences in recovery among the laboratory groups ($p=0.0000006$), with UNH having the highest overall recovery (83%), followed by UNC (60%), WSLH (58%), and TAMU showing the lowest overall recovery (40%). But there was no significant difference due to a possible matrix effect ($p=0.17$), leaving unexplained the previously observed differences among the laboratory groups.

Confirmation of Plaques in Method 1602

The laboratories also applied several modified versions of a confirmation procedure for plaques isolated from the SAL plates of Method 1602 when applied to the detection of coliphages in seeded water samples. Plaques were picked from the SAL plates using a variety of methods (i.e., with Pasteur pipettes, with Eppendorf pipettes, etc.), resuspended in Tryptic Soy Broth, and spotted onto pre-poured gridded plates of Tryptic Soy Agar containing host bacteria (as used in the Two-Step Enrichment procedure). The confirmation percentages are presented in Table 5a, which summarizes all data by experiment date, lab and host and in Table 5b, and which summarizes the descriptive statistics for the plaque confirmations. The results of these attempts at coliphage plaque confirmation ranged from excellent (average 99-100% at UNH) to moderate (38-68% at UNC). Overall, there are high likelihoods that the plaques detected on assay plates for Method 1602 are indeed produced by coliphages, with a 78% average or a nearly 4 out of 5

plaque confirmation rate. It is likely that confirmation rates can be further improved to give a greater confirmation efficiency by minor modifications in the plaque recovery and re-spotting procedure.

Table 5a: Percent Confirmation of Picked Plaques Isolated using Method 1602

Date	Matrix	Host	UNC	TAMU	WSLH	UNH
21-Feb-01	ground	C3000	33%	not done	95%	100%
	ground	CN13	85%	no data	100%	100%
	ground	Famp	15%	not done	90%	100%
27-Feb-01	ground	C3000	13%	not done	100%	100%
	ground	CN13	75%	no data	80%	100%
	ground	Famp	15%	not done	45%	100%
6-Mar-01	ground	C3000	60%	80%	100%	100%
	ground	CN13	45%	100%	60%	100%
	ground	Famp	30%	88%	67%	100%
13-Mar-01	ground	C3000	21%	not done	100%	100%
	ground	CN13	35%	no data	75%	95%
	ground	Famp	15%	not done	80%	95%
20-Mar-01	ground	C3000	78%	no data	95%	90%
	ground	CN13	50%	25%	90%	100%
	ground	Famp	50%	0%	80%	95%
27-Mar-01	ground	C3000	40%	no data	100%	95%
	ground	CN13	80%	0%	80%	100%
	ground	Famp	35%	53%	95%	95%
3-Apr-01	ground	C3000	50%	70%	100%	100%
	reagent	C3000	65%	90%	No data	100%
	ground	CN13	45%	80%	95%	100%
	reagent	CN13	75%	90%	No data	100%
	ground	Famp	0%	20%	100%	100%
	reagent	Famp	0%	10%	No data	100%
10-Apr-01	ground	C3000	93%	80%	100%	100%
	reagent	C3000	100%	80%	100%	100%
	ground	CN13	15%	90%	85%	100%
	reagent	CN13	90%	80%	95%	100%
	ground	Famp	10%	50%	90%	100%
	reagent	Famp	60%	30%	100%	100%
17-Apr-01	ground	C3000	80%	not done	100%	100%
	reagent	C3000	95%	not done	100%	100%
	ground	CN13	95%	no data	85%	100%
	reagent	CN13	70%	not done	100%	100%
	ground	Famp	70%	not done	100%	100%
	reagent	Famp	70%	not done	95%	100%
24-Apr-01	ground	C3000	90%	100%	100%	100%
	reagent	C3000	90%	100%	100%	100%
	ground	CN13	100%	100%	100%	100%
	reagent	CN13	95%	100%	100%	100%
	ground	Famp	85%	80%	95%	100%
	reagent	Famp	80%	90%	95%	100%

Table 5b: Descriptive Statistics for Plaque Confirmation of Coliphages Isolated by Method 1602

(Percent Confirmation Overall and by *E. coli* Host for each Laboratory)

	Overall				C3000			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**57%**	**67%**	**91%**	**99%**	**65%**	**86%**	**99%**	**99%**
Median	63%	80%	95%	100%	71%	80%	100%	100%
Mode	15%	80%	100%	100%	90%	80%	100%	100%
Std. Dev.	31%	34%	13%	2%	29%	11%	2%	3%
Variance	10%	11%	2%	0%	9%	1%	0%	0%
Minimum	0%	0%	45%	90%	13%	70%	95%	90%
Maximum	100%	100%	100%	100%	100%	100%	100%	100%
Count	42	25	39	42	14	7	13	14
95% CI	10%	14%	4%	1%	17%	10%	1%	2%
	CN13				Famp			
	UNC	TAMU	WSLH	UNH	UNC	TAMU	WSLH	UNH
Mean	**68%**	**74%**	**88%**	**100%**	**38%**	**47%**	**87%**	**99%**
Median	75%	90%	90%	100%	33%	50%	95%	100%
Mode	75%	100%	100%	100%	15%	NONE	95%	100%
Std. Dev.	26%	36%	12%	1%	30%	34%	16%	2%
Variance	7%	13%	1%	0%	9%	12%	3%	0%
Minimum	15%	0%	60%	95%	0%	0%	45%	95%
Maximum	100%	100%	100%	100%	85%	90%	100%	100%
Count	14	9	13	14	14	9	13	14
95% CI	15%	28%	7%	1%	18%	26%	10%	1%

Subsequent efforts to improve confirmation rates for plaques picked from SAL plates or lysis zones picked from spot plates of the enrichment method compared the original EPA conformation method described above to a modified method. In the modified method the picked material from plaques or lysis zones was re-enriched by culturing in host bacteria again. The picked plaque or lysis zone material was transferred to 5 mL of Tryptic Soy Broth to which had been added host bacteria and the mixture was incubated overnight at 37˚C. Volumes of 10 microliters were withdrawn from the resulting overnight enrichments and spotted onto prepoured lawns of host cells in nutrient agar media. The spot plates were incubate at 37˚C for a minimum for 4 hours and then observed for lysis zones indicative of coliphage positivity. The results of a side-by-side comparison of the original confirmation method with the modified method are

summarized in Table 5c.

Summary of Method 1602 Results

Results from a series of 10 replicate experiments by all four participating laboratories on the performance of the SAL method have been presented. The method was applied to replicate 100-mL volumes of groundwater seeded with sewage coliphages and detected with each of three *E. coli* host bacteria. The summarized results of these experiments (Table 6) show efficient coliphage detection (average 53%) and confirmation (average 78%) in 100-mL volumes of ground water. There were differences in recoveries based on the host used, and there were unexplained differences in recovery among the laboratories. Confirmation of plaque isolates gave success rates ranging from moderate to excellent among labs. Individual adaptations or modifications of confirmation methods somewhat improved low confirmation rates. Method 1602 gave generally acceptable detection of coliphages in seeded ground water and the majority of plaques detected by the method could be easily confirmed by a simple procedure. Overall, the results of these studies indicate that there is high likelihood of detecting even low levels of coliphages in 100-mL volumes of ground water using Method 1602.

Table 6. Coliphage Detection in 100-mL Volumes of Seeded Ground Water by Method 1602

Coliphage Group	Estimated Phages/100 mL	Coliphage Recovery (%)	Plaque Confirmation (%)
Somatic (*E. coli* CN13)	100	64	82
Male-specific (*E. coli* Famp)	100	58	68
Both (*E. coli* C3000)	100	38	87

Coliphage Recovery by Method 1601

The Two-Step Enrichment (SAL) validation study consisted of 8 replicate experiments performed by the four participating laboratories. In each experiment a small volume of the assayed sewage was added to a 30-liter volume of groundwater and mixed well to disperse the inoculum evenly. This inoculated groundwater was then dispensed into 30 1-liter bottles to which were added the enrichment media and the proper host bacteria (10 bottles per host). The bottles were incubated overnight, and small portions were spotted onto gridded TSA plates as described above. After incubation, these plates were examined for zones of lysis. Positive zones of lysis were considered positive for coliphage, and these were counted and recorded for each host. Based on the volume and the titer of the inoculated sewage, an expected coliphage titer per bottle was calculated for each host. Based on the number of positive bottles, the MPN (Most Probable Number) of coliphages per bottle was calculated using Thomas's MPN equation and taken as the number of coliphages recovered (observed number of coliphages). Using this observed MPN and the expected coliphage titer per bottle, percent recoveries were calculated.

The coliphage recovery rates for experiments in which replicate ten 1-liter volumes of groundwater were seeded with about 1.5 to 3 infectious units of coliphages are presented in Table 7a as percentage recoveries based on the observed (calculated) MPN coliphage concentrations per liter and in Table 7b as a comparison of expected and observed number of coliphage-positive 1-liter samples out of 10. Descriptive statistics presented in Tables 8a and 8b.

Table 7a: Recovery of Seeded Coliphages in 1-Liter Groundwater Samples by Method 1601

(Two-step enrichment)

Date	Host	UNC	TAMU	WSLH	UNH
9-May-01	C3000	4%	no data	13%	>63%
	CN13	134%	no data	>122%	131%
	Famp	26%	no data	19%	>70%
14-May-01	C3000	30%	281%	6%	62%
	CN13	193%	92%	>189%	9%
	Famp	>39%	348%	7%	>21%
21-May-01	C3000	10%	no data	134%	no data
	CN13	101%	no data	148%	no data
	Famp	26%	no data	35%	no data
4-Jun-01	C3000	41%	no data	23%	122%
	CN13	372%	no data	>359%	22%
	Famp	143%	no data	62%	99%
11-Jun-01	C3000	20%	79%	24%	82%
	CN13	215%	<=5%	>204%	304%
	Famp	44%	<=14%	15%	123%
18-Jun-01	C3000	15%	39%	28%	130%
	CN13	>137%	163%	>124%	77%
	Famp	13%	112%	10%	21%
25-Jun-01	C3000	no data	125%	6%	131%
	CN13	18%	122%	39%	220%
	Famp	no data	45%	56%	224%
9-Jul-01	C3000	261%	220%	24%	108%
	CN13	61%	191%	187%	230%
	Famp	<=518%	57%	33%	2317%

Table 7b: Descriptive Statistics for Recovery of Seeded Coliphages by Method 1601

	C3000			
	UNC	TAMU	WSLH	UNH
Mean	54%	149%	33%	109%
Median	20%	125%	24%	122%
Std. Dev.	92%	100%	42%	27%
Variance	84%	100%	18%	7%
Minimum	4%	39%	6%	62%
Maximum	261%	281%	134%	131%
Count	7	5	8	7
95% CI	85%	124%	35%	25%
	CN13			
	UNC	TAMU	WSLH	UNH
Mean	171%	114%	296%	142%
Median	163%	122%	247%	131%
Std. Dev.	117%	74%	208%	113%
Variance	136%	55%	431%	127%
Minimum	18%	0%	39%	9%
Maximum	372%	191%	718%	304%
Count	8	5	8	7
95% CI	97%	92%	174%	104%
	Famp			
	UNC	TAMU	WSLH	UNH
Mean	47%	112%	30%	424%
Median	26%	57%	26%	123%
Std. Dev.	49%	138%	21%	838%
Variance	24%	190%	4%	7018%
Minimum	0%	0%	7%	21%
Maximum	143%	348%	62%	2317%
Count	7	5	8	7
95% CI	45%	171%	17%	775%

The results in Tables 7a and 7b indicate that when 1-liter volumes of ground water seeded with 1-2 PFU of coliphages are analyzed by the enrichment method, there is a very high likelihood that coliphages will be detected with relatively high efficiency. Average coliphage recoveries from 8 replicate trials per coliphage host per lab ranged were 86% for combined coliphages on host *E. coli* C3000, 181% for somatic coliphages on host *E. coli* CN-13, and 153% for male-specific coliphages on host *E. coli* Famp. The results in Tables 7a and 7b indicate variability in coliphage recoveries from trial to trial. However, this extent of variability is to be expected

because coliphage recoveries are based on MPN estimates of the number of positive 1-liter enrichment culture bottles out of ten. Based on calculated 95% confidence intervals (CIs), the observed degree of variability was within the range expected for a 10-culture, single dilution Most Probable Number method. Examination of the 10-replicate single dilution MPN table in Standard Methods for the Examination of Water and Wastewater indicates that MPN estimates can have 95% CIs that vary by nearly 6-fold (600%) at low rates of positivity and almost always 3-fold (300%) or more at intermediate and high levels of positivity. Probably more important is that of the total 82 trials in which 10 1-liter bottles of seeded ground water were used per trial, coliphages were not detected in only 3 trials. Therefore, there is a very high probability of detecting low levels of (1 to 3) coliphages in 1-liter volumes of groundwater when using this method.

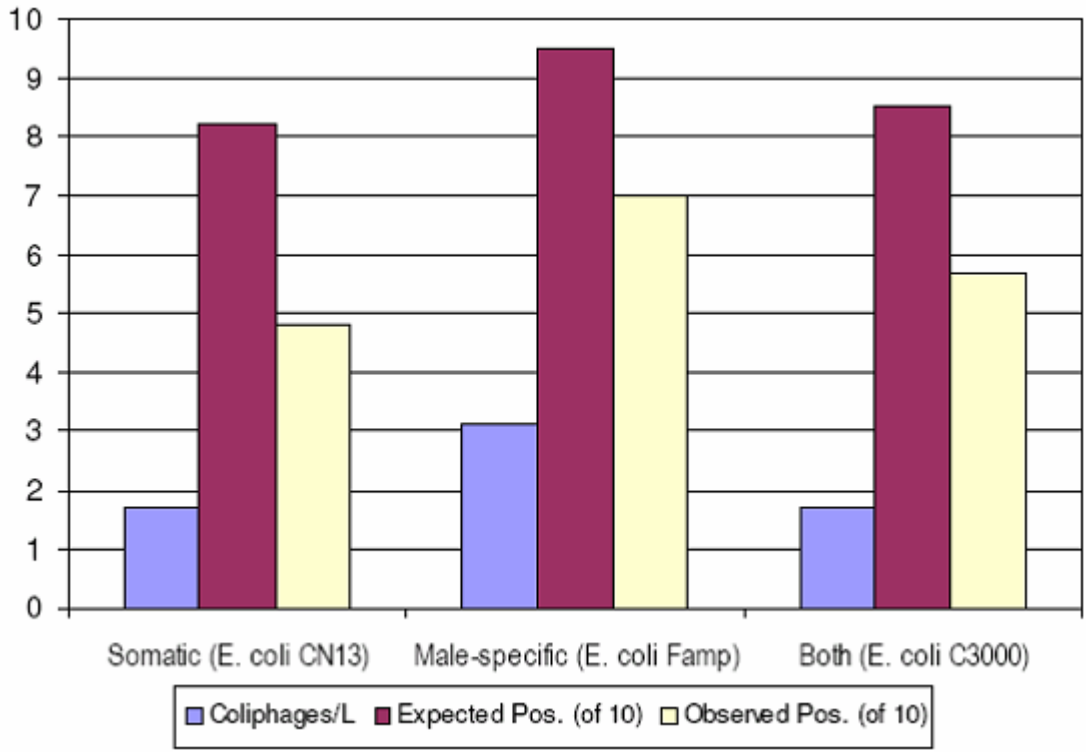
As in the statistical analyses for Method 1602 described earlier, the data for Method 1601 were subjected to ANOVA. This analysis detected no significant differences in recovery among the laboratories ($p=0.38$); nor did it detect any significant differences in recovery among the hosts ($p=0.41$).

The results from this series of 8 replicate experiments per lab are also summarized in Table 8 and Figure 1 based on the observed and expected number of positive 1-liter enrichment culture bottles out of 10**.** These results indicate sensitive coliphage detection that is close to the theoretical level of detection.

Table 8. Comparison of Observed and Expected Coliphage Detection in 1-Liter Volumes of Seeded Ground Water by Method 1601

Coliphage Group	Estimated Coliphages/L	# Positive Bottles out of 10	
		Expected*	Observed (Average)
Somatic (*E. coli* CN13)	1.7	8.2 (~8)	4.8 (~5)
Male-specific (*E. coli* Famp)	3.1	9.5 (9-10)	7.1 (~7)
Both (*E. coli* C3000)	1.7	8.5 (8-9)	5.7 (~6)

Figure 4. Coliphage Detection in 1-Liter Volumes of Seeded Ground Water by Method 1601



Based on direct analysis (plaque assay) of the sewage-derived coliphage stocks added to the ground water samples, the average concentrations of coliphages per 1-liter enrichment bottle were: 1.7 for somatic coliphages (detected on host *E. coli* CN13), 3.1 for male-specific coliphages (detected on *E. coli* Famp) and 1.9 for both groups of coliphages (detected on host *E. coli* C3000). According to these estimated coliphage concentrations per liter of seeded ground water, the expected numbers of positive enrichment bottles out of a total 10 enrichment bottles per coliphage host are computed. These estimates of expected numbers of positive enrichment bottles out of 10 were based on the principles of Poisson statistics (the Poisson Distribution and Poisson probabilities). The estimates were computed directly from the Poisson probability equation using the estimated coliphage concentrations per liter (based on direct assay of the sewage coliphages seeded into groundwater as an estimate of the mean number of coliphages per bottle. The expected numbers of positive bottles out of 10 were: about 8 for somatic coliphages, 9 to 10 for male-specific coliphages and 8 to 9 for both groups of coliphages. The experimentally observed numbers of positive enrichment bottles out of 10 for each group of coliphages are shown in Table 8 and Figure 1. Rounded to the nearest whole bottle, the numbers of positive enrichment bottles out of 10 were: 5 for somatic coliphages, 7 for male-specific coliphages and 6 for both groups of coliphages. These actual results indicate that the likelihood of detecting coliphages when analyzing 1-liter volumes of ground water containing only about 1.5-3 coliphages per liter by the enrichment method are very high and very close to the theoretically expected results. It is noteworthy that out of a total of 87 trials with 10 1-liter enrichment bottles per trial there were only 4 occasions when all 10 bottles were negative. This is well within the expected number of times when all 10 bottles would yield negative results,

based on statistical considerations.

The expected probability of getting 10 negative bottles in a 10-bottle enrichment test when there is an average of 1.5 to 3 infectious coliphages per bottle was actually higher than the observed numbers of 10 negative enrichment bottles in a test. It should be noted that the detection of both somatic and/or male-specific coliphages together on host *E. coli* C3000 was similar to the detection of either somatic or male-specific coliphages on their respective *E. coli* hosts. Although the numbers of positive enrichment bottles out of 10 were no higher on host *E. coli* C3000 than on the other two hosts, the ability to simultaneously detect both groups of coliphages using this host was not appreciably different than the detection of each coliphage group alone. Based on the number of times that all 10 bottles in an enrichment test were negative, *E. coli* C3000 was the same as or better than the other two hosts. The negativity rates per 30 trials of the method were 1, 1 and 2 per 30 trials for *E. coli* C3000 (both), CN-13(somatic) and Famp (male specific) respectively. These results indicate that *E. coli* C3000 can be successfully and reliably used to simultaneously detect low levels of both somatic and male specific coliphages in 1-liter volumes of ground water using the two-step enrichment method (Method 1601).

Summary of Method 1601 Results

In summary, recoveries of somatic, male-specific and total (somatic plus male-specific) coliphages from 1-liter volumes of ground water were efficient using Method 1601. Coliphage recoveries at input levels of about 1.5 to 3 infectious units per liter of ground water were somewhat variable but close to those expected based on the infectivity titer of the sewage seed

and to the expected number of positive 1-liter enrichment bottles out of a total of 10. The observed variability of coliphage detection was no more variable than would be predicted for a 10-sample MPN test. Coliphage recoveries were not significantly different among *E. coli* hosts and participating labs using ANOVA. Therefore, there is a high likelihood of detecting as few as 1-3 coliphages in 1-liter volumes of water using the two-step enrichment methods of Method 1601.

PHASE I CONCLUSIONS

In comparing the two coliphage recovery and detection methods, the participating laboratory groups tended to favor the Two-Step Enrichment over the Single Agar Layer method. This is because the former test was considered easier to perform, sensitive in detecting low numbers of coliphages and more consistent in its results. The Single Agar Layer (SAL) assay proved to be cumbersome when assaying multiple samples, and the time constraints imposed by the method were difficult to adhere to. The Two-Step Enrichment method is simpler to set up and much easier to carry out. The statistical analyses showed it to be more consistent among different laboratory groups. In addition, the results for the enrichment method showed that somatic and male-specific coliphages can be detected simultaneously on a single host, *E. coli* C3000, at a sensitivity comparable to detecting either somatic or male-specific coliphages individually. The simultaneous detection of both somatic and male-specific coliphages simplifies the method as well as lowers costs.

Further studies were done to characterize the performance of Methods 1601 and 1602 when applied to the detection of coliphages in unseeded samples of fecally contaminated ground water in Phase II of this study. The presence and concentrations of coliphages in field samples of groundwater were compared and also were to also be compared to the presence and concentrations of human enteric viruses in these fecally contaminated ground water samples. This information was to be used to determine if somatic, male-specific and total coliphages are

sensitive and reliable indicators of fecal contamination and the presence of human enteric viruses in groundwater.

PHASE II STUDIES

Statement of Work: Coliphage Method 1601 and 1602 Validation and Field Testing

Background. The US EPA's proposed Ground Water Rule may propose the examination of ground waters for coliphages. Coliphages have been found to be reliable indicators of fecal contamination of ground and surface water and of the fate of human enteric viruses in the subsurface environment. Recently, two different EPA methods were developed to detect somatic and male-specific coliphages in ground water. Method 1601 detects and quantifies coliphages by liquid enrichment culture method and Method 1602 detects and enumerates coliphages by a single agar layer (SAL) plaque assay. The original methods round robin studies analyzed for somatic and male-specific coliphages separately, and did not evaluate *E. coli* C3000 as host bacterium for detection and quantification of both somatic and male-specific coliphage. The SAL method for coliphage detection did not require a confirmation step for the plaques that were observed in the agar-host cell medium. There was some concern about the detection of "false positives" based on simply scoring plaques. Therefore, the methods needed to be further substantiated and validated and scientifically supportable to: (1) show that coliphages detected by these methods are of likely fecal origin and have characteristics and properties consistent with fecal origin, (2) reduce the cost and burden of using two different hosts to separately analyze somatic and male-specific coliphage by measuring both simultaneously in a single *E. coli* host bacterium, (3) include a simple confirmation step.

Purpose and Objectives of the Study. The purpose of this study was to further validate EPA method 1601 and 1602 and to test these methods in groundwater samples in four geographically representative regional laboratories in the USA (North Carolina, Minnesota, New Hampshire and Texas). The study was to determine if coliphages detected by Methods 1601 and 1602 can be confirmed and show properties and characteristics consistent with a fecal origin. The labs that conducted the studies were equipped and experienced to conduct the tasks required in the SOW. The study was headed by a coliphage expert who has at least 20 years experience in coliphage virology, who has experience in round robin testing, in developing research procedures and in method 1601 and 1602. The expert participated in the original EPA round robin testing for method 1601 and 1602, is knowledgeable about EPA programs, and the proposed Ground Water Rule.

Specific Objectives. The specific objectives of the study were to: (1) conduct a field validation of Methods 1601 and 1602 for coliphage detection, (2) determine the ability of coliphage indicators in predicting the presence of human enteric viruses in the same ground water samples, (3) confirm the presence of coliphages detected in ground water samples, (4) characterize the properties of these coliphages to confirm that they are of likely fecal origin, (5) determine the correlation and the reliability of such correlation in detecting fecal contamination and the presence of human enteric viruses in ground water, and (6) address some key questions about these coliphage methods and their use for coliphage detection in groundwater that arose in the April 2004 EPA International Workshop on Coliphages as Indicators of Fecal Contamination in Water and Other Environmental Media. An additional task was added to the study at the request

of the EPA project manager. This task was: (7) to determine the stability or survival of coliphages in groundwater samples that held for up to several days before analysis by Methods 1601 and 1602.

Task 1. Characterization and determination of properties of confirmed coliphage isolates. A total of 800 coliphages (200 from each of the four participating laboratories) were to be characterized of which 400 (100 from each laboratory) were to be from field samples and 400 (100 from each laboratory) were to be from sewage-seeded ground water samples used in methods validation studies in Phase I. The contractor was to subject confirmed coliphage isolates to the analyses described below.

(a) bacterial host range analyses. Determine the ability of test coliphage isolates to be grown in both *E. coli* and non-*E. coli coliform* hosts and other bacteria by spotting onto pre-poured agar medium-host cell lawns of the following 23 different host bacteria, if available: *E. coli* strains C, CN13, C3000, K12F, K12F and Famp*, S. typhimurium* WG45 and WG49*, Klebsiella pneumoniae* ATCC strains 23356 and 23357, *Enterobacter cloacae* ATCC strain 223355, *Citrobacter braakii* (formerly *Citrobacter freundil)* ATTC strain6570 ATCC strain 12012, *Serratia marcescens* ATCC strain 14764, *Shigella sp.* ATCC 23354, *Shigella flexneri* ATCC 12661, *Yersinia pseudotuberculosis* ATCC strain 23207, *Proteus mirabilis* ATCC strain 9921, *Yersinia enterocolitica* ATCC strains 9610, 29913, *Pseudomonas aeruginosa* ATCC s train 12175, *Aeromonas hydrophila* ATCC strain 23211.

(b) growth temperature range. Test the coliphage isolates for their ability to grow at temperatures of 25, 36, 42 and 44.5˚C on *E. coli* hosts.

(c)nucleic acid analyses. Examine coliphage isolates for taxonomy. Male specific coliphages were be tested to determine the type of nucleic acid as either DNA or RNA.

The contractor shall analyze 32 geographically representative ground water samples (8 per laboratory in four geographically representative laboratories) for coliphages and human enteric viruses by combined cell culture and nucleic acid amplification methods.

Task 2. Cell culture RT-PCR or cell culture-PCR. The contractor shall analyze 32 geographically representative ground water samples (8 per laboratory in four geographically representative laboratories) for coliphages and human enteric viruses by combined cell culture and nucleic acid amplification methods.

Task 3. Coliphages and enteric viruses from groundwater. Each of the four participating laboratories shall analyze an additional 8 samples of ground water for culturable human enteric viruses and for coliphages. Four different ground waters shall be analyzed on two different occasions. A total of 36 ground water samples shall be analyzed for coliphages and human enteric viruses. The extent to which somatic and male-specific coliphages detected by Methods 1601 (in 1-liter sample volumes) and 1602 (in 0.1-liter sample volumes) are associated with the occurrence of human enteric viruses in 100-1000-liter sample volumes of fecally contaminated

ground water will be determined. The contractor shall statistically analyze data on the occurrence of coliphages and human enteric viruses in field ground water samples to determine if there is a co-occurrence and the extent to which they co-occur.

Task 4. Coliphage and bacterial analyses. Each lab will sample, process, and analyze ground water by Methods 1601 and 1602 using hosts *E. coli* Famp, *E. coli* CN-13 and *E. coli* C3000 according to the established methods. In parallel to the coliphage analysis, each lab will sample and process the same fecally contaminated ground waters by the EPA ICR methods for human enteric viruses. Also, in parallel each lab will sample and process the same fecally contaminated ground waters for *E. coli* and enterococci.

Task 5. Enteric virus analyses. Processed ground water samples will be analyzed for culturable human enteric viruses by observation for cytopathogenic effects (CPE) in BGMK cells according to EPA ICR method. In addition, the inoculated cell cultures also will be examined for non-cytopathogenic enteroviruses, caliciviruses, adenoviruses, hepatitis A, rotaviruses, reoviruses by combined cell culture and nucleic acid amplification methods, as previously described. The labs also will analyze the samples for culturable human enteric viruses in CaCo2 cells. The data on the occurrence and concentrations of coliphages and concentrations of human enteric viruses as detected by CPE and by PCR will be statistically analyzed to determine co-occurrence with coliphages.

Task 6. **Report.** The contractor shall prepare a consolidated draft report of all the data generated

in all the tasks in all 4 laboratories and statistically analyzed. The report shall include an interpretation of the results and recommendations to EPA. The report shall be submitted in 3 double spaced hard copies and a 3½ diskette in WordPerfect, version 9/8.0 for Windows. A summary fact sheet of the study and results shall accompany the draft report.

Task 7. Peer review. The contractor shall incorporate internal and external peer review comments and a workshop input comments in a final report.

The EPA Work Assignment Manager will give technical direction in this study. The contractor shall not cite, quote or distribute the results of this EPA study until EPA publishes it. Publications from any aspect of this EPA research study will be subjected to EPA review and will be published jointly with EPA. A monthly conference call shall be scheduled by the contractor until completion of the study. An on site visit will be conducted by the EPAWAM on a mutually acceptable date with the technical lead.

Schedule of Deliverables

The deliverables will include a consolidated 1st draft report with data from all the completed 7 tasks listed above and a final peer review report.

NOTE: These project objectives were not fully met due to extenuating circumstances. Specifically, Task 1 could not be completed due to circumstances beyond the control of the project investigators. Although more than 800 coliphage isolates were obtained during this

study, the vast majority of the inventory of these coliphages were lost to due failures of ultracold freezers in which the isolates were stored at the UNC laboratories for subsequent characterization. These freezer failures were due to both freezer malfunctions and to power outages at the laboratories that were due to natural disasters (ice storms and hurricanes) beyond the control of the project investigators. Furthermore, these freezer failures caused the loss of the majority of bacterial hosts on which the coliphages isolates were to be characterized as to host range in order to fulfill Task 1a. Most of these bacterial strains had been previously purchased from the American Type Culture Collection and the costs of replacing them were prohibitive and had not been included in the project budget.

To compensate for the loss of these coliphage isolate samples and their further characterization, the project labs undertook additional work in support of meeting other project objectives and tasks. Specifically, the participating labs analyzed more samples of groundwater in the Phase II studies than were originally specified. Contract specifications called for the analysis of a total of 64 samples (16 per laboratory) for coliphages, bacterial indicators and human enteric viruses. The eventual number of samples analyzed was actually 106 samples (27 by three laboratories and 25 by the fourth laboratory). It was believed that the analysis of extra field samples would provide more representative data for determining if coliphages were effective indicators of fecal contamination of groundwater and of human enteric viruses. Additionally, the analysis of human enteric viruses in groundwater samples was expanded to include astroviruses, which were not originally included in the specifications for human enteric viruses to be analyzed. Hence, this

additional task was taken on to further improve the opportunities to detect human enteric viruses in groundwater as part of the effort to obtain more definitive data.

At the request of the UNC project manager, the UNC lab also took an additional task that was not in the original scope of work or its budget. This additional task was to determine the survival of coliphages in groundwater samples to be analyzed for viruses by Methods 1601 (two-step enrichment spot plate) and Method 1602 (SAL plaque assay). Groundwater samples seeded with mixed populations of sewage-derived viruses were analyzed for coliphages initially (on day zero) and also after 2, 3 and 6 days of storage at 4°C. These survival experiments were done to determine if collected samples held for several days prior to analysis due shipping and storage still had most of their initial coliphages that still could be detected by Methods 1601 and 1602.

PHASE II METHODS AND MATERIALS

Groundwater Samples and Wells

The original goal of this study was for each of the four, regionally representative laboratories (southeast, northeast, upper midwest and southwest) to collect and analyze 27 ground water samples from public water supply wells. Efforts were made to identify candidate public water supplies that previously had coliform bacteria violations or other evidence of vulnerability to fecal contamination. In some cases candidate wells were prescreened by bacteriological and coliphage analyses for evidence of fecal contamination. Because not all participating labs could identify and get access to 27 public water supply wells, some labs also included non-public and private wells in their sampling. Three labs obtained 27 ground water samples and one lab obtained a total of 25 samples for a total of 106 samples overall. The characteristics of the wells that were sampled are presented below, by region.

Southeast. Of the 27 wells in the Southeast, 13 were in North Carolina and 4 were in Florida. The Florida wells were all public water supply wells. Florida Well UNC #1 is in Orange County, FL. There is no history of that well ever being disinfected. Florida Well UNC #2 is in Orange County, Fl. The pump was taken out of service for repairs (rebuilt pump), and it was disinfected in January - February of 2002. Prior to placing the well back into production it was disinfected with chlorine. Approximately 30 gallons of 12% liquid bleach was placed into the well for 24 hours (100ppm). Then water was discharged for a minimum of 4 hours and bacteriological samples were taken to confirm their absence. Theses Florida wells were samples in June and

September, 2002, which was 4 and 7 months after Well #2 had been chlorinated.

Two other Florida wells, designated UNC #3 and UNC #4 and located in Ocala County also were sampled. Both wells had periodic coliform positivity during the and prior to the study period (2002). They serve a population of about 57,000 in the Ocala area. UNC #3 and #4 were not disinfected prior to or during sampling for this study. However, the utility currently (year 2004) adds calcium hypochlorite (granular chlorine) to control the total coliforms they are getting (and have been getting a lot more since the hurricanes this year - 2004). The chlorine is now added weekly to both wells to achieve the CT for 4 log virus removal through their treatment process, including chlorination.

There were 13 wells in North Carolina, and the characteristics of these wells are summarized below.

Type	County	Well Identification
Private industrial	Cartaret	BF
Community water supply	Cartaret	BMHP
Community water supply	Carteret	SB MHP
Non-community water supply (private campgrounds)	Pamlico	Camp DL
Non-community water supply (private campgrounds)	Pamlico	Camp SF #1
Non-community water supply (private campground)	Pamlico	Camp SF #2

Private	Carteret	TGUMC
Private	Carteret	GL
Private	Pender	SC
Private	Pender	VE
Private	Duplin	RH
Private	Duplin	KC
Private	Duplin	TH

Southwest. Only PWS wells were investigated in this study, and a total of eleven different PWS wells were included. The sampling sites were located in the San Antonio region of Texas (wells RS, KK, and HCR) and along the US-Mexico border in southern New Mexico (wells MHPa, MHPb, MHPc, FVE, AVC, SME, and LME). The wells in the San Antonio region were part of a karst aquifer and were previously implicated in a documented groundwater contamination event. Also, during the initial pre-screening of the wells some of the samples were positive for somatic and male-specific coliphages. The wells in southern New Mexico were identified as being vulnerable to groundwater contamination based on parameters such as closeness to septic tanks, proximity to the Rio Grande river and the aquifer in question. These wells were part of a previous EPA-funded project on the microbiological quality of wells in the shallow aquifer along the US-Mexico border during which some of the wells in the sampling area were positive for enterococci, *E . coli*, male-specific coliphages and somatic coliphages. The wells were in the 100-150 feet depth range. The static water levels were around 10-20 feet and in terms of their hydrogeologic setting, they were located in the Rio Grande alluvium/Hueco-Tularosa aquifers.

Groundwater samples were collected between June 2002 and January 2003. Multiple samples were collected from each of the wells to be representative of the aquifer and the sampling location. During each sampling adequate volumes were collected for the coliphage analysis as well as for the enteric virus analysis. Grab samples were collected for the coliphage and bacterial analysis while the 1MDS filters were used for collecting the large volume enteric virus samples.

Upper Midwest. A total of 27 groundwater samples were collected from 25 wells. Two wells were tested twice. Details of these wells are provided in the report of the Upper Midwest lab, which is in the Appendix). All wells except 6 private ones in Minnesota were considered non-community public water supplies by the State of Minnesota and none were disinfected. Noncommunity transient public water supplies (i.e., groundwater) are monitored for nitrate and total coliform bacteria as required by the SDWA. Private water systems including those places of business not meeting the federal definition of PWS have no long term monitoring requirements.

Northeast. All sample sites were located in New England. Eight well sites were public water sources and 17 were private wells. A total of 25 wells samples were collected instead of 27 due to a very severe and harsh winter. NH had its first snowfall at the end of October and a second snowfall at the beginning of November, 2002. Plans to sample two additional wells as soon as the weather permitted could not be carried out because New England experienced one of the snowiest winters ever. Therefore, only 25 well samples were collected and analyzed. Of the 25 wells, there were 12 sample sites in New Hampshire, two of which were from public wells that were approximately 500 and 700 ft deep, respectively. None of these wells had any form of

disinfection. The other wells from NH were all private wells. These wells also were not disinfected. One well from NH was a private, very shallow well, less then 35 feet deep and lined with stone. This was not considered a potable well but was used for farm irrigation. Four sites in Maine were all privately owned wells and not disinfected. Three sites were in Vermont, and they were all privately owned wells and not disinfected. All of the privately owned wells were drilled wells, excepted for the one in NH as indicated above, and they were of varying depths that were unknown to the homeowner at the time samples were collected. There were 6 samples from public water supply wells in Massachusetts. The public water supplies in Massachusetts were chosen due to positive results previously found for total and fecal coliforms, enterococci, and male-specific coliphages. Additionally 3 of the 6 locations had positives previously reported for rotavirus and enterovirus, by molecular methods.

Coliphage Analysis of Groundwater

Groundwater samples were analyzed by Method 1601, the two-step spot-plate enrichment method and by Method 1602, the Single Agar Layer (SAL) plaque assay using sample volumes of 1 liter and 100 mL, respectively, for each target group of coliphages (male-specific, somatic and "total" coliphages). Host bacteria for the target groups of coliphages were *E. coli* CN-13 for somatic coliphages, *E. coli* Famp for F+ coliphages and *E. coli* C3000 for "total" (somatic plus F+) coliphages. Coliphage analyses were performed according to the EPA-approved methods, except lysis zones from enrichment spot plates and plaques from SAL plates were confirmed using the proposed EPA confirmation method. In this method, material from individual SAL plaques or lysis zones on spot plates was removed (aspirated) with a Pasteur pipette, micropipette

tip, or other device and the recovered material was resuspended in 0.5 mL of tryptic soy broth. These suspensions were held briefly for coliphages to diffuse out of the agar and then the samples were vortex mixed vigorously to disperse the coliphages. Then, 10µl aliquots were removed from the suspension and spotted onto pre-poured spot-plates of the appropriate *E. coli* host bacterium as in the enrichment procedure. The spot-plates were incubated overnight and checked for zones of lysis. Any spots showing lysis were scored as confirmed coliphages.

Coliphage Isolate Characterization

A total of 800 coliphages (200 from each of the four participating laboratories) were to be characterized for their properties to determine if they were of likely fecal origin. For each of the four participating laboratories 100 coliphage isolates from the phase I studies with groundwater samples seeded with sewage-derived coliphages and another 100 isolates from the unseeded field groundwater samples of each laboratory were to be subjected to characterization by bacterial host range analysis, growth temperature range analysis and determination of type of nucleic acid (for F+ coliphages).

For bacterial host range analyses coliphage isolates were to be tested for their ability to grow in both *E. coli* and non-*E. coli coliform* hosts and other bacteria by spotting onto pre-poured agar medium-host cell lawns of the following 23 different host bacteria if available: *E. coli* strains C, CN13, C3000, K12F, K12F and Famp*, S. typhimurium* WG45 and WG49*, Klebsiella pneumoniae* ATCC strains 23356 and 23357, *Enterobacter cloacae* ATCC strain 223355, *Citrobacter braakii* (formerly *Citrobacter freundil)* ATTC strain6570 ATCC strain 12012,

Serratia marcescens ATCC strain 14764, *Shigella sp.* ATCC 23354, *Shigella flexneri* ATCC 12661, *Yersinia pseudotuberculosis* ATCC strain 23207, *Proteus mirabilis* ATCC strain 9921, *Yersinia enterocolitica* ATCC strains 9610, 29913, *Pseudomonas aeruginosa* ATCC s train 12175, *Aeromonas hydrophila* ATCC strain 23211. Spotted plates are incubated at 37^0C overnight and observed for evidence of lysis of the host bacteria in each spot as evidence of growth on each host bacterium.

For growth temperature range characterization, coliphage isolates were to be tested for their ability to grow at temperatures of 25, 36, 42 and 44.5˚C on *E. coli* hosts. Coliphage isolates were to be serially diluted 10-fold and several dilution were to be spotted in 10 uL amounts onto replicate pre-poured lawns of *E. coli* host bacteria in agar media Petri dishes. Each replicate plate was to be incubated at the aforementioned temperatures overnight and then the spots n these plates were to be observed and quantified for coliphage growth at each of the 4 test temperatures. Coliphage growth at temperatures of not only 36ºC but also growth at the temperatures of 42 and or 44.5oC was considered evidence of thermotolerance and of a likely fecal origin.

For nucleic acid analyses of F+ coliphages, isolates were to be examined for taxonomy as F+ DNA or F+ RNA coliphages using previously described methods (Hsu et al., 1995). Briefly, 10 µl volumes of F+ coliphage suspensions were to be spotted onto duplicate pre-poured lawns of *E. coli* host bacteria in agar medium. One plate contained Rnase at 100 µg/mL and the other plate did not. Plates were to be incubated overnight at 37ºC and then they were to be observed for lysis or the appearance of plaques in the spots of applied coliphage suspensions. Presence of a

lysis zone or plaques in the spot on the plate without Rnase and the absence of such lysis or plaques in the spot on the plate with Rnase were considered evidence of an RNA coliphage. The presence of lysis or plaques in the spots of plates with and without Rnase was considered evidence of an F+ DNA coliphage.

As indicated above, these coliphage characterization activities were not completed due to extenuating circumstances beyond the control of the project investigators. Ultracold freezer failures caused the loss of archived coliphage isolates to be characterized and also the loss of most of the bacterial hosts that were to be used for host range characterization studies of these coliphage isolates.

Bacteriological Analysis of Groundwater

Field groundwater samples were analyzed for *E. coli* and enterococci and in some cases for fecal coliforms using EPA-approved methods. For *E. coli*, some labs used mFC agar for fecal coliforms, with incubation at 44.5ºC for 20-22 hours, followed by transfer or membranes to nutrient agar-MUG medium, re-incubation for several hours, and observation for colonies fluorescing blue under long-wavelength UV light as evidence of *E. coli* colonies. Another lab used mEC medium for *E. coli* with incubation at 44.5 ºC (APHA, 1998). Another lab used mColiBlue agar for simultaneous detection of total coliforms and *E. coli*, according to USEPA-approved methods. For enterococcus analysis, labs used standard membrane filter methods and with either modified ME agar or MEI agar and incubation conditions as specified in the EPA method (APHA, 1995; Levin et al., 1975; USEPA, 2002). Samples for *E. coli* analysis were 100

mL, although one lab also analyzed volumes of 1000 mL. Data for the results of this larger 1000 mL volume were not included in the compilation and analysis of data for all labs, as no other lab analyzed this volume and it is not a standard volume used for bacteriological analysis of water. The results for this larger volume are in the report from this participating laboratory, which is in the Appendix to this report.

Analysis of Groundwater for Human Enteric Viruses

Primary virus concentration from groundwater. Ground water from candidate wells was sampled using the EPA ICR method, with minor modifications (USEPA, 1996). Groundwater sample volumes of 1,500 liters (397 gallons) were to be filtered through a 1 MDS pleated cartridge filter (CUNO) at pH 6-8. The filter was eluted with 1.5% beef extract (Becton Dickinson #212303) buffered with 0.05 M glycine at pH 9.5. Viruses in the resulting beef extract eluate were further concentrated by organic flocculation (acid precipitation) as specified by the ICR methods. The only significant change to the ICR procedure was that the acid precipitate was resuspended in 20 about mL of sodium phosphate rather than 30 mL in order to reduce the concentrate volume and the number of cell cultures required to assay the concentrate. The entire concentrate was filter-sterilized using a 0.2 micrometer pore size Gelman Serum Acrodisc filter (#4525) which has been pretreated with a small volume of beef extract eluent to minimize viral adsorption.

The filter-sterilized concentrate was subdivided into the following aliquots prior to being frozen at -80 :

Equivalent of 500 L of water sample for assay of viruses in Caco-2 cell cultures

Equivalent of 500 L of water sample for assay of viruses in BGMK cell cultures, further subdivided into a 1.5 mL subsample, and the remainder. The 1.5 mL subsample was used in a pre-test for cytotoxicity in BGMK cultures.

Equivalent of 100 L of water sample for assay of HAV in FRhK-4 cell cultures.

Equivalent of 100 L of water sample for assay by direct RT-PCR for caliciviruses (noroviruses).

The remainder of the sample concentrate (20%, equivalent to 300 L) was archived at - 80 C.

Virus isolation in cell cultures. Three cell lines were used to detect a range of infectious enteric viruses. The BGMK cell line was used to propagate adenoviruses, enteroviruses, and reoviruses according to the procedure of (Chapron et al. (2000). Caco-2 cells were used for the detection of astroviruses and rotaviruses according to Chapron et al. (2000). The FRhK-4 cell line was used to detect hepatitis A virus (HAV).

Cell culture infectivity assays were performed in a minimal number of 75 cm^2 flasks (generally 4 or 5). A pretest to screen each sample concentrate for cytotoxicity was performed in 25 cm^2 BGMK culture flasks, with one flask being inoculated with 1.0 mL of sample concentrate and a second flask being inoculated with 0.5 mL of sample concentrate. The pretest cultures were observed microscopically for evidence of cytotoxicity (or CPE) for one week, before the remainder of the sample was inoculated into cell cultures.

Sample concentrates inoculated into BGMK and Caco-2 cultures were pre-activated by treatment with the proteolytic enzyme trypsin prior to inoculation. This was done because previous studies had indicated enhanced enteric viruses detection using this trypsin pre-treatment. Each

concentrate was mixed with a solution of type IX trypsin (Sigma T-0303) yielding a final 10 µg/mL concentration, then incubated 30 minutes at 37 C. Pre-activation was not necessary and therefore not employed for HAV propagation in FRhK-4 cultures. Because divalent cations enhance attachment of HAV and many other enteric viruses to cells, sample concentrates were diluted with an equal volume of Dulbecco's PBS before being inoculated into FRhK-4 cultures.

Cell cultures in 75 cm^2 (confluent monolayers for BGMK cells and 90-95% confluency for Caco-2 and FRhK-4 cell cultures) were drained and rinsed three times with Dulbecco's phosphate buffered saline (Sigma #D-8662, Gibco #14040 or equivalent) to remove residual serum. FRhK-4 cell cultures were rinsed once. Replicate cultures were inoculated with sample concentrates, and incubated at 37 C for 90 minutes, while being rocked every 15-20 minutes to re-distribute the inoculum, to allow for virus adsorption to cells. One negative control flask was inoculated with PBS before any flasks were inoculated with sample concentrates, and a second negative control culture was similarly be inoculated at the end of the sample inoculation step. No enteric virus positive control flasks were to be prepared at this time in order to avoid possible laboratory virus contamination. Maintenance medium was then added to each flask, and the cultures were incubated at 37 C. The maintenance medium for BGMK and Caco-2 cultures consisted of serum-free Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 5 µg/mL trypsin. The maintenance medium used in FRhK-4 cultures consisted of Eagle's minimum essential medium with Earle's salts supplemented with 2% serum and 30 mM $MgCl_2$.

Cultures were observed microscopically on days 1 and 2, then at least every other day following inoculation. The occurrence of cytopathology or cytopathic effects (CPE) on the first two days was tentatively assumed to be evidence of sample cytotoxicity or the release of cells from the bottom surface of the tissue culture flask by the action of the trypsin. If cytotoxicity thought to be associated with sample inocula was not too far advanced, the affected cultures were given a change of maintenance medium to saved them from possible destruction by sample cytotoxicity. Alternative approaches for cytotoxicity reduction included removing the inoculum following the 90 minute incubation period and then rinsing the cell layer with PBS, diluting the sample concentrate in Dulbecco's PBS, or inoculating less concentrate into each cell culture. Every reasonable effort was made to reduce sample cytotoxicity and maximize enteric virus detection.

BGMK and Caco-2 cultures were incubated for 7 at 37^0C days following inoculation. All cultures were freeze-thawed, and 10% of the lysate from each flask was inoculated into fresh cultures for a second 7-day passage. If a flask exhibited possible viral cytopathology (CPE), the lysate was passed through a 0.22µm pore size, sterilizing filter into a fresh culture to confirm the presence of viruses and the absence of bacterial or fungal contamination. FRhK-4 cultures were incubated for two 14-day passages, with the maintenance medium being changed after seven days, to maximize the detection of typically slow-growing HAV.

At the end of the final cell culture passage, flasks were frozen and thawed twice. A 1-mL aliquot from each of the first and second passage BGMK and Caco-2 flasks inoculated with a given sample was pooled in a centrifuge tube. A half-volume of chloroform was added, and the tube was vortex mixed at high speed for two minutes. The tube was centrifuged at 1,200-1,800 x g

for 20 minutes, then the supernatant extract was removed and split into aliquots for viral analysis or archiving.

Virus detection by nucleic acid amplification. Chloroform-extracted cell culture lysate pools were examined for adenoviruses, astroviruses, enteroviruses, HAV, reoviruses and rotaviruses by RT-PCR or PCR. Viral nucleic acids were extracted from lysates using the QIAamp Viral RNA Mini Kit (Qiagen #52904). By modifying two steps of the QIAamp protocol, adenovirus DNA could be efficiently recovered without compromising extraction of viral RNA:

In step #8 of the Qiagen protocol, The sample column was incubated for one minute after adding buffer AW1, before centrifuging the column.

In step #9, the sample column was incubated for one minute after adding buffer AW2, before centrifuging the column.

Prior to RNA extraction, enteric viruses in 2-milliliter aliquots of pooled, chloroform-extracted cell culture lysates were concentrated using polyethylene glycol (PEG) precipitation. Polyethylene glycol (Sigma #P2139, molecular weight = 8,000) was added to a final 8% concentration. Sodium chloride was added to a 0.3 M concentration, and the sample was mixed until the additives dissolved. The solution was incubated for two hours at room temperature and then centrifuged at 6,700 x g for 20 minutes at 4 C. The pellet was resuspended in 300 µL of Dulbecco's PBS, then extracted with 300 µL of chloroform.

An aliquot of each water sample concentrate was assayed directly for human caliciviruses (noroviruses) by nucleic acid amplification using RT-PCR because these viruses cannot be propagated in cell cultures. Each sample was reconcentrated using polyethylene glycol precipitation. PEG was added to a final 10% w/v concentration. Sodium chloride was added to a final 0.3 M concentration. Since the sample has previously been adjusted to pH 7.0-7.5, no further pH adjustment was necessary. The mixture was shaken until the additives had dissolved. The solution was incubated at room temperature for two hours or at 4 C overnight. The solution was centrifuged at 6,000 to 10,000 x g for 15 minutes, and the supernatant was removed by aspiration and discarded. The pellet, which may not be visible, was resuspended in a maximum of 140 µL of Dulbecco's PBS containing magnesium and calcium ions.

Prior to RNA extraction, the resuspended pellet was extracted with chloroform. A 100-µL volume of chloroform was added, and the sample vortex mixed for one minute. The sample was centrifuged at about 3,000 x g for 5-10 minutes. The supernatant was removed by aspiration and recovered. Viral RNA was extracted from the recovered, chloroform-extracted supernatant using the standard QIAamp Viral RNA Mini Kit protocol.

Nucleic acid amplification by (RT-)PCR

Introduction. Viruses in chloroform-extracted cell culture lysates that had been inoculated with water sample concentrates and human caliciviruses (noroviruses) in aliquots of water sample concentrates were analyzed by either PCR for adenoviruses or RT-PCR for astroviruses, caliciviruses, enteroviruses, hepatitis A virus (HAV), reoviruses, and rotaviruses. The

procedures for combined cell culture and (RT-)PCR were based on those previously used by Chapron et al. (2000) with minor modifications. The nucleic acid extraction procedures and the (RT-)PCR primers and amplification procedures applied to each virus or virus group are described in more detail in the sections that follow and in the individual report of the other three participating laboratories (see Appendix). RT-PCR for HAV and caliciviruses was done by the University of North Carolina lab (Southeast), RT-PCR for enteroviruses was done by the University of Minnesota lab (Upper Midwest), RT-PCR for reovirus and rotavirus was done by the TAMU lab (Southwest), and (RT-)PCR for adenoviruses and astroviruses was done by the University of New Hampshire lab (Northeast). The details of the virus (RT-)PCR procedures of the participating laboratories are given below and also in more detail in the individual project reports of the other three participating labs, which appear in the Appendix of this report.

Enterovirus RT-PCR

The primers for RT-PCR amplification of enteroviruses were:

3' pan-enterovirus primer: 5'-ACC GGA TGG CCA ATC CAA

5' pan-enterovirus primer: 5'-CCT CCG GCC CCT GAA TG

Random hexamers may also be used as the primer for enteroviruses for reverse transcription.

The reaction mixtures for a 3.5 μL sample were as follows. (For larger sample volumes the amounts were increased proportionally). (Note: These mixtures utilized reagents from the GeneAmp RNA PCR Core Kit, Applied Biosystems #N808-0143.)

RT master Mix	Stock conc.	reaction/reaction	Final conc.
MgCl2	25 mM	4	5 mM
10x PCR Buffer II, pH 8.3	10x	2	1x
each dNTP	10 mM	2 each	1 mM
3' primer or random hexamers	50 µM _____	0.5	1.26 µM
MuLV reverse transcriptase	50 U/µL	0.9	45 units
Rnase inhibitor	20 U/ L	0.9	18 units

The RT conditions were: 95 for 5 minutes, 42 for 60 minutes, and 95 for 5 minutes.

PCR master mix	Stock conc.	reaction/reaction	Final conc.
MgCl2	25 mM	4	2 mM
10x PCR Buffer II, pH 8.3	10x	8	1x
Water (Sigma #W-4502)		66	
Ampli-Taq DNA polymerase	5 U/µL	0.5	2.5 units
5' primer	50 µM	0.5	0.25 µM
(and 3' primer if used random hexamers)			

The PCR conditions were per cycle: 95 C for 1.5 minutes, 55 C for 1.5 minutes, and 72 C for 1.5 minutes, for a total of 40 cycles. The expected product (amplicon) size was 197 bp. The internal oligonucleotide probe for hybridization was: 5'-TAC TTT GGG TGT CCG TGT TTC. Hybridization was at 55 C

Hepatitis A virus RT-PCR

The primers for RT-PCR amplification of HAV were:

3' HAV primer: 5'-CTC CAG AAT CAT CTC CAA C

5' HAV primer: 5'-CAG CAC ATC AGA AAG GTG AG

(VP1-VP3 capsid protein interface region)

The RT-PVR reaction mixtures and reaction conditions were the same as for enteroviruses, and the expected product (amplicon) size was192 bp. The internal oligonucleotide probe for HAV was: 5'- TGC TCC TCT TTA TCA TGC TAT G. and the hybridization temperature was 55 C

Rotavirus RT-PCR

The primers for RT-PCR amplification of rotaviruses were those for Group A, gene 9:

3' rotavirus primer: 5'-GGT CAC ATC ATA CAA TTC T

5' rotavirus primer: 5'-GAT ATA ACA GCT GAT CCA ACA AC

The reaction mixtures and reaction conditions were the same as for enteroviruses, and the expected product (amplicon) size was 208 bp. The internal probe that could be used for product confirmation by hybridization was as follows: 5'-AAT TGG AAA AAA TGG TGG CAA GT. The hybridization temperature was 55 C.

Adenovirus and Astrovirus (RT-)PCR

Nested PCR was performed on UNH, UNC, UMN and TAMU samples for both astrovirus and adenovirus type 40 and 41. The equivalent volume of original water sample examined for each virus was 500 liters. Positive controls were at the level of (RT-)PCR. Virus was added to cell culture lysate to act as a positive control for (RT-PCR)PCR

Astrovirus. All molecular techniques were done as specified in the methods and materials developed by the project team in communication with the EPA project manager. Astrovirus RT-PCR was done according to Chapron et al. (2000). The primers used were specific for human astrovirus:

RT primer 5'-GTAAGATTCCCAGATTGGT-3', and
PCR primer 5'-CCTGCCCCGAGAACAACCAAG-3'.

An 11-µL sample of the combined (pooled) cell lysate was denatured with 0.5 µL each of 0.05 M EDTA and downstream primer at 99˚C for 8 min. Eighteen µL of the RT mixture was then added and run for 42 min. at 42˚C to reverse transcribe, followed by 5 min. at 99 C. The RT mixture per sample consisted of 2.5 µL 10X buffer II, 8.5 µL of 25mM $MgCl_2$, 1.25 µL of each 10mM dNTP, 0.5 µL of 100mM DTT (Promega), 10 units of Rnasin, and 50 units of RT.

After the RT step, 28.5 µL of a PCR master mix was added. The PCR mixture per sample consisted of: 3 µL of 10X buffer II, 1 µL of the PCR primer, 0.5 µL of the RT primer, 24 µL of

molecular grade water, and 2.5 units of Ampli-Taq DNA polymerase. The PCR amplification parameters were 95 C for 5 minute hot start, followed by 35 cycles of: 95 C for 30 seconds, 56˚C for 30 seconds, 72 C for 30 seconds, with a final extension at 72 C for 5 minutes. These primers yielded a 193 and/or 243 bp amplicon.

For nested PCR, 1 L from each RT-PCR reaction was added to a new tube containing 90 L of a nested PCR reaction mixture, which contained 8 mM $MgCl_2$, 10 µL 10x buffer, 1mM of each dNTP, 2.5 units of Ampli-Taq DNA polymerase and 1 µM of each primer. The primers used were: 5'-CCTTGCCCCGAGCCAGAA-3' and 5'-TTGTTGCCATAAGTTTGTGAATA-3'. These primers yield a 143 and/or 183-bp amplicon. Twelve l of each RT-PCR product as well as 12 µL of the nested PCR product was resolved and sized by electrophoresis on an 1.8% agarose gel, stained with ethidium bromide. Molecular weights were determined by comparison with a 1 Kb DNA ladder (Life Technologies). Astrovirus serotype 2 was used as a positive control.

Adenovirus. All molecular techniques were done as specified in the methods and materials developed by the project team and communicated to the EPA project manager. Adenovirus Hexon PCR was done generally according to the procedures of Xu et al. (2000).

The PCR primers used were:

Ad1 5'-CCCTGGTA(G/T)CC(A/G)AT(A/G)TTGTA-3' and

Ad2 5'-TTCCCCATGGC(Inosine)CA(C/T)AACAC-3'.

A 5μL sample of the combined cell lysates was added to 47.5 μL final volume PCR master mix. Final concentrations in the PCR master mix per sample were 1.5mM $MgCl_2$, 1x (10x Buffer II), 0.2mM dNTP mix, 0.6μM of each primer, and 2.5 units of Ampli-Taq DNA polymerase. The PCR parameters were 95 C for 5 minutes, followed by 40 cycles of: 94 C for 1 minute, 55 C for 1 minute, and 72 C for 2 minutes, with a final extension at 74 C for 5 minutes. These primers yielded a 482 bp amplicon.

For nested PCR, 1 l from each PCR reaction was added to a new tube containing 90 l of a nested PCR reaction mixture, which contained 8 mM $MgCl_2$, 10 μL 10x buffer, 1mM of each dNTP, and 1 μM of each primer. The primers used were:

5'-GCCACCGAGACGTACTTCAGCCTG-3' and

5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3.

These nested primers were specific for Adenovirus type 40 and 41. Samples were run for 35 cycles of: 95 C for 30 seconds, 55 C for 30 seconds, 72 C for 30 seconds, yielding a 142 bp amplicon. Twelve μL of each nested PCR product was resolved and sized by electrophoresis on 1.8% agarose gels and stained with ethidium bromide. Molecular weights were determined by comparison with a 1 Kb DNA ladder (Life Technologies). Adenovirus 40 and 41 were used as positive controls.

Reovirus RT-PCR

The primers for RT-PCR amplification of reoviruses and kindly provided by Shay Fout of the US EPA, Cincinnati, were:

3' pan-reovirus primer: 5'-GTG CTG AGA TTG TTT TGT CCC AT

5' pan-reovirus primer: 5'-ACG TTG TCG CAA TGG AGG TGT

Reaction mixtures for 5 µL samples were**:**

RT master mix	Stock conc.	reaction/reaction	Final conc.
$MgCl_2$	25 mM	1.8	1.5 mM
10X PCR Buffer II	10X	3	1X (Applied Biosystems)
dNTP mix	10 mM each	2	0.7 mM
3' primer	10 µM	5	1.7 µM
Water		11.45	

The initial RT-PCR reaction conditions were: 99 C for 5 minutes; then tubes were placed in ice.

Enzyme mix	Stock conc.	reaction/reaction	Final conc.
RNasin	30 units/µL	0.75	22 units (Promega N2511)
MuLV RT	50 units/µL	1	50 units (Applied Biosystems)

The RT reaction conditions were: 43 C for 60 minutes, 95 C for 5 minutes, and then tubes were placed in ice.

PCR master mix	Stock conc.	reaction/reaction	Final conc.
$MgCl_2$	25 mM	4.2	1.5 mM
10X PCR Buffer II	10X	7	1X
5' primer	10 μM	5	0.5 μM
AmpliTaq Gold		1	(pH 8.3 buffer only)

The PCR reaction conditions were per cycle: 95 C for 1 minute, 55 C for 1.5 minutes, and 72 C for 1.5 minutes, for a total of 40 cycles. The expected product (amplicon) size was125 bp. Internal oligonucleotide probes for the individual reovirus types 1, 2 and 3 were kindly provided by Shay Fout, and the hybridization temperature was 51 C.

Calicivirus direct RT-PCR

Calicivirus (Norovirus) RT-PCR analysis of concentrated virus samples from groundwater was done with the modified generic primers designated JV12/JV13 (Vinje et al., 2001; Hamidjaja et al. 2004).

3' RegA primer: 5'-CTC (A/G)TC ATC (Inosine)CC ATA (A/G)AA (Inosine)GA

5' MJV12 primer: 5'-TA(C/T) CA(C/T) TAT GAT GC(A/C/T) GA(C/T) TA

The RT-PCR reaction mixture for 5μl samples had the following composition:

Antisense mix	Stock conc.	reaction/reaction	Final conc.
RegA primer	50 μM	1.2	60 pM
Water		2.8	

The initial RT reaction conditions were as follows: 94 C for 2 minute and then chilling in ice.

RT master mix	Stock conc.	reaction/reaction	Final conc.
MgCl2	25 mM	1.8	3 mM
10X PCR Buffer II	10X	1.5	1X (pH 8.3)
dNTP mix	10 mM each	1.5	1 mM
Water		0.2	
AMV-RT	10 units/μL	0.5	5 units
Rnase inhibitor	40 units/μL	0.5	20 units

The subsequent RT reaction conditions were: 42 C for 60 minutes and then 94 C for 5 minutes, followed by chilling in ice.

PCR master mix	Stock conc.	reaction/reaction	Final conc.
MgCl2	25 mM	2.4	1.5 mM
10X PCR Buffer (pH 9.0)	10X	4.5	
dNTPs	10 mM each	0.5	0.2 mM
MJV12 primer	50 μM	1	50 pM
RegA primer	50 μM	0.6	50 pM
Taq polymerase	5 units/μL	0.5	2.5 units
Water		35.5	

The PCR reaction conditions were an initial 94 C for 3 minutes, followed by 40 cycles with each cycle consisting of: 94 C for 1 minute, 50 C for 1.5 minutes, and 74 C for 1 minute. This was followed by 74 C for 7 minutes. The expected product (amplicon) size was 327 bp.

The internal oligonucleotide probe for human caliciviruses was a mixture of one Group 1 probe (GGI) and three Group II probes as follows:

GGI probe: 5'-ATG GA(CT) GTT GG(CT) GA(C/T) TAT GT (20 pM)

GGIId probe: 5'-TGG AAC TCC ATC GCC CAC TGG (40 pM)

GGIIe probe: 5'-TGG AAC TCC ATC ACA CAT TGG (80 pM)

GGLeeds probe: 5'-TCA CCA GAT GTT GTC CAA GC

The hybridization temperature was 42 C.

MS-2 RT-PCR

Coliphage MS2 was used as the positive control for RT-PCR analyses by some laboratories to avoid introduction of potential human enteric virus contamination. About 100 pfu of MS2 per reaction tube was to be used and RT-PCR was done according to Meschke and Sobsey (1998).

The RT-PCR primers for MS2 were as follows:

3' (downstream) MS2 primer: 5'-CCC TAC AAC GAG CCT AAA TTC

5' (upstream) MS2 primer: 5'-GCA ACC TCC TCT CTG GCT AC

Random hexamers could be used as the primer MS2 reverse transcription. Reaction mixtures and reaction conditions for MS2 were the same as for enteroviruses. The expected PCR product size was 220 bp.

RT-PCR and PCR controls

To reduce the possibility of cross-contamination of field samples during nucleic acid amplification, RNA coliphage MS-2 was selected to act as a positive control for RT-PCR procedures. This positive control with its own pair of primers was included in each set of reactions done by some participating labs. Other labs already had their own RT-PCR and PCR controls and they used those existing QA/QC control procedures and reagents that were already in place. The details of those measures can be found in the reports of the other 3 participating laboratories, which are in the Appendix to this report.

The minimal negative control samples that were to be run as part of each set of RT-PCR and PCR samples included the following: (1) combined master mixes, enzymes and water done twice, one tube placed at the beginning of the set, and one tube placed at the end of the set, and (2) a cell culture negative control. Because the same pooled cell cultures were tested for multiple groups of human enteric viruses, cell culture negative control RNA extracts needed to be assayed using all of the appropriate virus primer pairs used by a given participating laboratory. RT-PCR for HAV and Caliciviruses was done by the UNC lab (Southeast), RT-PCR for enteroviruses was done by the University of Minnesota lab (Upper Midwest), RT-PCR for reovirus and rotavirus was done by the TAMU lab (Southwest), and (RT-)PCR for adenoviruses and astroviruses was done by the University of New Hampshire lab (Northeast). Further details of the virus (RT-)PCR

methods of the participating laboratories are given in their individual project reports, which appear in the Appendix of this report.

Confirmation of presumptive (RT-)PCR positive samples

Amplified PCR products from non-nested protocols were to be examined by agarose gel electrophoresis. If cDNA bands of the appropriate size were detected, the presence of enteric virus sequences was to be confirmed using a labeled oligonucleotide probe internal to the original amplicon, as specified above. If enteric virus cDNA was detected, it was to be preserved for possible nucleotide sequencing.

PHASE II RESULTS AND DISCUSSION

Introduction

The Phase II studies of the project consisted of both additional lab studies as well as field studies. Lab studies were performed to further characterize and improve the method to confirm coliphage isolates from plaques on SAL plates and lysis zones on spot plates from the enrichment method. Lab studies also were conducted to determine the survival of coliphages in groundwater held at 4oC for up to 6 days prior to coliphage assay by Methods 1601 and 1602. Field studies consisted of the analysis of groundwater samples from wells for F+, somatic and "total" coliphages (by Methods 1601 and 1602), fecal indicator bacteria *(E. coli* and enterococci), and human enteric viruses by each of the four regional labs. Each lab collected and analyzed groundwater samples in its region. The samples concentrated for recovery of human enteric viruses by each of the 4 labs were divided into aliquots so that individual aliquots could be sent to other participating labs for centralized analysis of one or two the different target groups of human enteric viruses. The data for coliphages and fecal indicator bacteria in groundwater were analyzed to determine if the analysis of both coliphages and fecal indicator together in the same sample of groundwater gave greater detection of fecally contaminated groundwater than the analysis of only one indicator, either a bacterium or a coliphage.

Results of Field Sample Analysis of Coliphage and Bacterial Indicators in Groundwater

Table 9 contains all of the data for the presence and concentrations coliphages and fecal indicator bacteria in samples of groundwater from all four laboratories.

Table 9. Coliphages Detected by Methods 1601 and 1602 and Indicator Bacteria in Groundwater

Lab* and Samples	SAL (#/100 mL)			Enrichment (1 L)			Bacteria/100 mL)		
	Famp	CN-13	C3000	Famp	CN-13	C3000	Fec. Colif.	*E. coli*	Ent.
TAMU-RS (1)	0	0	0	0	**1**	0	No Data	0	0
TAMU-HCR (1)	0	0	0	0	0	0	No Data	0	0
TAMU-RS (2)	0	0	0	0	0	0	No Data	0	0
TAMU-BM (1)	0	0	0	0	0	0	No Data	0	0
TAMU-KK (1)	0	0	0	0	0	0	No Data	0	0
TAMU-RS (3)	0	0	0	0	0	0	No Data	0	0
TAMU-KK (2)	0	0	0	0	**1**	0	No Data	0	0
TAMU-HCR (2)	0	**1**	0	0	**1**	**1**	No Data	0	**1**
TAMU-RS (4)	0	0	0	0	0	0	No Data	0	0
TAMU-RS(5)	0	0	0	0	0	0	No Data	**1**	0
TAMU-MHP1a	0	0	0	0	0	0	No Data	**1**	**5**
TAMU-MHP1b	0	0	0	0	0	0	No Data	0	0
TAMU-AVC1	0	0	0	0	**1**	0	No Data	0	0
TAMU-FVE1	0	0	0	0	0	0	No Data	0	0
TAMU-AVC2	0	0	0	0	0	0	No Data	0	0
TAMU-FVE2	0	0	0	0	0	0	No Data	0	0
TAMU-FVE3	0	0	0	0	0	0	No Data	0	0
TAMU-AVC3	0	0	0	**1**	**1**	**1**	No Data	0	0
TAMU-MHP1c	0	0	0	0	0	0	No Data	0	0
TAMU-MHP2a	0	0	0	0	0	0	No Data	0	0
TAMU-MHP2c	0	0	0	0	0	0	No Data	0	0
TAMU-MHP3a	0	0	0	0	0	0	No Data	0	0
TAMU-MHP2b	0	0	0	0	0	0	No Data	0	0
TAMU-SME1	0	0	0	0	0	0	No Data	0	0
TAMU-SME2	0	0	0	0	0	0	No Data	0	0
TAMU-LME1	0	0	0	0	0	0	No Data	0	0
TAMU-MHP3b	0	0	0	0	0	0	No Data	0	0
UNH-1	0	0	0	0	0	0	**1**	0	0
UNH-2	0	0	0	0	0	0	0	0	0
UNH-3	**4**	0	0	**1**	0	**1**	0	0	0
UNH-4	0	0	0	0	0	0	0	0	0
UNH-5	0	0	0	0	0	0	0	0	0
UNH-6	0	0	0	0	0	0	0	0	0
UNH-7	0	0	0	0	0	0	0	0	0
UNH-8	0	0	0	0	0	0	0	0	0
UNH-9	0	0	0	0	0	0	0	0	0
UNH-10	0	0	0	0	0	0	0	0	0
UNH-11	0	0	0	0	0	0	0	0	0
UNH-12	0	0	0	0	0	0	0	0	0
UNH-13	0	0	0	0	0	0	0	0	0
UNH-14	0	0	0	0	0	0	0	0	0
UNH-15	0	0	0	0	0	0	**35**	0	0
UNH-16	0	0	0	0	0	0	0	0	0
UNH-17	0	0	0	0	0	0	0	0	0
UNH-18	0	0	0	0	0	0	0	0	**1**
UNH-19	0	0	0	0	0	0	**2**	0	**13**

UNH-20	0	0	0	0	0	0	0	0	0
UNH-21	0	0	0	0	0	0	0	0	**2**
UNH-22	0	0	0	0	0	0	0	0	0
UNH-23	0	0	0	0	0	0	**5**	**0**	**2**
UNH-24	0	0	0	0	0	0	0	0	0
UNH-25	0	0	0	0	0	0	0	0	0
MN-01 Amu	0	**TNTC**	0	0	0	**No data**	0	0	0
MN-02 Ger	0	**4**	**5**	0	0	0	0	0	0
MN-03 Rou	**4**	**2**	**4**	0	0	0	0	0	0
MN-04 Tur	**2**	**2**	**3**	0	**1**	**1**	0	0	0
MN-05 Bro	0	0	0	0	0	0	0	0	0
MN-06 OG	0	0	0	0	0	0	0	0	0
MN-07 KM	**1**	**58**	**4**	0	0	0	0	0	0
MN-08 KM	**40**	**12**	**7**	**1**	0	**1**	0	0	**1**
MN-09 Ham	0	0	0	0	0	0	0	0	0
MN-10 Nor	**9**	**28**	0	0	0	0	0	0	0
MN-11 Pre	0	0	0	0	0	0	0	0	0
MN-12 Imm	0	0	0	0	0	0	**30**	0	**1**
MN-13 Cen	0	0	0	0	0	0	0	0	0
MN-14 His	0	0	0	0	0	0	**1**	0	0
MN-15 Nor	0	0	0	0	0	0	0	0	0
MN-16 Lak 1	**234**	**574**	0	**1**	0	0	**1**	0	0
MN-17 Lak M	0	0	0	0	0	0	0	0	0
MN-18 Lak M	0	0	0	0	0	0	0	0	0
MN-19 Al	0	0	0	0	0	0	0	0	0
MN-20 Day	0	0	0	0	0	0	0	0	0
MN-21 Lak M	0	**9**	**1**	0	0	0	0	0	0
MN-22 GF	0	**2**	**2**	0	0	0	0	0	**1**
MN-23 TA	**3**	**2**	**7**	0	**1**	**1**	**1**	0	0
MN-24 Mil	**11**	**6**	**1**	0	0	0	**17**	**15**	**12**
MN-25 Jay	0	**6**	**3**	0	0	0	**3**	**1**	**1**
MN-12 ChR	**3**	**3**	**4**	0	0	0	0	0	0
MN-16 Cem R	**6**	**5**	**6**	0	0	0	0	0	0
MN-24 Mil R	**2**	**5**	0	0	0	0	**248**	**3**	**20**
UNC-1-BMH	0	0	0	0	0	0	0	0	0
UNC-2-GL	0	0	0	0	0	0	0	0	0
UNC-3-VE	0	0	**0.4**	0	0	0	0	0	0
UNC-4-KC	0	0	**0.4**	0	**1**	0	0	0	**0.5**
UNC-5-OC-FL#1	0	0	0	0	0	0	0	0	0
UNC-6-OC-FL#2	0	0	0	0	0	0	0	0	**1.5**
UNC-7-KC	0	**0.4**	0	0	0	0	0	0	0
UNC-8-VE	0	**0.4**	0	0	0	0	0	0	0
UNC-9-BF	0	0	0	0	0	0	0	0	0
UNC-10_SB MHP	0	0	0	0	0	0	0	0	0
UNC-11-BMH1	0	0	0	0	0	0	0	0	0
UNC-12_GL	0	0	0	0	0	0	0	0	0
UNC-13_OC-FL#1	0	0	0	0	0	0	0	0	0
UNC-14_OC-FL #2	0	0	0	0	0	0	0	0	0
UNC-15_GL	0	0	0	0	0	0	0	0	0
UNC-16-CDL	0	0	0	0	0	0	0	0	0

UNC-17-BF	0	0	0	0	0	0	0	0	0
UNC-18-SB MHP	0	0	0	0	0	0	0	0	0
UNC-19-CDL	0	0	0	0	0	0	0	0	0
UNC-20-CSF #1	0	0	0	0	0	0	0	0	0
UNC-21-CSF #2	0	0	0	0	0	0	0	0	0
UNC-22-Oca FL#1	0	0	0	0	0	0	0	0	0
UNC-23-Oca FL#2	**0.4**	0	0	0	0	0	0	0	0
UNC-24-CSF #1	0	0	0	0	0	0	0	0	0
UNC-25-CSF #2	0	0	0	0	0	0	0	0	0
UNC-26-Oca FL #1	0	0	0	0	0	0	0	0	0
UNC-27-Oca FL #2	0	0	0	0	0	0	0	0	0

*TAMU = Texas Agricultural and Mechanical University, UNH = University of New Hampshire,

MN = University of Minnesota and UNC = University of North Carolina

Table 10 summarizes these data on the presence of fecal indicator microbes, including somatic coliphages, male-specific coliphages, "total" coliphages (detected on host *E. coli* C3000), fecal coliform bacteria, *E. coli* and enterococci in groundwater samples in this study on the basis of positive samples, regardless of microbe concentration. A total of 107 samples were analyzed and these samples correspond to the samples that were also analyzed for human enteric viruses. Additional groundwater samples were analyzed by some laboratories in the initial screening of groundwater wells for possible inclusion in the study. However, these samples are not included in the table because not all microbial indicators were measured by all methods during this pre-screening analysis effort and there was no concurrent analysis of human enteric viruses for possible comparison.

Table 10. Frequency of Occurrence of Fecal Indicator Microbes in Field Ground Water Samples

Indicator	#. Pos./# Tested at:				Total # Pos./ Total # Tested, All Labs	% Positive
	TAMU	UMN	UNC	UNH		
Somatic Coliphage - SAL	1/27	16/28	2/27	0/25	19/116	16.4%
F+ Coliphage by SAL	0/27	11/28	1/27	1/25	13/116	11.2%
"Total" Coliphage" - SAL	0/27	12/28	2/27	0/25	14/116	12%
Somatic Coliphage Enrichment	5/27	2/28	1/27	0 /25	8/116	6.9%
F+ Coliphage Enrichment	1/27	2/28	0/27	1/25	4/116	3.4%
"Total" Coliphage Enrichment	2/27	3/28	0/27	1/25	6/116	5.2%
Fecal Coliform	Not done	7/28	0/27	4/25	11/80	13.8%
E.coli	2/7	3/28	0/27	0/25	5/116	4.3%
Fecal Coliform and/or *E. coli*	2/27	7/28	0/27	4/25	13/116	11.2%
Enterococci	2/27	6/28	2/27	4/25	14/116	12.1%

As shown in Table 10, The frequency of detection of any single fecal indicator microbe was highest for somatic coliphages as measured by the SAL method at 16.4% and second highest for fecal coliform at 13.8%. However, the frequency of detection of somatic coliphage by the SAL method and of fecal coliform was not significantly different (P = 0.768 by Mann-Whitney U-test). Interpretation of these statistical results for comparative detection of somatic coliphages by SAL and fecal coliforms is limited. This is because not all samples were analyzed for both of these fecal indicators and therefore a paired statistical analysis of the results was not possible.

Enterococci and "total coliphage" by the SAL method were tied for third in detection frequency at 12.1%. Overall, these results indicate the rate of detection of any single fecal indicator was higher for coliphages, specifically somatic coliphage detected by SAL, than any other single indicator tested. It is also noteworthy that the simultaneous detection of both somatic and male-specific coliphages as "total coliphages" by the SAL method on a single host bacterium, *E. coli*

C3000, gave a high frequency of detecting fecal contamination at 12.1%, making it one of the best indicators tested.

Examination of the results of coliphage analyses in Table 10 indicate that each coliphage group was detected more frequently by the single agar layer (SAL) method than by the two-step enrichment spot plate method. This finding is striking given that the sample volume for the SAL method was only 100 mL and for the enrichment method it was 1 liter. The comparative detection of coliphages by SAL and enrichment methods was 16.4% versus 6.9% for somatic coliphage, 11.2% versus 3.4% for F+ coliphages and 12.1% versus 5.2% for "total" coliphages. The results for the frequency of detection of the different coliphage groups by the SAL or enrichment method were statistically compared by a non-parametric, paired t-test (Wilcoxon matched-pairs signed-ranks test). The detections frequencies between SAL and enrichment methods were significantly different for somatic coliphages ($P = 0.137$) and for F+ coliphages ($P = 0.0351$) and they were nearly significant for "total" coliphages ($P = 0.580$). Overall, these results indicate that the SAL method gave significantly better detection coliphages than did the enrichment method. The reasons for this are not known and probably deserve further investigation. It should be remembered that both methods were highly efficient in detecting coliphages when tested in phase I studies on seeded samples of groundwater.

Comparative Detection of Two Indicators in Groundwater Samples

It was of interest to consider the simultaneous detection of two indicators in groundwater samples. This is because the proposed groundwater rule has considered the possibility of measuring only one indicator in a sample (either bacterium or a coliphage indicator) versus

measuring both a bacterial and a coliphage indicator. Therefore, a fundamental consideration is whether or not the dual measurement of two indicators improves the detection of fecal contamination in groundwater samples by increasing the frequency of fecal indicator (a positive sample). The results for selected pairs of fecal indicators that gave the highest detection frequencies are summarize in Table 11.

Table 11. Frequency of Occurrence of Dual Indicators in Field Ground Water Samples

Indicator Pair	#. Pos./# Tested at:				Total # Pos./ Total # Tested, All Labs	% Positive
Somatic and/or F+ Coliphage - SAL	1/27	16/28	3/27	1/25	21/116	18.1%
Somatic and/or F+ Coliphage - Enrichment	5/27	4/28	1/27	1/25	11/116	9.5%
Enterococci and Fecal Coliform and/or *E. coli*	3/27	9/28	2/27	6/25	20/116	17.2%
Somatic Coliphage - SAL and/or Enterococci	2/27	16/28	2/27	4/25	24/116	20.7%
Somatic Coliphage - SAL and/or Fecal Coliform or *E. coli*	3/27	17/28	2/27	4/25	26/116	22.4%
Fecal Coliform and/or *E. coli*	2/27	7/28	0/27	4/25	13/116	11.2%
Enterococci	2/27	6/28	2/27	4/25	14/116	12.1%
Enterococci and Fecal Coliform and/or *E. coli*	3/27	9/28	2/27	6/25	20/116	17.2%
Somatic Coliphage - SAL and/or Enterococci	2/27	16/28	2/27	4/25	24/116	20.7%
Somatic Coliphage - SAL and/or Fecal Coliform or *E. coli*	3/27	17/28	2/27	4/25	26/116	22.4%

Dual versus individual detection of somatic and F+ coliphages by SAL. As a first case of comparing the detection of positive samples with pairs of indicators versus single indicators is the SAL detection of somatic and/or male-specific coliphages in groundwater samples. This coliphage indicator pair was considered because the measurement of both of these two groups of coliphages in an option in the proposed groundwater rule. Currently, there is no clear basis for choosing one coliphage group over the other and therefore, the measurement of both coliphage groups in a sample on their respective *E. coli* hosts is an option. The frequency of detecting a positive sample (positive for one or the other or both) was 18.1% (Table 11). For each group

alone, the SAL detection frequency was 16.4% for somatic coliphages and 11.2% for F+ coliphages (Table 10). The SAL detection of either or both of these coliphage indicators in a sample by dual analysis (18.1%) was compared to the frequency of detection of each of them alone (F+ SAL = 11.2% and somatic SAL = 16.4%) using the Friedman test, a non-parametric Analysis of Variance (ANOVA). The P value was very significant (0.0055), indicating the detection frequencies were significantly different and were highest for the detection of either or both coliphage groups when both are measured in a sample.

When the individual SAL detection frequencies of F+ coliphages (11.2%) and somatic coliphages (16.4%) were compared by a non-parametric t-test (Wilcoxon matched-pairs signed-ranks test), there was no significant difference (P= 0.105), indicating equivalent detection of either of these two coliphage groups alone. Furthermore, when the SAL detection frequencies of F+ coliphages alone (11%) were compared to the dual detection of either F+ coliphages and/or somatic coliphages (18.1%) by the Wilcoxon matched-pairs signed-ranks test, the difference was very significant (P = 0.0078). This indicates that SAL detection of both coliphage groups is better than detecting F+ coliphages alone. A similar comparison for SAL detection of somatic coliphages alone (16%) versus the SAL detection of either or both coliphages when both are measured in a sample (18.1%) indicated no significant difference because the sample size was too small. Overall, SAL detection of both groups of coliphages (F+ and somatic) is better than SAL detection of either group alone.

Dual versus individual detection of bacterial indicator pairs. When the detection of two fecal indicator bacteria such as fecal coliforms and/or *E. coli* versus enterococci is considered because both groups are measured simultaneously, the frequency of detecting a positive sample (positive for one or the other or both) was also high at 17.2% (Table 11). For each group alone, the detection frequency was 12.1% for enterococci and 11.2% for fecal coliforms and/or *E. coli* (Table 10). The dual detection of either or both of these bacterial indicator groups in a sample (18.1%) was compared to the frequency of detection of each of them alone (enterococci = 12.1% and fecal coliforms and/or *E. coli* = 11.2%) using the Friedman test. The P value was significant (0.0366), indicating the detection frequencies were significantly different and were highest for the detection of either or both indicator bacteria groups when both are measured in a sample. When the individual detection frequencies of enterococci (12.1%) and fecal coliforms and/or E. coli (11.2%) were compared by the non-parametric Wilcoxon matched-pairs signed-ranks test, there was no significant difference (P= 0.839), indicating equivalent detection of either of these bacterial indicator groups alone. Furthermore, when the detection frequencies of enterococcus alone (12.1%) was compared to the dual detection of either or both enterococcus and/or fecal coliforms and/or *E. coli* (17.2%) by the Wilcoxon matched-pairs signed-ranks test, the difference was significant (P = 0.031). This indicates that dual detection of both bacterial indicator groups is better than detecting enterococci alone. A similar comparison for detection of fecal coliforms and/or *E. coli* alone (11.2%) versus the dual detection of either or both enterococcus and/or fecal coliforms and/or *E. coli* (17.2%) by the Wilcoxon matched-pairs signed-ranks test also was significant (P = 0.016). This indicates that detection of both bacterial indicator groups is better than detecting fecal coliforms and/or *E. coli* alone. Overall, detection of both groups of bacteria is better than detection of either group of bacteria alone.

Dual versus individual detection of bacterial and coliphage indicator pairs. The extent to which dual detection of a coliphage indicator and bacterial indicator versus individual detection of either one alone was considered. This is because the detection of both a coliphage indicator and a bacterial indicator in a groundwater sample has been an option for the proposed groundwater rule. The first pair of coliphage and bacteria indicators to compare was somatic coliphages detected by the SAL method (16.4%) and enterococci (12.1%) (Table 10). This pair was chosen because these were the individual coliphage and bacterial indicators measured in all samples and detected most frequently. For SAL somatic coliphages and/or enterococcus being measured together in samples, the frequency of detecting a positive sample (positive for one or the other or both) was 20.7% (Table 11), which was higher than either indicator alone or any coliphage pair or bacterial pair. Furthermore, a statistical comparison of measuring either indicator alone or both indicators together showed significant improvement in detecting fecal contamination of groundwater. The detection of either or both of these indicator groups in a sample (20.7%) was statistically compared to the frequency of detection of each of them alone (enterococci = 12.1% and SAL somatic coliphages = 16.4%) using the Friedman test. The P value was significant (0.028), indicating detection frequencies were significantly different and were highest for the detection of either or both indicator groups (enterococci and/or SAL somatic coliphages) when both are measured in a sample. When the individual detection frequencies of enterococci (12.1%) and SAL somatic coliphages (16.4%) were compared by the non-parametric Wilcoxon matched-pairs signed-ranks test, there was no significant difference (P= 0.355), indicating equivalent detection of either of these indicator groups alone. Furthermore, when the detection frequencies of enterococcus alone (12.1%) were compared to the detection of either or both enterococcus and/or SAL somatic coliphages (20.7%) by the Wilcoxon matched-pairs

signed-ranks test, the difference was very significant (P = 0.002). This indicates that detection of both indicator groups is better than detecting enterococci alone. A similar comparison for detection of SAL somatic coliphages alone (16.4%) versus the detection of either or both enterococcus and/or SAL somatic coliphages (20.7%) by the Wilcoxon matched-pairs signed-ranks test was not significantly different (P = 0.206). Overall, the detection of both groups of indicators (SAL somatic coliphages and enterococci) was generally better than detection of either indicator group alone.

When the coliphage with the highest detection frequency, which was SAL somatic coliphages (16.4%), and the bacterial indicator with the highest detection frequency (and measured in any project samples), which was fecal coliforms and/or E. coli (11.2%), were being measured together in samples, the frequency of detecting a positive sample (positive for one or the other or both) was 22.4%% (Table 11). This indicator detection frequency was even higher than either indicator alone or any coliphage pair or any bacterial pair. Furthermore, a statistical comparison of measuring either indicator alone or both indicators together showed significant improvement in detecting fecal contamination of groundwater. The detection of either or both of these indicator groups in a sample (SAL somatic coliphages and/or fecal coliforms and/or *E. coli* = 22.4%) was statistically compared to the frequency of detection of each of them alone (SAL somatic coliphages = 16.4% and fecal coliforms and/or *E. coli* = 11.2%) using the Friedman test). The P value was very significant (0.003), indicating the detection frequencies were significantly different. When the individual detection frequencies of fecal coliforms and/or *E. coli* (11.2%) and SAL somatic coliphages (16.4%) were compared by the non-parametric Wilcoxon matched-pairs signed-ranks test, there was no significant difference (P= 0.276),

indicating equivalent detection of either of these indicator groups alone. Furthermore, when the detection frequency of SAL somatic coliphages alone (16.4%) was compared to the combined detection of either or both fecal coliforms and/or *E. coli* and/or SAL somatic coliphages (22.4%) by the Wilcoxon matched-pairs signed-ranks test, the difference was significant (P = 0.039). This indicates that detection of both indicator groups is better than detecting SAL somatic coliphages alone. A similar comparison for detection of fecal coliforms and/or *E. coli* alone (11.2%) versus the combined detection of either or both fecal coliforms and/or E. coli and/or SAL somatic coliphages (22.4%) by the Wilcoxon matched-pairs signed-ranks test was extremely significantly different (P = 0.0002). Therefore, the dual detection of both a coliphage indicator and a bacteria indicator in groundwater samples is better than detecting either indicator group alone.

Because there is interest in using a single coliphage host to simultaneously detect both somatic and male-specific (or "total") coliphages, it also was of interest to examine the dual detection of total coliphages and a bacterial indicator. When "total" coliphages detected by the SAL method (detection frequency = 12.1%) and the bacterial indicator of enterococci (detection frequency = 12.1%) are considered together, the frequency of detecting a positive sample (for either or both indicators) was 19.8% (Table 11). A statistical comparison of measuring either indicator alone or both indicators together showed significant improvement in detecting fecal contamination of groundwater. The detection of either or both of these indicator groups in a sample (19.8%) was statistically compared to the frequency of detection of each of them alone (SAL total coliphages = 12.1% and enterococci = 12.1%) using the Friedman test. The P value was significant (0.011), indicating the detection frequencies were significantly different. When the individual detection

frequencies of enterococci (12.1%) and SAL total coliphages (12.1%) were compared by a non-parametric t-test (Wilcoxon matched-pairs signed-ranks test), there was no significant difference (P>0.999), indicating equivalent detection of either of these indicator groups alone. Furthermore, when the detection frequencies of SAL total coliphages alone (12.1%) was compared to the dual detection of either or both SAL total coliphages and enterococci (19.8%) by the Wilcoxon matched-pairs signed-ranks test, the difference was very significant (P = 0.0039). This indicates that detection of both indicator groups is better than detecting SAL total coliphages alone. A similar comparison for detection of enterococci alone (12.1%) versus the dual detection of either or both enterococcus and/or SAL total coliphages (19.8%) by the Wilcoxon matched-pairs signed-ranks test the difference also was very significant (P = 0.0039). Therefore, dual detection of both a coliphage indicator ("total" coliphages by SAL) and a bacteria indicator (enterococci) in groundwater samples is better than detecting either indicator group alone. This is the case even for the single coliphage indicator capable of detecting both male-specific and somatic coliphages ("total" coliphages).

Detection of "total" coliphages by SAL (detection frequency = 12.1%) and the bacterial indicator of fecal coliforms and/or *E. coli* (detection frequency = 11.2%) also were considered together, and the frequency of detecting a positive sample (for either or both indicators) was 20.7% (Table 11). A statistical comparison of measuring either indicator alone or both indicators together showed significant improvement in detecting fecal contamination of groundwater. The detection of either or both of these indicator groups in a sample (20.7%) was statistically compared to the frequency of detection of each of them alone (SAL total coliphages = 12.1% and fecal coliforms/*E. coli* = 11.2%) using the Friedman test. The P value was very significant (0.005),

indicating the detection frequencies were significantly different and were highest for the detection of either or both indicator groups (fecal coliforms/*E. coli* and SAL total coliphages) when are both measured in a sample. When the individual detection frequencies of the bacterial indicator (fecal coliforms/*E. coli* =12.1%) and SAL total coliphages (11.2%) were compared by the non-parametric Wilcoxon matched-pairs signed-ranks test, there was no significant difference (P = 0.865), indicating equivalent detection of either of these indicator groups alone. Furthermore, when the detection frequencies of SAL total coliphages alone (12.1%) was compared to the detection of either or both SAL total coliphages and/or fecal coliforms/*E. coli* (20.7%) by the Wilcoxon matched-pairs signed-ranks test, the difference was very significant (P = 0.002). This indicates that detection of both indicator groups is better than detecting SAL total coliphages alone. A similar comparison for detection of fecal coliforms/*E. coli* alone (11.2%) versus the dual detection of either or both fecal coliforms/*E. coli* and/or SAL total coliphages (20.7%) by the Wilcoxon matched-pairs signed-ranks test also was extremely significant (P = 0.001). Therefore, these results again show that detection of both a coliphage indicator ("total" coliphages) and a bacterial indicator (fecal coliforms and/or *E. coli*) in groundwater samples is better than detecting either indicator group alone.

It is noteworthy that the frequency of detecting fecal contamination in a groundwater sample was nearly as high with "total" coliphages detected by SAL and a bacteria indicator (with either enterococci or fecal coliforms/*E. coli* at 19.8 and 20.7%, respectively) as with somatic coliphages detected by SAL with a bacteria indicator (with either enterococci or fecal coliforms/*E. coli*; 20.7% and 22.4%, respectively). Overall, these results indicate that the measurement of both a coliphage indicator and a bacterial indicator together in a groundwater sample gives a higher

frequency or likelihood of detecting fecal contamination than measuring any single indicator alone or even measuring pairs of bacterial indicators or pairs of coliphage indicators.

Statistical Comparisons of Fecal Indicators in Groundwater Samples

Additional statistical analyses of data on indicator occurrence in groundwater samples were performed for the data set of all groundwater samples for which human enteric viral analysis was done. Initially, statistical analysis was done on samples for which there were results for all of the fecal indicators tested by all methods. Because fecal coliforms were not analyzed by one of the 4 participating labs, they were excluded from some analyses that required complete sample and indicator pairing or matching (no missing data for any indicator in any sample). An analysis of all indicator data was done by a repeated measures ANOVA (Friedman Test), which assumes that the data in each row (which represents a water sample) is matched (a reasonable assumption because it is for a specific sample). The analysis gave a P-value of <0.0001, which is highly significant, and therefore, indicates that variation among column medians (for microbial indicators and coliphage methods) is significantly greater than expected by chance. Hence, the different indicators and coliphage methods gave significantly different results in detecting fecal contamination. Interestingly all post-tests for significant differences between each possible combination of indicator pair were not significant, with all P-values >0.05.

The data for all indicators, including fecal coliforms, also were analyzed by the Kruskal-Wallis Test (Nonparametric one-way ANOVA), which assumes no matching and does not require a complete matrix, thereby allowing for missing data for some samples. This analysis can be justified on the basis of simply considering an unknown distribution of microbial indicators of

fecal contamination in groundwater with unknown variability over time and space. The P value was significant at 0.0086, indicating that variation among column medians was significantly greater than expected by chance. This analysis again shows that different indicators and coliphage methods gave significantly different results in detecting fecal contamination.

These indicator data for groundwater samples were re-analyzed based on sample positivity instead of microbe concentrations by dichotomizing the data as positive and negative samples and assigning a value of 1 to a positive sample and keeping 0 for a negative sample. This analysis was again done by a repeated measures ANOVA (Friedman Test), which assumes that the data in each row (which represents a water sample) is matched (a reasonable assumption because it is results for the same specific sample). The analysis gave a P-value of <0.0001, which is highly significant, and therefore, indicates variation among column medians (for microbial indicators and coliphage methods) is significantly greater than expected by chance. Hence, the different indicators and methods gave significantly different results in detecting fecal contamination. Interestingly all post-tests for significant differences between each possible combination of indicator pair were not significant, with all P-values >0.05. When these data were re-analyzed by the Kruskal-Wallis Test (Nonparametric one-way ANOVA), which assumes no matching and does not require a complete matrix, thereby allowing for missing data in some samples, the P value also was significant at 0.0086. This result indicates that variation among column medians (for the different fecal indicators and coliphage methods) was significantly greater than expected by chance.

These indicator data for sample positivity in groundwater samples also were re-analyzed by a non-parametric repeated measures Analysis of Variance (Friedman Test) by excluding the fecal coliform data and including only those samples that were analyzed for human enteric viruses. The P value for this test was 0.0001, considered extremely significant, and indicating that variation among column means (for the different fecal indicators and coliphage methods) was significantly greater than expected by chance. As a post-test to the Friedman Test, the Tukey-Kramer multiple comparisons test was used to determine if there were significant differences in sample positivity between pairs of indicators. Significant differences in sample positivity were observed for the indicator pairs of: SAL somatic versus Enrichment F+ ($P<0.01$), SAL somatic versus Enrichment "total" coliphage (*E. coli* C3000) ($P<0.05$), and SAL somatic versus *E. coli* ($P<0.01$). None of the other indicator pairs were significantly different ($P>0.05$).

Analysis for Enteric Viruses in Groundwater

A total 106 samples of groundwater were analyzed for enteric viruses and no enteric viruses were detected in any of the samples analyzed. All but three (3) of the groundwater samples were 1500 liters in volume. There were 3 well samples from the Northeast for which the sample volume was less than 1500 liters due to a lack of water in the well or to clogging of the filter used to concentrate viruses from water. These Northeast wells were: Well #2 = 592 L, Well #4 = 229 L, and Well #14 = 400 L.

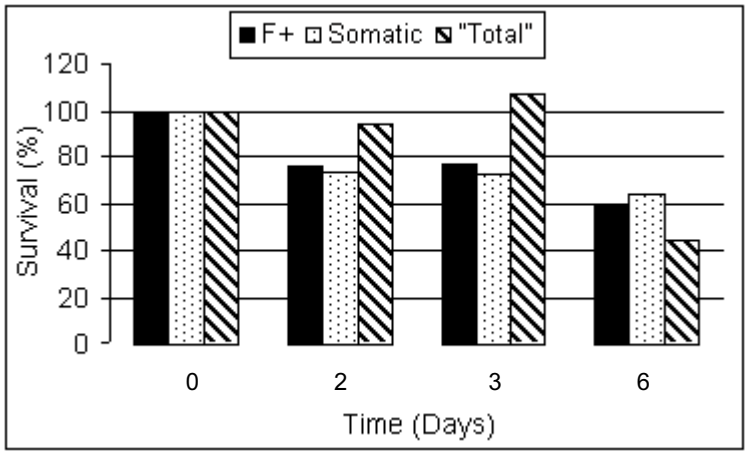
None of the 106 groundwater samples collected and analyzed were positive for human enteric viruses by cell culture and (RT-)PCR for adenoviruses, astroviruses, enteroviruses, reoviruses, rotaviruses or hepatitis A virus or, in the case of caliciviruses, by direct RT-PCR analysis.

Presumptive positive results observed in one participating lab for detachment of cells from flasks of all samples were due to non-viral effects such as the action of the trypsin in the medium or to cytotoxicity from the sample concentrate inocula. Trypsin can cause the cells to dislodge from the surface of the flask and appear abnormal. Trypsin effects are the most likely explanation because cell detachment was also observed in the negative control cultures. Regardless of the cause of this effect, it was not due to the presence of any enteric viruses, based on the negative results from sample analysis by nucleic acid amplification methods. Therefore, despite well developed protocols and the analysis of large sample volumes for a range of human enteric viruses, none were found in any of the samples analyzed. It is also noteworthy that no laboratory experienced any episode of false positive viral contamination in negative control samples or other types of virus-free control samples. Hence, no viral contamination occurred that could have compromised the interpretation of positive results had there been any virus-positive field samples.

Survival of Coliphage in Seeded Groundwater

Coliphage survival in groundwater after storage at 4^{o}C for times periods of 0-6 days was determined for samples seeded with sufficient coliphages (as filtered raw sewage) to give about 30-70 PFU per 100 mL when assayed by the SAL method. Duplicate experiments were done and the average results of these experiments are summarized in Figure 5 below.

Figure 5. Survival of F+, Somatic and "Total" Coliphages in Seeded 100-mL Samples of Groundwater Held at 4°C and Assayed by the SAL Method on Days 0, 2, 3 and 6.



As shown in Figure 5, F+, somatic and "total" coliphages survived relatively well for 2 or 3 days, with average survivals of >70% compared to day zero when detected by the SAL method (Method 1602). By day 6, SAL coliphage titers were somewhat lower, with average survivals of about 40-60% compared to day zero. Overall, these results indicate that samples of groundwater for coliphage analysis by SAL can be held for periods of 2 or 3 days with only relatively minor losses in coliphage titer (<30%) and with high probabilities of detecting coliphages that were initially present when the samples were collected. These coliphage survival data were subjected to statistical analyses by both parametric and non-parametric analysis of variance (ANOVA). The coliphage titers were not significantly different at the 5% level ($p > 0.05$) among the days of analysis (days 0, 2, 3 and 6). These results indicate that coliphage titers in groundwater as measured by SAL did not significantly decrease over the 6-day holding period.

Coliphage survival in groundwater after storage at 4°C for times periods of 0-6 days was also determined for groundwater samples seeded with sufficient coliphages (as filtered raw sewage) to give about 5 infectious units per sample bottle mL of 1000 mL when assayed by the enrichment method (Method 1601). As shown in Table 12, F+, somatic and "total" coliphages survived relatively well for as long as 6 days, with the number of positive enrichment bottles out of 10 remaining at high levels of 7 to 10.

Table 12. Survival of Coliphages in Seeded Groundwater Held at 4°C and Assayed by the Two-Step Enrichment Spot-plate Method

Time (Days)	No. of Positive Enrichment Bottles of 10 for Indicated Coliphage Group		
	F+ Coliphages	Somatic Coliphages	"Total" Coliphages
0	8	10	10
2	10	8	10
3	9	8	10
6	9	8	7

Overall, these results indicate that samples of groundwater for coliphage analysis by the enrichment method can be held for periods of as long as 6 days with no appreciable loss losses in coliphage titer and with high probabilities of detecting coliphages that were initially present when the samples were collected. These coliphage survival data were subjected to statistical analyses by both parametric and non-parametric analysis of variance (ANOVA). The coliphage titers were not significantly different at the 5% level ($p > 0.05$) among the days of analysis (days 0, 2, 3 and 6). These results indicate that low coliphage titers in groundwater (about 5 infectious

units per liter) as measured by enrichment did not significantly decrease over the 6-day holding period.

Comparison of Coliphage, Bacterial Indicator and Enteric Virus Detection in This Study and in Previous Studies in the USA

Only a few previous studies have examined coliphages and bacteria in groundwater of the USA. In one study by Abbaszadegan et al. (1999), coliphages were analyzed in the equivalent of about 15-liter samples of water using eluates from adsorbent filters used to concentrate enteric viruses from groundwater samples. Coliphages were assayed on the following host bacteria: *E. coli* C for somatic coliphages (this host is similar t E. coli CN-13), Salmonella WG49 for F+ coliphages and E. coli C3000 for both somatic and male-specific ("total" coliphages). Of the 444 samples analyzed the percentages of positive samples were: 4.1% on *E. coli* C, 10.8% on *E. coli* C3000 and 9.5% on Salmonella WG49. The rates of coliphage positivity in this previous study are lower than the rates of positivity in this current study. In the current study 16.4% of samples were positive for somatic coliphages detected in 100-mL sample volumes by the SAL method on *E. coli* CN-13 (Method 1602) compared to 4.1% positive for somatic coliphages detected on *E. coli* C. In the current study 11.2% of samples were coliphage positive for F+ coliphages on host *E. coli* Famp by SAL compared to 9.5% positive for F+ coliphages on Salmonella WG49. In the current study 12.1% of samples were positive for "total" coliphages on E. coli C3000 compared to 10.8% positive on this host in previous studies. The percent of samples positive for any of the three coliphage hosts was 20.7% and for all three hosts together it was 0.2%. In the current study the percent of samples positive for the coliphage host pair of *E. coli* CN-13 (somatic coliphages) and *E. coli* Famp (F+ coliphages) was 20.7%. Thus the rate of positivity of two hosts in the

current study was the same as the rate of positivity for 3 hosts in this previous study. Furthermore, these results for the current study employed 100-mL samples assayed by SAL compared to 15-liter samples assayed as filter eluate concentrates by the double agar layer plaque assay in sample concentrate volumes of 2.5 or 5 mL per plate. The recovery efficiency and lower detection limit of the coliphage assay method used in the previous study was not reported.

In the same study by Abbaszadegan et al. (1999), culturable human enteric viruses were analyzed in the equivalent of 160-gallon (605-liter) samples of water by CPE in BGM cell cultures. In 442 samples, 4.8% of sample sites and 4.1% of total samples were positive for culturable viruses by CPE. In comparison, no culturable human enteric viruses were detected in any of the 106 groundwater samples, each of 1500-liter (400-gallon) volume, analyzed in the current study.

In a later study Karim et al. (2004) sampled 20 groundwater wells monthly for 12 months from 11 states for coliphages, bacterial indicators and human enteric viruses. Sixteen of the wells were known to be fecally contaminated. Wells were monitored for the presence of culturable viruses, enteric virus nucleic acid (enterovirus, hepatitis A, norwalk virus, rotavirus, and adenovirus) by (RT-)PCR, coliphages using USEPA Methods 1601 and 1602, double agar layer method (DAL), and RT-PCR, and indicator bacteria (total coliforms, *E. coli*, enterococci, and *Clostridium perfringens* spores). A total of 231 to 235 samples were analyzed per well. The percentages of (RT-)PCR-positive samples for enteric viruses were: 2.1% for enteroviruses, 0% for HAV, 5.6% for rotavirus, 4.3% for Norwalk Virus, and 0.4% for adenovirus. For culturable viruses by CPE, positivity was 3.9%. As previously indicated, no human enteric viruses were detected in this current study.

For coliphage indicators detected by the enrichment method (1601) the percentage of positive samples was 0% for somatic coliphages in 100 mL and 1000 mL volumes, and 0.4 and 2.2% for F+ coliphages in 100 mL and 1000 mL, respectively. These are lower rates of sample positivity than were obtained in this current study, which were 6.9% for somatic and 3.4% for F+ coliphages in 1000 mL volumes. For coliphage indicators detected by the SAL method (1602) in 100-mL sample volumes, the percentage of positive samples was 0.9% for somatic coliphages and 5.6% for F+ coliphages. These also are lower rates of positivity than in this current study, which were 16.4% and 11.2% for somatic and F+ coliphages, respectively. For enterococcus, the percentage of positive samples was 0.4 and 5.5% for 100 mL and 1000 mL volumes respectively. In this current study enterococcus positivity in 100- mL samples was much higher at 12.1%. For *E. coli*, the percentage of positive samples was 4.3% and 11.1% for 100 mL and 1000 mL samples, respectively. In this current study, the frequency of *E. coli*-positive 100-mL samples was 4.3%, which is the same *E. coli*-positivity the as the in previous study.

The results of the previous study suggested that dual monitoring for both a bacterial indicator and coliphage would be useful for detecting fecal contamination of groundwater. As in this current study, monitoring coliphages and bacteria together detected fecally contaminated wells more frequently than either a coliphage or a fecal bacterial indicator alone. As a single indicator, total coliforms in 1-L sample volumes were found to occur most frequently (80% of the wells and 38.3% of the samples). However, total coliforms are not fecal indicator bacteria and in our opinion would not seem to be appropriate or useful as a single fecal indicator organism. The authors of the previous study concluded that the dual measurement of both a coliphage and a bacterial indicator would increase the detection of fecally contaminated groundwater. No single

fecal indicator alone was as effective in detecting fecal contamination as the dual use of a coliphage and a bacterial indicator. These previous findings are consistent with those of this current study, which also found that the dual measurement of two indicators and especially a coliphage and a bacterial indicator increased the likelihood of detecting fecally contaminated groundwater.

Responses to Questions and Comments of the April 2004 Coliphage Workshop

Several questions about coliphage methods were identified by participants at an "International Workshop on Coliphages as Indicators of Fecal Contamination in Water and Other Environmental Media," that was sponsored by US EPA and held in Arlington, VA, April 20-21, 2004. These questions and our responses to them are given below in this section of the report.

1. Costs of the coliphage tests?

Response. The four participating laboratories have estimated the costs of coliphage testing and these costs are listed in the Table below.

Table 13. Costs of Coliphage Analysis by the Four Study Laboratories – November 2004

Coliphage Assay Costs

UNC

SAL 100 mL water sample assayed for each host

	C3000	Famp	CN13	Famp+CN13
Labor and materials	$54	$55	$55	$84
46% indirect costs	$25	$25	$25	$38
Total	$79	$80	$80	$122
Enrichment	C3000	Famp	CN13	Famp + CN13
Labor and materials	$67	$68	$68	$92
46% indirect costs	$31	$31	$31	$42
Total	$98	$99	$100	$134

TAMU

Actual Costs of doing coliphage analysis.

Single Agar Layer (per host bacterium) per sample

Total Labor time: (3.5 hours @ $ 20.00/hour): $70.00

Material costs: $10.00

Total cost: $ 80.00

2-step Enrichment

Total Labor Time: 3 hours @ $20.00/hour: 20.00

Material costs: $ 10.00

Total Cost: $ 70.00

These are the costs per sample, per host bacterium and does not include "overhead" or other costs.

The labor includes media preparation, analysis time and data recording.

UNH - All Methods

C3000	Famp	CN13	Famp + CN13
$68	$68	$68	$103
$32	$32	$32	$47
$100	$100	$100	$150

U of Minn

SAL 100 mL water sample assayed for each host

	C3000	Famp	CN13
Labor and materials	$80	$80	$80
49% indirect costs	$39	$39	$39
Total	$119	$119	$119
Enrichment			
	C3000	Famp	CN13
Labor and materials	$100	$100	$100
49% indirect costs	$49	$49	$49
Total	$149	$149	$149

2. The need for and effectiveness of the method of confirming coliphage-positives in the tests?

In this study the plaque conformation procedure was carefully studies. I was found that the plaque confirmation rate based on the development of lysis or plaques on spots of host lawns in

agar medium averaged nearly 80%. It was concluded this was a simple and sufficiently reliable confirmation method and would be adequate for routine use by labs doing coliphage analysis

3. The issue of using a singe indicator, such as a bacterial indicator or a coliphage indicator, versus using both a bacterial indicator and a coliphage indicator in detecting a positive ground water sample? Specifically, the extent to which there is increased detection of positives when using only one indicator such as a bacterial indicator versus two indicators - a coliphage indicator and a bacterial indicator?

The results of the phase II field studies of this project show quite clearly that the dual measure of two indicators, especially a coliphage indicator and a bacterial indicator significantly increases the frequency of getting a positive sample. Measuring either a coliphage or a bacterial indicator alone gave significantly lower detection of positive samples. Therefore, the results of this study support the use of both coliphage and bacterial indicators together in the analysis of groundwater samples for evidence of fecal contamination.

4. The choice of coliphages to detect: somatic, male-specific or "total" coliphages?

The results of the current study provide data showing that the frequency of detecting a coliphage in ground water is highest for somatic coliphages and nearly as high for "total" coliphages using either the SAL (Method 1602) or enrichment (Method 1601) methods. Therefore, it is concluded from these results that either of these coliphage groups are likely to give greater detection of coliphages than the measurement of F+ coliphages. However, F+ coliphages also are important indicators of fecal contamination. Because they have the ability to distinguish human from animal fecal contamination F+ coliphages and especially F+ RNA coliphages also have merit as

coliphage indicators of fecal contamination of groundwater.

5. Whether or not study wells were subjected to treatment (disinfection)?

The wells of the current study were not disinfected. Most were non-community public water supplies, some were private wells and some were public water supplies. These wells were not required to disinfect or otherwise treat in the States where the wells were located. Because two of the wells in one state had periodic coliform violations, they are now routinely chlorinated. However, at the time of the study they were not being chlorinated.

SUMMARY AND CONCLUSIONS

In initial studies Methods 1601 and 1602 were evaluated for their ability to detect somatic, male-specific (F+) and total (somatic plus F+) coliphages in groundwater samples seeded with mixed, natural populations of coliphages from sewage. The SAL method (Method 1601) was applied in 10 experiments to replicate 100-mL volumes of groundwater seeded with sewage coliphages for coliphage detection with each of three *E. coli* host bacteria: *E. coli* CN-13 for somatic coliphages, *E. coli* Famp for male-specific (F+) coliphages and E. coli C3000 for somatic plus F+ ("total") coliphages. There was efficient coliphage detection (average 53%) and plaque confirmation (average 78%) in 100-mL volumes of ground water. Overall, the results of these studies indicate that there is high likelihood of detecting even low levels of coliphages in 100-mL volumes of ground water using Method 1602.

For evaluation of the enrichment method (Method 1601), recoveries of somatic, F+ and total coliphages from 10 replicate 1-liter volumes of seeded ground water in eight replicate experiments were efficient at coliphage input levels of about 1.5 to 3 infectious units/L. Recoveries were somewhat variable but close to those expected based on the expected number of positive 1-liter enrichment bottles out of a total of 10. There is a high likelihood of detecting as few as 1-3 coliphages in 1-liter volumes of water using the two-step enrichment methods of Method 1601. For both Method 1601 and 1602, the results of studies with seeded samples of groundwater showed that holding samples at 4°C for up to 3 days did not significantly reduce the ability to detect low levels of coliphages. Hence samples can be collected, shipped and stored prior to assay.

These improved and validated versions of Methods 1601 and 1602 were further validated and evaluated studies of coliphage detection and occurrence in more than 100 field samples of groundwater from wells located in 4 different geographic regions of the USA (Northeast, Southeast, Southwest and upper Midwest). Overall, the results for fecal indicator occurrence in the field groundwater samples analyzed in this study indicated that coliphages are reliable indicators for detecting fecal contamination and can detect fecal contamination as frequently or more frequently than do bacterial indicators. In more than 100 groundwater samples collected from wells coliphages were detected with greater or similar frequency than were fecal indicator bacteria. The percentages sample positivity for coliphages were 11 to16% by the SAL method (Method 1602) and 6.9 to 3.4% by the two-step enrichment spot plate method (Method 1601). By comparison, the percentages of sample positivity for bacteria (fecal coliforms, E. coli or enterococci) ranged from 13.8 to 4.3%.

Coliphage detection in groundwater was higher using the SAL assay (Method 1602) on 100-mL sample than using the two-step enrichment spot plate method (Method 1601) on 1-liter samples. Additionally, coliphage detection by either method was highest for somatic coliphages, next highest for "total" coliphages and lowest for F+ coliphages. The relatively high detection of "total" coliphages by the SAL method indicates that a single host, *E. coli* C3000, can be used to detect either somatic or male-specific coliphage or both with a high degree of sensitivity.

The results from the analyses of these groundwater samples indicate that there is a significantly greater likelihood of detecting fecal contamination if two indicators are analyzed in the same sample than if only one indicator is analyzed. Detection of two indicators was higher with a

coliphage and a bacteria indicator pair (as high as 20.7 and 22.4% positivity) than with either pairs of coliphage indicators (as high 18% positivity) or pairs of bacterial indicators (17.2% positivity). Therefore, rates or frequencies of detecting fecal contamination in groundwater are higher when using two fecal indicators than using a single fecal indicators, and highest when using a coliphage and a bacterial indicator together. These findings clearly support the position of determining groundwater vulnerability to fecal contamination by measuring **both** a coliphage indicator **and** a bacterial indicator, rather than measuring either one alone.

Human enteric viruses, including adenoviruses, astroviruses, enteroviruses, hepatitis A virus, reoviruses and rotaviruses were not detected by combined cell culture and (RT-)PCR in any of the 106 samples analyzed. Human Caliciviruses (Noroviruses) were not detected by direct RT-PCR of virus concentrates from the same 106 samples groundwater. Therefore, it was not possible to compare or look for associations in occurrence of coliphages and/or bacteria relative to human enteric viruses in groundwater samples.

It is recommended that EPA adopt these improved methods for coliphage detection for the forthcoming Groundwater Rule. It is also recommended that the Groundwater Rule require the analysis of both coliphages and fecal indicator in the same sample of groundwater in order to significantly increase the likelihood of detecting fecal contamination. Examination of groundwater samples for a single indicator, either a virus or a bacterium, will significantly reduce the chances of detecting fecal contamination.

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APPENDICES

APPENDIX I

EPA Coliphage Groundwater Study

Summary Report of the Northeast Region

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and

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November, 2004

Introduction

The purpose of this study was to validate and apply US EPA Methods 1601 and 1602 for detection coliphages in water by applying them to field samples of groundwater. The goal was to examine 27 samples of groundwater, preferably from public water supply wells, for somatic, F+ and total coliphages, fecal indicator bacteria and human enteric viruses in the Northeast United States.

Methods and Materials

Sampling sites

All sample sites were located in New England. Eight well sites were public water sources and 17 were private wells. A total of 25 wells were sampled instead of 27 due to a very severe and harsh winter. NH had its first snowfall at the end of October and a second snowfall at the beginning of November, 2002. Plans to sample two additional wells as soon as the weather permitted could not be carried out because New England experienced one of the snowiest winters ever. Therefore, only 25 well samples were collected and analyzed. Of the 25 wells, there were 12 sample sites in New Hampshire, two of which were from public wells that were approximately 500 and 700 ft deep, respectively. None of these wells had any form of disinfection. The other wells from NH were all private wells. These wells also were not disinfected. One well from NH was a private, very shallow well, less then 35 feet deep and lined with stone. This was not considered a potable well but was used for farm irrigation. Four sites in Maine were all privately owned wells and not disinfected. Three sites were in Vermont, and they were all privately owned wells and not disinfected. All of the privately owned wells were drilled wells, excepted for the one in NH as indicated above, and they were of varying depths that were unknown to the homeowner at the time samples were collected. There were 6 samples from public water supply wells in Massachusetts. The public water supplies in Massachusetts were chosen due to positive results previously found for total and fecal coliforms, enterococci, and male-specific coliphages. Additionally 3 of the 6 locations had positives previously reported for rotavirus and enterovirus, by molecular methods.

Sampling

All groundwater samples were collected using the EPA ICR method between June and November 2002 (US EPA, 1996). At each well site, 1500 L of well water was collected through a sterile 1MDS filter setup. For a few samples, the filter clogged or the well ran dry, before 1500 liters could be processed. In these cases the successfully filtered volume is reported. Additionally a 10 L grab sample was collected from each well in a sterile container for bacteriological and coliphage analyses. Enteric virus sampling equipment was sterilized between well sites with 0.1% Bleach solution followed by successive 2% sodium thiosulfate and distilled water rinses. All samples were kept at 4^0C and analyzed within 48 hours.

Bacteriological analysis

All bacterial analysis of fecal coliforms and enterococcus was done as specified in EPA-approved methods. The samples were analyzed by membrane filtration using mFC and MEI agars.

Coliphage analysis

All analysis of male-specific (F+), somatic coliphages and total coliphages was done as specified in EPA-approved methods. The US EPA Methods 1601 (enrichment) and 1602 (single agar layer) were used with the host bacteria *E .coli* Famp for F+ coliphages, *E. coli* CN-13 for somatic coliphages, and *E. coli* C3000 for "total" coliphages.

Enteric virus recovery and analysis

The 1MDS filters used to adsorb viruses from samples of ground were eluted and concentrated as

specified in the EPA ICR method. The only change was that the final resuspended acid (flocculated beef extract) precipitate was suspended in 20 mL of phosphate buffered saline (Sigma D8662) rather than 30 mL of sodium phosphate. Samples were filtered through a 37 mm diameter, 0.2 micrometer pore size, beef extract pre-treated Gelman Serum Acrodisc filter (4525). The filter-sterilized concentrate was divided into 5 aliquots. Aliquots were: 2/ 6.7 mL portions for UNH Caco-2 and BGMK cell cultures, 2/ 1.3 mL portions for UNC FRhK-4 cell culture and Calicivirus (norovirus) analysis, and 4 mL was archived. These sample concentrate volumes are equivalent to 500 liters of initial water for inoculation into Caco-2 and BGMK cells, respectively, the equivalent of 100 liters of initial water for FRhK-4 (HAV) and Calicivirus (norovirus) analysis, and 300 liters of initial water for archiving. All aliquots were frozen at -80^{0}C prior to shipment and analysis.

Tissue culture protocol for virus isolation in BGMK and CaCo-2 cells

UNH screened concentrates for BGMK cytotoxicity on 25 cm^2 flasks prior to inoculation of samples onto 75 cm^2 flasks. Sample concentrates were pre-activated for 30 minutes at 37^{0}C with 10 µg/mL of type IX trypsin (Sigma T-0303) for both Caco-2 and BGMK inoculates. BGMK and Caco-2 cell cultures were rinsed three times with PBS before inoculation. Inoculated cultures were incubated at 37^{0}C for 90 minutes with rocking every 15-20 minutes. Only negative controls were run. Serum free maintenance media containing 5 µg/mL trypsin was added to cultures after incubation. Cultures were incubated at 37^{0}C for 7 days. The cultures were checked microscopically daily for the first two days and then every other day thereafter. After 7 days all cultures were freeze thawed and 10% of the lysate was filtered through a 0.22 um filter and inoculated onto new cells for a second passage. At the end of the second passage cultures were

freeze thawed twice. Lysates were pooled and divided into aliquots for further analysis and shipping. Samples were not chloroform extracted. Sample aliquots were sent to UMN for enterovirus analysis and TAMU for rotavirus and reovirus analysis.

RT-PCR Analysis for Astrovirus and Adenovirus

Nested PCR was performed on UNH, UNC, UMN and TAMU samples for both Astrovirus and Adenovirus type 40 and 41. The equivalent volume of original water sample examined for each virus was 500 liters. Positive controls we did were at the level of (RT-)PCR. Virus was added to cell culture lysate to act as a positive control for (RT-PCR)PCRNested PCR was performed on UNH, UNC, UMN and TAMU samples for both Astrovirus and Adenovirus type 40 and 41. The equivalent volume of original water sample examined for each virus was 500 liters. Positive controls we did were at the level of (RT-)PCR. Virus was added to cell culture lysate to act as a positive control for (RT-PCR)PCRNested PCR was performed on UNH, UNC, UMN and TAMU samples for both Astrovirus and Adenovirus type 40 and 41. The equivalent volume of original water sample examined for each virus was 500 liters. Positive controls we did were at the level of (RT-)PCR. Virus was added to cell culture lysate to act as a positive control for (RT-PCR)PCRNested PCR was performed on UNH, UNC, UMN and TAMU samples for both Astrovirus and Adenovirus type 40 and 41. The equivalent volume of original water sample examined for each virus was 500 liters. Positive controls we did were at the level of (RT-)PCR. Virus was added to cell culture lysate to act as a positive control for (RT-PCR)PCR

Astrovirus. All molecular techniques were done as specified in the methods and materials developed by the project team in communication with the EPA. Astrovirus RT-PCR was done according to Chapron et al. 2000. The primers used were specific for human astrovirus, RT primer 5'-GTAAGATTCCCAGATTGGT-3' and PCR primer 5'-CCTGCCCCGAGAACAACCAAG-3'. An 11-µL sample of the combined cell lysates was denatured with 0.5 µL each of 0.05 M EDTA and downstream primer at 99˚C for 8 min. Eighteen µL of the RT mixture was then added and run for 42 min at 42˚C to reverse transcribe and then 5 min at 99˚C. The RT mixture per sample consisted of 2.5 µL 10X buffer II, 8.5 µL of 25mM $MgCl_2$, 1.25 µL of each 10mM dNTP, 0.5 µL of 100mM DTT (Promega), 10 units of Rnasin, and 50 units of RT. After the RT step 28.5 µL of a PCR master mix was added. The PCR mixture per sample consisted of 3 µL of 10X buffer II, 1 µL of the PCR primer, 0.5 µL of the RT primer, 24 µL of molecular grade water, and 2.5 units of Ampli-Taq DNA polymerase. The parameters were 95˚C, 5 minute hot start, followed by 35 cycles of 95˚C for 30 seconds, 56˚C for 30 seconds, 72˚C for 30 seconds, with a final extension at 72˚C for 5 minutes. These primers yielded a 193 and/or 243 bp amplicon.

For nested PCR, 1 µL from each RT-PCR reaction was added to a new tube containing 90 µL of a nested PCR reaction mixture, which contained 8 mM $MgCl_2$, 10 µL 10x buffer, 1mM of each dNTP, 2.5 units of Ampli-Taq DNA polymerase and 1 µM of each primer. The primers used were 5'-CCTTGCCCCGAGCCAGAA-3' and 5'-TTGTTGCCATAAGTTTGTGAATA-3'. These primers yield a 143 and/or 183-bp amplicon. Twelve µL of each RT-PCR product as well as 12 µL of the nested PCR product was run and sized by electrophoresis on 1.8% agarose gel, stained with ethidium bromide. Molecular weights were determined by comparison with a 1 Kb DNA

ladder (Life Technologies). Astrovirus serotype 2 was used as a positive control.

Adenovirus. All molecular techniques were done as specified in the methods and materials provided by EPA. Adenovirus Hexon PCR was done according to Xu et al. 2000. The primers used were Ad1 5'-CCCTGGTA(G/T)CC(A/G)AT(A/G)TTGTA-3' and Ad2 5'-TTCCCCATGGC(Inosine)CA(C/T)AACAC-3'. A 5µL sample of the combined cell lysates was added to 47.5µL final volume PCR master mix. Final concentrations in the PCR master mix per sample were 1.5mM $MgCl_2$, 1x (10x Buffer II), 0.2mM dNTP mix, 0.6µM of each primer, and 2.5 units of Ampli-Taq DNA polymerase. The PCR parameters were 95˚C for 5 minutes, followed by 40 cycles of 94˚C for 1 minute, 55˚C for 1 minute, 72˚C for 2 minutes, with a final extension at 74˚C for 5 minutes. These primers yielded a 482 bp amplicon.

For nested PCR, 1 µL from each PCR reaction was added to a new tube containing 90 µL of a nested PCR reaction mixture, which contained 8 mM $MgCl_2$, 10 µL 10x buffer, 1mM of each dNTP, and 1 µM of each primer. The primers used were 5'-GCCACCGAGACGTACTTCAGCCTG-3' and 5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'. These nested primers were specific for Adenovirus type 40 &41. Samples were run for 35 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 30 seconds yielding a 142 bp amplicon. Twelve µL of each nested PCR product was run and sized by electrophoresis on 1.8% agarose gels and stained with ethidium bromide. Molecular weights were determined by comparison with a 1 Kb DNA ladder (Life Technologies). Adenovirus 40 & 41 were used as positive controls.

Data management

Results of all analyses were entered into the attached excel spreadsheet as well as a laboratory notebook.

Results and Discussion Summary

The objectives of this project were: 1) evaluate EPA methods 1601 and 1602 for the recovery and detection of male specific coliphage, somatic coliphage and total)somatic and male-specific) coliphage from well water; 2) Compare the efficiency of using a single host, C3000 for the detection of both phages; 3) Compare EPA methods 1601 and 1602 for phage detection using all three hosts; 4) Determine if there is a correlation between the detection of coliphage using either EPA Method 1601 or 1602 and indicator bacteria (fecal coliforms and Enterococcus) and 5) Determine if there is any correlation between the detection of indicator bacteria, coliphage (using either method) and certain enteric viruses detected by the Polymerase Chain Reaction Assay (PCR). For this work, 25 wells in the Northeast, some from New Hampshire, Vermont, Maine and Massachusetts, were sampled and evaluated for each organism.

An entire summary of the results can be found in the accompanying data spreadsheet. All samples were negative for Adenovirus and Astrovirus using an integrated cell culture Polymerase Chain Reaction Assay followed by a nested PCR assay.

Overall, 9 of 25 samples or 36% were positive for one or more fecal indicator microbe, either a coliphage or a bacterial indicator. Only one well of 25 (4%) was positive for coliphage (Table 1).

This coliphage-positive was by the enrichment assay on hosts *E. coli* Famp and C3000. The same well was positive for 4 plaques/100 mL using Method 1602 on F+ host *E. coli Famp*, but it was negative for coliphage plaques on *E. coli* C3000. The one well that was positive for coliphage was negative for both indicator bacteria and enteric viruses. No well was positive for coliphage by either method with any other microorganism tested.

Eight wells of 25 (32%) were positive for indicator bacteria. Two wells were positive for both fecal coliforms and Enterococcus, 3 wells were positive for fecal coliforms only and negative for Enterococcus and 3 wells were positive for Enterococcus only but negative for fecal coliforms.

Since so many samples from community wells were negative for all microorganisms, the decision was made to include private drilled wells in the study. It was hoped that because these wells, on average, were probably less deep then the community wells, that there would be an increased chance of detecting indicator organisms as well as enteric viruses. While some of the wells were positive for indicator bacteria and one was positive for coliphage (though both coliphage and bacteria were not the same well), no wells were positive for enteric viruses. Two of these wells in VT were less then 100 ft deep. These wells were included on the study in further efforts to increase the probability of detecting enteric viruses. Both of these wells were negative for viruses and coliphages while one of the wells was positive for 1 fecal coliform colony in the 1 L volume assayed. To further increase the probability of detecting enteric viruses, a stoned lined, non-potable well, which was only approximately 35 feet deep was also sampled. This well was negative for all indicator bacteria, coliphage and virus.

There were 6 samples from public wells in Massachusetts. The public water supplies in Massachusetts were chosen due to positive results previously found for total and fecal coliforms, enterococci, and male-specific coliphages. Additionally 3 of the 6 locations had positives previously reported for rotavirus and enterovirus by molecular methods. All of these 6 samples were negative for coliphage and enteric virus. One of these samples was positive for both fecal coliforms and enterococcus, 2 of the remaining 5 wells were positive for enterococcus but for no other microorganism.

One of the key study objectives was to evaluate coliphage occurrence, by either method, as an indicator for the presence of enteric virus. The results of this study indicate that, overall, the wells were not contaminated by enteric viruses at the time they were sampled. However, the results did yield positive results for coliphage presence in groundwater in the absence of detectable bacterial indicators in that sample. However, bacterial indicators were found more frequently than coliphages (8 samples versus 1 sample) and they were found in the absence of detectable coliphages in these samples. Therefore, coliphages and bacteria were not detected together in any of the samples analyzed. This finding of a lack of co-occurrence of coliphages and bacteria in the same sample supports the measurement of both coliphages and bacteria in groundwater samples as a way to increase the likelihood of detecting fecal contamination.

Table 1. UNH Coliphage Results for Northeast Groundwater

Well Sample Number and Type	Samples positive for phage by SAL (100 mL)			Samples positive for phage by enrichment (1 L)		
	Famp	Cn-13	C3000	Famp	Cn-13	C3000
Well # 3, Private	0	0	0	1	0	1
Wells 1, 2 and 4-25	0	0	0	0	0	0

Table 2. UNH Groundwater Wells Positive for Bacterial Indicators

Positive UNH Bacterial Results for Groundwater Samples	Fecal Coliforms		Enterococcus	
Well Type and Number	100 ML	1 L	100 ML	1 L
Community, Well # 1	1	200	0	0
Private; shallow, Well #4	0	1	0	0
Private Well #9	0	0	0	3
Private, Well #15	35	TNTC	0	0
Private, Well # 18	0	0	1	93
Private, Well # 19	2	10	13	89
Community, #23	0	0	2	87
Community, #23	5	69	2	32

Table 3. Results of UNH Groundwater Samples Analyzed for Adenovirus and Astrovirus

Well #	Results
1	Negative for all viral analyses
2	Negative for all viral analyses
3	Negative for all viral analyses
4	Negative for all viral analyses
5	Negative for all viral analyses
6	Negative for all viral analyses
7	Negative for all viral analyses
8	Negative for all viral analyses
9	Negative for all viral analyses
10	Negative for all viral analyses
11	Negative for all viral analyses
12	Negative for all viral analyses
13	Negative for all viral analyses
14	Negative for all viral analyses
15	Negative for all viral analyses
16	Negative for all viral analyses
17	Negative for all viral analyses
18	Negative for all viral analyses
19	Negative for all viral analyses
20	Negative for all viral analyses
21	Negative for all viral analyses
22	Negative for all viral analyses
23	Negative for all viral analyses
24	Negative for all viral analyses
25	Negative for all viral analyses

Table 4. Summary of UNH Samples Positive for Coliphages, Bacterial Indicators, Adenoviruses and/or Astroviruses

Well Number and Type	Samples positive for:						
	Coliphage	Bacterial Indicators			Phage and Bacterial Indicators	Coliphage and Virus	Bacterial Indicators and Virus
		FC	Ent	Any			
Well # 3, Private	1	0			0	0	0
Well # 1, Community	0	1	0	1	0	0	0
Well # 9, Private	0	0	1	1	0	0	0
Well # 15, Community	0	1	0	1	0	0	0
Well # 18, Community	0	0	1	1	0	0	0
Well # 19, Community	0	1	1	1	0	0	0
Well # 21, Private	0	0	1	1	0	0	0
Well # 22, Private	0	1	1	1	0	0	0
Well # 23,	0	1	1	1	0	0	0
All other wells	0	0	0	0	0	0	0

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APPENDIX II

EPA OST Groundwater Coliphage Project

Coliphage, Bacteria and Human Enteric Virus Isolation from Ground Water–

Southwest Region Laboratory

Texas A&M University – Prof. Suresh Pillai, Co-PI

Introduction and Background

Ground water samples for microbial analyses.

The original goal of this study was for each of the four, regionally representative laboratories (southeast, northeast, upper Midwest and southwest) to collect and analyze 27 ground water samples from public water supply wells. Efforts were made to identify candidate public water supplies that previously had coliform bacteria violations or other evidence of vulnerability to fecal contamination. In some cases candidate wells were prescreened by bacteriological and coliphage analyses for evidence of fecal contamination. Because not all participating labs could identify and get access to 27 public water supply wells, some labs also included non-public and private wells in their sampling. Three labs obtained 27 ground water samples and one lab obtained a total of 25 samples for a total of 106 samples overall. The characteristics of the wells that were sampled are presented in data tables in the Results section of this report. This report describes the methods, materials, coliphage and bacterial indicator and enteric virus results for samples from the southwest region. The results for bacteria and coliphage analyses of these samples are also

presented in an Excel spreadsheet that accompanies this document and in the PowerPoint presentation that was delivered at the April, 2004 EPA Coliphage Workshop.

Methods and Materials

Sampling sites and wells in Texas and New Mexico: Only PWS wells were included in this study, and a total of eleven different PWS wells were identified for this study. The sampling sites were located in the San Antonio region of Texas (wells RS, KK, and HCR) and along the US-Mexico border in southern New Mexico (wells MHPa, MHPb, MHPc, FVE, AVC, SME, and LME). The wells in the San Antonio region were part of a karst aquifer and were previously implicated in a documented groundwater contamination event. Also, during the initial pre-screening of the wells some of the samples were positive for somatic and male-specific coliphages. The wells in southern New Mexico were identified as being vulnerable to groundwater contamination based on parameters such as closeness to septic tanks, proximity to the Rio Grande river and the aquifer in question. These wells were part of a previous EPA-funded project on the microbiological quality of wells in the shallow aquifer along the US-Mexico border during which some of the wells in the sampling area were positive for enterococci, *E. coli*, male-specific coliphages and somatic coliphages. The wells were in the 100-150 feet depth range. The static water levels were around 10-20 feet and in terms of their hydrogeologic setting, they were located in the Rio Grande alluvium/Hueco-Tularosa aquifers.

Sampling: Groundwater samples were collected between June 2002 and January 2003. Multiple samples were collected from each of the wells to be representative of the aquifer and the sampling

location. During each sampling adequate volumes were collected for the coliphage analysis as well as for the enteric virus analysis. Grab samples were collected for the coliphage and bacterial analysis while the 1MDS filters were used for collecting the large volume enteric virus samples.

Microbiological Analysis: The USEPA methods 1601 and 1602 were used for the coliphage analysis. The host bacteria used in these analyses included *E . coli* Famp for F+ coliphage, *E .coli* CN-13 for somatic coliphages and *E. coli* C-3000 for "total" coliphages. The samples were also analyzed for *E . coli* and *Enterococcus* spp using the membrane filtration protocol and M-coli Blue and MEI agars, respectively.

Virus concentration from ground water samples. Viruses were concentrated from 1500-liter ground water samples by filtering the water at its ambient pH using standard 1-MDS electropositive cartridge filters (ZetaPor Virosorb, Cuno Product No.45144-01-1MDS) and using procedures described by the US Environmental Protection Agency (2001). In cases where the sample size is not 1500 liters, the filtered volume is specified in the results section of this report.

Filter elution and concentration. Human enteric viruses were eluted from the Cuno 1-MDS cartridge filters with a solution of 1.5% beef extract (Becton Dickinson) plus 0.05 M glycine at pH 9.5. The eluent was allowed to contact the filter cartridge for a minimum of six minutes. Viruses in the beef extract-glycine eluate were subsequently concentrated into a smaller volume by acid precipitation (organic flocculation). Briefly, the eluates were adjusted to pH 3.5, stirred slowly for 30 minutes, then centrifuged at 6,200 x g for 20 minutes. The resulting pellets were resuspended with 15 mL of 0.15 M Na_2HPO_4, adjusted to pH 9.0-9.5, and centrifuged at 6,200 x g

for 15 minutes to remove residual particulates. The resulting supernatants were adjusted to pH 7.0-7.5, and filtered through 0.2µm pore size, serum Acrodisc syringe filters (Pall) to remove bacterial and fungal contaminants. Each concentrated sample of 18-20 mL was subdivided into aliquots for subsequent detection of specific enteric virus groups, then stored at –80˚. Aliquots of the concentrate were shipped to participating labs for their respective viral analyses by integrated cell culture and (RT-)PCR (for hepatitis A virus [HAV], enteroviruses, adenoviruses, rotaviruses, reoviruses and astroviruses) and direct RT-PCR for noroviruses (human, Norwalk-like caliciviruses).

Infectivity assays in BGMK and Caco-2 cell cultures. One-third of each sample concentrate, equivalent to 500 L of source water, was inoculated into cultures of the Buffalo Green Monkey Kidney (BGMK) continuous cell line, and another third was inoculated into cultures of the Caco-2 continuous cell line. Another portion, corresponding to 100 liters of ground water , was used for cell culture plus RT-PCR analysis of hepatitis A virus (HAV), and another portion, also corresponding to 100 liters of ground water, was used for direct RT-PCR of human caliciviruses (noroviruses). The remaining one-fifth of the sample was archived as a contingency for possible future analysis. Sample concentrates for cell culture inoculation were pre-activated by adding type IX trypsin (Sigma T-0303) to a 10 µg/mL concentration, and incubating at 37˚ for 30 minutes prior to inoculation. Newly confluent layers of each cell type in 75 cm^2 tissue culture flasks were rinsed three times with Dulbecco's phosphate buffered saline (PBS) supplemented with magnesium and calcium (Gibco) to remove residual calf serum associated with the cell growth medium. The cultures were inoculated with trypsin pre-activated concentrate, and incubated at 37˚ for 80 minutes. Serum-free maintenance MEM medium with Earle's salts supplemented with

5µg/mL type IX trypsin was added. Inoculated cultures were incubated for 7 days at 37˚ with periodic microscopic examination for evidence of viral cytopathology.

After seven days, the inoculated cultures were frozen and thawed twice. Newly confluent PBS-rinsed layers of the same cell line were inoculated with 10% of the cell culture lysate from each first passage (initial) culture, calf serum-free medium supplemented with trypsin was added, and the cultures were incubated at 37˚ for a second passage of the sample material. The second passage cultures were periodically observed microscopically, then frozen seven days after inoculation.

All first and second passage cell cultures were frozen and thawed twice. A single lysate pool of about 35 mL was prepared for each ground water sample by combining 10% of the lysate from both first and second passage BGMK and Caco-2 cultures that had been inoculated with a specific water sample concentrate. A 10-mL portion of each lysate pool was extracted with 5 mL of chloroform, and centrifuged at 1,800 x g for 15 minutes. Each sample extract was subdivided into aliquots for isolation of viral nucleic acid and viral nucleic detection using the nucleic acid amplification methods of either polymerase chain reaction (PCR) for DNA viruses (adenoviruses) or reverse transcription PCR (RT-PCR) by other participating laboratories, and stored at –80˚.

Tissue culture protocol for virus isolation in BGMK and CaCo-2 cells at Texas A&M University. The groundwater concentrates (equivalent to 500L) were initially pre-tested for cytotoxicity after an initial pre-activation. (No cytotoxicity tests were done prior to the CaCo-2 cell cultures since none of the samples were positive for cytotoxicity on BGMK cells).

Preactivation was done using 0.5 mL and 1.0 mL of the groundwater concentrate. The 0.5ml sample was added to 5µLof trypsin and the 1.0 mL sample was added to 10ul of trypsin. The samples were incubated for 30 minutes and then refrigerated prior to the cytotoxicity tests. The T25 flasks (having 80% confluency) were washed twice with 5 mL of Hanks Balanced Salt Solution (HBSS). The cells were inoculated with 0.5ml of the preactivated sample, and incubated for 90 minutes with cells being rocked every 15 minutes. After the 90-minute incubation, 5ml of MEM complete (serum free with 0.25µL/mL of trypsin) was added and the cells were observed for 2 days. Cytotoxicity was evaluated using a sterile HBSS -inoculated "negative control."

Each of the T75 flasks were washed with 15 mL of HBSS two times. The HBSS was siphoned off and the flasks were inoculated with the remainder of the sample across 3 flasks. Two negative controls (1 before inoculation of sample and 1 after inoculation of sample) were also included. The flasks were incubated for 90 minutes at 37°C with 5% CO_2 and rocking every 15 min. After the 90-minute incubation, 15 mL of MEM (serum free with 0.25µL/mL of trypsin) was added. The flasks were incubated at 37°C for 7 days and observed every day for cytopathic effects (CPE). The same procedure was followed for CaCo-2 cells as well

The samples were passaged a second time by freeze thawing once and removing approximately 10% of the lysate from the original flaks and placed in new 100% confluent flasks that were washed as mentioned previously. The samples were incubated for 90 minutes, rocking every 15 minutes and 15 mL of MEM (serum free containing 25µL/mL of trypsin) was subsequently added. The samples for incubated for another 7 days and observed by microscopy daily.

The samples were passaged a third time by removing the lysate from the second passage, filter sterilized through a 100 mm diameter, 0.22 µm pore size cellulose ester filter into T75 flasks that were prepared as before. The flasks were placed in the incubator at 37°C for 5-7 days and observed for cytopathic effects.

Table 1. Tissue Culture Results for Virus Isolation from Ground Water Samples Based on Microscopic Observation Only

Sample ID	Sample volume		CPE Results (BGMK and CaCo-2)		
	BGMK cells	CaCo-2 cells	Passage # 1	Passage # 2	Passage # 3
RS (1)	4.75	5.5	+	+	+
HCR (1)	*Groundwater concentrate sample lost due to centrifuge tube breakage*				
RS (2)	*Groundwater concentrate sample lost due to centrifuge tube breakage*				
BM (1)	5.0	6.0	+	+	+
KK (1)	4.75	6.0	+	+	+
RS (3)	5.0	5.5	+	+	+
KK (2)	5.0	5.5	+	+	+
HCR (2)	6.0	6.5	+	+	+
RS (4)	5.0	5.5	+	+	+
RS(5)	5.0	4.5	+	+	+
MHP1a	5.0	5.5	+	+	+
MHP1b	5.0	6.5	+	+	+
AVC1	5.0	6.0	+	+	+
FVE1	5.0	6.0	+	+	+
AVC2	4.0	4.5	+	+	+
FVE2	4.5	6.0	+	+	+
FVE3	6.5	7.75	+	+	+
AVC3	4.5	5.0	+	+	+
MHP1c	5.5	6.0	+	+	+
MHP2a	4.75	5.5	+	+	+
MHP2c	4.8	5.0	+	+	+
MHP3a	5.3	5.5	+	+	+
MHP2b	6.1	6.5	+	+	+
SME1	4.8	5.0	+	+	+
SME2	6.0	6.0	+	+	+
LME1	4.6	5.25	+	+	+
MHP3b	4.3	4.5	+	+	3

+ = indicates possible cytopathic effect.

Viral RNA Extraction for Rotavirus and Reovirus detection by RT-PCR

The cell culture extracts from the BGMK cells (from passage # 1 and passage # 3) (1 mL each) were combined with 2 mL from CaCo-2 cell lysates and to this was added to 2 mL of chloroform. The mixture was vortexed for 2 min at high speed, then centrifuged at 18K rpm for 20 minutes. The top layer was pipetted out and aliquotted into 4 cryo-tubes (1 mL each). (Samples 1-9 that was sent from Texas A&M University contained only extracts from CaCo-2 cells due to a laboratory error). One cryo-tube of each sample was shipped to UNC, Univ. of Minnesota and UNH.

The QiAmp viral RNA extraction kit was used for RNA extraction from the cell culture lysates per the manufacturer's recommended protocols (Qiagen, Valencia, CA). The final extract was resuspended in 80 L of buffer, which was stored at -80C until the RT-PCR analyses.

RT-PCR Analysis for Rotaviruses and Reoviruses

Rotavirus analysis. For Rotavirus, 3.5 µL of the RNA extract was used. Separate RT and PCR amplifications were performed with 10 and 50 µL total reaction volumes, respectively. The final concentrations in the RT step were 5mM (1X PCR Buffer II), 5mM $MgCl_2$, 1mM of each dent, 1.26 µM of 3' rotavirus primer, 45 units of Reverse Transcriptase, RNAse inhibitor (18 units). The sample was "hot-started" (95ºC for 5 min) and when the temperature reached 60ºC, reverse transcriptase, RNAse inhibitor and dNTP were added. The RT step was conducted at 42ºC for 60 minutes. A wax layer was used to prevent accidental aerosolization of samples when the tubes were subsequently opened. The samples were heated at 95ºC for 5 minutes. The sample was maintained at 80ºC. The PCR master mix was then added to this sample. The final concentration

in the sample after the addition of the PCR master mix was 2mM $MgCl_2$, 1X PCR Buffer II, 2.5 units of Taq DNA polymerase, and 0.25µM of 5' Rotavirus primer. The cycling conditions were 95ºC for 1.5 min, 55C for 1.5 min and 72C for 1.5 minutes. Forty PCR cycles were performed. The PCR products were run on a pre-made (6 %) Novex TBE gels (Invitrogen, Valencia, CA) for detection of the 208 bp product.

The controls included a Rotavirus RNA-spiked positive control and a water negative control. Additionally, MS2 RNA was spiked into a select number of samples to detect any possible sample inhibition. Primers directed to the capsid gene of the MS2 RNA were used for this purpose (Valenzuela and Pillai, 1998).

Reovirus Analysis. A volume of 5ul of the RNA extract was used for RT-PCR analysis. Separate RT and PCR amplifications were performed, with 10 µl and 50 µl reaction volumes respectively. The final concentration of the RT components were 1.5 mM $Mgcl_2$, 1X of PCR Buffer II, 0.7 mM of each dNTP and 1.7µM of the 3' Reovirus primer. A wax layer was used to prevent accidental aerosolization during subsequent handling. The samples were heated at 99ºC for 5 minutes and then placed on ice. Once the samples were cooled, RNAse inhibitor (22 units), and 50 units of Reverse Transcriptase were added. The RT conditions were 43ºC for 60 minutes. The samples were subsequently heated for 5 minutes at 95ºC and placed on ice. The PCR master mix was then added to this sample, giving a final volume of 50 microliters. The final concentrations of the PCR mix ingredients were 1.5mM of $MgCl_2$, 1X PCR Buffer II, 05 µM of the 5' Reovirus primer and 5.0 units of the Taq DNA polymerase. The PCR amplification conditions were 95ºC for 1 minute, 55ºC for 1.5 minutes, 72ºC for 1.5 minutes. Forty PCR cycles were performed. The PCR products

were resolved on a 6% TBE premade Novex gels.

RESULTS

Enteric viruses

None of the 27 groundwater samples from either Texas or New Mexico were positive by cell culture and (RT-)PCR for adenoviruses, astroviruses, enteroviruses, reoviruses, rotaviruses or hepatitis A virus or, in the case of caliciviruses, by direct RT-PCR analysis. The presumptive positive results for cytopathic effects shown in Table 1 must have been due to non-viral effects such as the action of the trypsin in the medium. Trypsin can cause the cells to dislodge from the surface of the flask and appear abnormal, or to cytotoxicity from the sample concentrate inocula. Regardless of the cause of this effect, it was not due to the presence of any of the viruses for which samples were analyzed by nucleic acid methods.

Bacterial and Coliphage Indicators in Groundwater.

The results for bacterial and coliphage indicators in positive samples are summarized in Table 2. In all, 7 of 27 samples (26%) were positive for at least one indicator microbe.

Table 2: Summarized data showing groundwater wells that were positive for bacterial and/or viral (coliphage) indicators.

Sample	Entero-cocci (Number/ 100 mL)	*E. coli* Number/ 100 mL	Method 1602 Number/100 mL			Method 1601 Positive (+) or Negative (-) per Indicated Volume					
						Famp		CN-13		C3000	
			Famp	CN-13	C3000	100 mL	1000 mL	100 mL	1000 mL	100 mL	1000 mL
RS(1)	0	0	0	0	0	-	-	+	-	-	-
KK (2)	0	0	0	0	0	-	-	-	+	-	-
HCR(2)	1	0	0	1	0	-	-	+	+	+	-
MHPa(1)	5	1	0	0	0	-	-	-	-	-	-
RS (5)	0	1	0	0	0	-	-	-	-	-	-
AVC(1)	0	0	0	0	0	-	-	-	+	-	-
AVC(3)	0	0	0	0	0	+	-	+	-	-	1000

Bacterial Indicators. Out of 27 samples that were analyzed, only 2 sample (7.4%) were positive for *E. coli* and 2 samples (7.4%) were positive for Enterococci. There was only 1 sample that was positive for both *E. coli* and Enterococci. The maximum density of *E. coli* in a sample was 1 CFU/100 mL compared to Enterococci, which showed a maximum density of 5 CFU/100 mL.

Viral (Coliphage) Indicators. Out of 27 samples, 5 samples were positive for coliphages. There was only 1 sample that was positive for male-specific coliphages (based on detection of a plaque on *E. coli* host Famp). This is in contrast to 5 samples that were positive for somatic coliphages (based on plaques on *E. coli* host CN-13 or growth in enrichment cultures) while 2 samples were positive for "all" coliphages based on *E. coli* host C-3000. Four samples were positive for coliphages when 1000 mL was analyzed compared to 3 samples that were positive when only 100 mL samples were analyzed. Two of the samples were positive when 100 mL and 1000 mL

aliquots of the sample were screened for coliphages.

Comparison of Bacterial and viral Indicator Results: Table 2 shows the results from the bacterial and viral (coliphage) indicator analyses so that the two types of indicators can be compared. Out of 27 samples that were analyzed for bacterial and viral indicators, 7 (25.9%) were positive for either bacterial or viral indicators. Only 3 of the samples (11.1%) were positive for either of the bacterial indicators (*E. coli* or enterococci) while 5 samples (18.5%) were positive for coliphages (either by Method 1601 or Method 1602). Four samples (14.8%) were positive for coliphages but negative for bacterial indicators. This is in comparison to only 2 samples (7.4%) that were positive for bacterial indicators but negative for viral indicators.

These results suggest that coliphages can be used as a tool for screening ground water samples for the presence of fecal contamination. The results strongly suggest that coliphage analysis should be conducted along with or in addition to conventional bacterial indicator analysis. This is because the inclusion of coliphages increases the likelihood of detecting a contaminated samples, based on the presence of either bacteria or coliphage indicators. The total absence of human enteric viruses in the presence of the selected indicator organisms suggest that it is highly unlikely that pathogens would be detected routinely. It is possible that only under heavily contaminated conditions would there be a direct correlation or co-occurrence between the presence of viral pathogens and fecal indicator organisms.

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APPENDIX III

EPA Coliphage Method Validation Project Report: Detection of Coliphages, Indicator Bacteria and Enteric Viruses in Groundwater

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STUDY COMPLETED ON

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Purpose: To determine if FRNA phages are useful indicators of fecal contamination and human enteric viruses by testing well water samples for the presence of fecal coliforms, *Escherichia coli,* Enterococcus, somatic coliphages, FRNA coliphages, "total" coliphages and human enteric viruses.

Materials and Methods

Source of samples. Ground water samples were collected from 27 candidate wells (address with contact numbers are provided in Table 1). All wells except 6 private ones in Minnesota are considered public water supplies by the State of Minnesota and none are disinfected.

Recovery of Enteric Viruses. From each well 1,500 liters of water was pumped through a 1-MDS filter cartridge followed by virus elution in 1.5% beef extract-0.05 M glycine solution. Another 5 liter sample of water was collected from each well in a sterile container for bacteriological and coliphages analysis. These samples were maintained at 4^0C until analyzed, usually within 24 hrs of collection. The results are given in Table 2.

Bacteriological evaluation. Grab samples of water were analyzed for fecal coliforms, *E. coli* and *Enterococcus* using Membrane Filter (MF) technique as recommended in chapter 9 of Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1998). Briefly, a 100 mL volume of a water sample was filtered through a 0.45 μm pore size, 47 mm diameter membrane filter. These filters were then placed on plates of selective mFC agar for fecal coliforms, mEC for *E. coli* and mE media for Enterococcus. For fecal coliforms, the plates

were incubated at 44.5^{0}C for fecal coliforms and *E. coli* and at 41.5^{0}C for Enterococcus.. The number of characteristic colonies was counted following incubation for 24 hrs and concentrations are expressed as colony-forming units per 100 mL. All media were obtained from Becton Dickinson, Cockeysville, MD.

Coliphages analyses. All 27grab samples were analyzed for the presence of FRNA (male-specific) coliphages, somatic coliphages and "total" coliphages using single agar layer procedure and enrichment method (Methods 1601 and 1602; Environmental Protection Agency, 2001a; 2001b). The host bacteria were *E. coli* F_{amp} (ampicillin and streptomycin resistant mutant of *E. coli*; ATCC 700891) for FRNA coliphages, CN13 (nalidixic acid resistant mutant of *E coli*; ATCC 700609) for somatic coliphages, and *E. coli* C3000 (ATCC 15597) for "total" coliphages. A log phase culture of the host bacterium was prepared by inoculating a stock of the bacteria into 30 mL of trypticase soy broth followed by incubation for 4 hrs at 37^{0}C on a shaker platform. To 100 mL aliquots of water samples were added 0.5 mL of 4 M $MgCl_2$, 10 mL of log phase culture of host bacteria, and 100 mL of molten and cooled double strength tryptic soy agar. The sample was thoroughly mixed and poured into four 150-mm Petri plates followed by incubation at 37^{0}C for 24 hrs. Positive results were indicated by circular zones of lysis in contrast to opaque lawn of host bacterial growth. Plaques from all four plates were counted for each sample. Plaques were confirmed by picking them, resuspending the picked material in 100 ul of TSB, spotting onto prepoured lawns of the respective host bacterium, incubating for 4 hours at 37^{0}C, and observing the spots for evidence of coliphage presence as lysis zones or plaques.

In the other method of coliphages testing, the enrichment-spot plate method, 12.5 mL of $MgCl_2$, 50 mL of 10X TSB and 10 mL of ampicillin/streptomycin or nalidixic acid and 5 mL of host culture *E. coli* Famp, CN13 or C3000 were added to 1-liter aliquots of water. After incubation at 37^0C for 24 hrs, 10 µL of the culture was spotted on freshly prepared Spot plates of the respective host culture (*E. coli* Famp, N13 or C3000). Positive results were indicated by circular zones of lysis in contrast to opaque lawn of host bacterial growth. . ((Plaques were confirmed by picking them, resuspending the picked material in 100 µL of TSB, spotting onto prepoured lawns of the respective host bacterium, incubating for 4 hours at 37^0C, and observing the spots for evidence of coliphage presence as lysis zones or plaques. This method is qualitative in nature because it scores sample volumes as either positive or negative for coliphages.

Enteric virus isolation. Viruses were isolated from groundwater samples using the US EPA ICR Method with minor modifications (US EPA, 1996). After filtering 1,500 liters of water through the CUNO 1-MDS filter, adsorbed viruses were eluted from the filter with 1.5% beef extract-0.05 M glycine solution (pH 9.5). The eluate was further concentrated using the acid precipitation method. All concentrates were suspended in the same volume (22 mL) of sodium phosphate buffer. The final sample was filter sterilized using a 25 mm diameter 0.22 micrometer pore size Gelman Acrodisc filter. Two aliquots of 2 mL each were sent to UNC for detection of human caliciviruses (noroviruses) and Hepatitis A viruses. Two aliquots of 7.5 mL each, corresponding to 500 liters of groundwater, were used for culturable virus isolation by inoculation of BGM and Caco-2 cell lines. All samples were passaged twice in BGM and Caco-2 cells, with incubation periods of one week per passage. The culture fluids were pooled separately (one sample passaged twice in BGMK and Caco-2 was pooled). Pooled lysates were chloroform extracted and aliquots

of 2 mL each, corresponding to 100 liters of groundwater, were sent to UNH and TAMU for detection of adenoviruses, reovirus, rotavirus and astrovirus. Cell culture lysates from UNC, UNH, and TAMU were also received for detection of enteroviruses by the cell culture and RT-PCR methods described here. A volume of concentrated sample corresponding to 100 liter of groundwater was also examined for human caliciviruses (noroviruses) by direct RT-PCR at UNC

RT-PCR. Approximately 5 mL volumes of all cell culture lysates were concentrated to 300 L using PEG 8000. Of this, 140 µL was used for RNA extraction using Qiagen RNA extraction kit. The remaining 160 µL was archived. The primers used for amplification of enterovirus nucleic acid are shown below (Schwab et al., 1996).

3' Primer: 5' ACC GGA TGG CCA ATC CAA 3'

5' Primer: 5' CCT CCG GCC CCT GAA TG 3'

RT-PCR conditions were according to those previously used and were: RT - 42^0C for 60 min, followed by inactivation of RT at 95^0C for 15 min. Denaturation - 95^0C for 90 sec; Annealing – 55^0C for 1.5 min; extension - 72^0C for 1.5 min; final extension - 72^0C for 10 min. No. of cycles-40 (3). The RT-PCR products were analyzed by agarose gel electrophoresis and confirmed by ethidium bromide staining for observation of DNA amplicons of the correct size. For positive amplification, an amplicon of 197 bp was expected.

Results Summary. Of the 27 wells tested, fecal coliforms were detected in 7 (26%), *E. coli* in 3 (11%) and Enterococcus in 6 (8 positive samples) (22%). Three of 27 wells contained fecal

coliforms, *E. coli* and Enterococci while one well was positive for both fecal coliforms and *E. coli*. Somatic coliphages were detected in 16 wells (59%) male-specific FRNA phages in 11 wells (41%) (12 positive samples), and "total" coliphages in 12 samples. None of the samples showed cytopathological effects (CPE) characteristic of enteric viruses during their passages in BGM and Caco-2 cell lines. None of the samples was positive for enteric viruses by RT-PCR or PCR.

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Table 1. Details of Groundwater Wells Selected/Screened During the Study

Date on Wells for EPA groundwater study on coliphage methods						
Sample	**Well**	**Well Name**	**Date of sampling**	**Well Address**	**Contact person**	**Contact Phone**
1	01 Amundson	Amundson Farms	4/11/2002	Amundson Farms, RR1 Box 25, Chattfield, MN 55923	Brad	507-867-3396
2	02 Gervais	Lake Gervais	4/23/2002	Lake Gervais, 2500 Ederton St., Maplewood, MN	Richard (Dick) Haus	651-748-2500
3	03 Round	Round Lake Park	4/23/2002	Round Lake Park, 910 Frost, St. Paul, MN	Richard (Dick) Haus	651-748-2500
4	04 Turtle	Turtle Lake	4/23/2002	Turtle Lake, 4079 Hodgson Rd., Shoreview, MN	Dick	651-748-2500
5	05 Brookdale	Brookdale Park	4/30/2002	Brookdale Park, 7650 June Ave. North, Brooklyn Park, MN55443	Layne	763-493-8350
6	06 Oak Groove	Oak Groove Park	4/30/2002	Oak Grove Park, 6941 102nd Avenue N., Brooklyn Park, MN55443		763-493-8350
7	07 Keller Golf	Keller Golf Course	5/3/2002	2166 Maplewood Drive, Maplewood, MN 55109		651-766-4173
8	08 Keller Golf	Keller Main Park	5/3/2002	Keller Main Park, Hwy. 61, Maplewood, MN 55109	Dick	651-748-2500
9	09 Hamilton	Hamilton Park	5/7/2002	6101 Candlewood drive, Brooklyn Park, MN		763-493-8350
10	10 Norwood	Norwood Park	5/7/2002	8100 Newton Ave. N., Brooklyn Park, MN		763-493-8350

11	11 Presbyterian	Presbyterian Church Maple Plain	5/9/2002	558 County Rd. 110, Maple Plain, MN 55359		763-479-2158
12	12 Immanuel	Immanuel United Methodist Church	5/9/2002	10095 County Rd. 101, Cocoran, MN		763-420-2585
13	13 Central	Central Park	5/13/2002	8440 Regent Ave. Brooklyn Park, MN		763-493-8350
14	14 Historical	Brooklyn Park Historical Farm	5/13/2002	4345 101st Avenue N., Brooklyn Park		763-493-8350
15	15 Northwood	Northwood Park	5/13/2002	107th Quebeck Ave. N., Brooklyn Park, MN		763-493-8350
16	16 Lakewood	Lakewood	5/13/2002	3600 Hennepin Ave.	Ron Gjerde	612-822-2171
17	17 Lakewood Mausoleum	Lakewood Cemetery	5/16/2002	3600 Hennepin Ave.	Ron Gjerde	612-822-2171
18	18 Lakewood Maintenance	Lakewood Cemetery	5/16/2002	3600 Hennepin Ave.	Ron Gjerde	612-822-2171
19	19 Al	Alan Ducommun's Father	5/20/2002	5435 152nd Ave., Anoka, MN 55303	Alan Ducommun	
20	20 Dayne	Dayne Ducommun	5/20/2002	4841 Salish Circle, Ramsey, MN55303	Dayne Ducommun	763/753/5090
21	21 Lake Maria	Lake Maria	5/29/2002	11411 Clementa, Monticello, NN 55362	Tom/Mark	763-878-2325
22	22 Gibbs Farm	Gibbs Farm Museum	5/29/2002	Larpentar – Cleveland Av.		
23	23 Tom Arendt	Tom Arendt Home	6/1/2002	9871 John Trail, Chisago City, MN55193	Tom Arendt	651-257-2295
24	24 Milena	Milena's house	6/3/2002	58585 222nd Street, Litchfield, MN55355	Milena	320-693-6754
25	25 Jay	Jay Keil home	6/3/2002	18076 68th Ave, Darwin, MN 55355	Milena	320-693-6754
26	12 Immanuel	Immanuel United Methodist	6/14/2002	3600 Hennepin Ave.	Ron Gjerde	612-822-2171

		Church				
27	24 Milena	Milena's house	6/17/2002	58585 222nd Street, Litchfield, MN55355	Milena	320-693-6754

Table 2. Bacteriological, Coliphage and Virological Analysis of 27 Well Water Samples from Minnesota

EPA PROJECT – COMPLETE RESULTS OF WATER SAMPLE TESTING

	Bacteriological analysis			COLIPHAGES (Method 1601, 1602)				Virus Isolation in cells		RT-PCR on Pooled cell lysate for Entero-virus
Well#	Fec. Col.	E. coli	Enter-ococci	SOM-ATIC	F+	TOTAL	Method	BGMK	Caco-2	
01 Amundson	Neg.	Neg.	Neg.	Pos., (tntc)	Neg.	NT	1601 & 1602	2 passages, no cpe	2 passage, no cpe	Negative
02 Gervais	Neg.	Neg.	Neg.	Pos., 4/100	Neg.	Pos., 5/100	1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
03 Round	Neg.	Neg.	Neg.	Pos., 2/100	Pos., 4/100	Pos., 4/100	1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
04 Turtle	Neg.	Neg.	Neg.	Pos., 4/100	Pos., 2/100	Pos., 3/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
05 Brookdale	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
06 Oak Groove	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	3 passages, no cpe	2 passages, no cpe	Negative
07 Keller Main	Neg.	Neg.	Neg.	Pos., 1/100	Pos., 58/100	Pos., 4/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
08 Keller Main	Neg.	Neg.	Pos., 1/100	Pos., 12/100	Pos., 40/100	Pos., 7/100	Method 1601 & 1602	3 passages, no cpe	3 passages, no cpe	Negative
09 Hamilton	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
10 Norwood	Neg.	Neg.	Neg.	Pos., 28/100	Pos., 9/100	Neg, 0/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
11 Presbyterian	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
12 Immanuel	Pos., 30/100	Neg.	Pos., 1/100	Neg.	Neg.	Neg.	Method 1601 & 1602	3 passages, no cpe	2 passages, no cpe	Negative
13 Central	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
14 Historical	Pos. 1/100	Neg.	Pos. 1/100	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method	2 passages,	2 passages,	Negative

Northwood							1601 & 1602	no cpe	no cpe	
16 Lakewood 1	Pos. 1/100	Neg.	Pos., 1/100	Pos., 574/100	Pos. , 234/100	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
17 Lakewood Mausoleum	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	3 passages, no cpe	3 passages, no cpe	Negative
18 Lakewood Mausoleum	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
19 Al	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	3 passages, no cpe	Negative
20 Dayne	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
21 Lake Maria	Neg.	Neg.	Neg.	Pos., 9/100	Neg.	Pos., 1/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
22 Gibbs Farms	Neg.	Neg.	Pos., 1/100	Pos., 2/100	Neg.	Pos., 2/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
23 Tom Arendt	Pos. 1/100	Neg.	Neg.	Pos., 574/100	Pos., 3/100	Pos., 7/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
24 Milena	Pos. 17/100	Pos., 12/100	Pos., 2/100	Pos., 574/100	Pos., 11/100	Pos., 1/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
25 Jay	Pos. 3/100	Pos., 1/100	Pos., 15/100	Pos., 574/100	Neg.	Pos., 3/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
12 Church Repeat	Neg.	Neg.	Neg.	Pos., 574/100	Pos., 3/100	Pos., 4/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
16 Cemetery Repeat	Neg.	Neg.	Neg.	Pos., 574/100	Pos., 6/100	Pos., 6/100	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative
24 Milena Repeat	Pos. 248/100	Pos., 3/100	Pos., 20/100	Pos., 574/100	Pos., 2/100	Neg.	Method 1601 & 1602	2 passages, no cpe	2 passages, no cpe	Negative

Positive UNH Bacterial Results for Groundwater Samples

Well Type and Number	Fecal Coliforms		Enterococcus	
	100 ML	1 L	100 ML	1 L
Community, Well # 1	1	200	0	0
Private; shallow, Well #4	0	1	0	0
Private Well #9	0	0	0	3
Private, Well #15	35	TNTC	0	0
Private, Well # 18	0	0	1	93
Private, Well # 19	2	10	13	89
Community, #23	0	0	2	87
Community, #23	5	69	2	32

Table 3. Results of UNH Groundwater Samples Analyzed for Adenovirus and Astrovirus

Well #	Results
1	Negative for all viral analyses
2	Negative for all viral analyses
3	Negative for all viral analyses
4	Negative for all viral analyses
5	Negative for all viral analyses
6	Negative for all viral analyses
7	Negative for all viral analyses
8	Negative for all viral analyses
9	Negative for all viral analyses
10	Negative for all viral analyses
11	Negative for all viral analyses
12	Negative for all viral analyses
13	Negative for all viral analyses
14	Negative for all viral analyses
15	Negative for all viral analyses
16	Negative for all viral analyses
17	Negative for all viral analyses
18	Negative for all viral analyses
19	Negative for all viral analyses
20	Negative for all viral analyses
21	Negative for all viral analyses
22	Negative for all viral analyses
23	Negative for all viral analyses
24	Negative for all viral analyses
25	Negative for all viral analyses

Table 4. Summary of UNH Samples Positive for Coliphages, Bacterial Indicators, Adenoviruses and/or Astroviruses

Well Number and Type	Samples positive for:						
	Coliphage	Bacterial Indicators			Phage and Bacterial Indicators	Coliphage and Virus	Bacterial Indicators and Virus
		FC	Ent.	Any			
Well # 3, Private	1	0			0	0	0
Well # 1, Community	0	1	0	1	0	0	0
Well # 9, Private	0	0	1	1	0	0	0
Well # 15, Community	0	1	0	1	0	0	0
Well # 18, Community	0	0	1	1	0	0	0
Well # 19, Community	0	1	1	1	0	0	0
Well # 21, Private	0	0	1	1	0	0	0
Well # 22, Private	0	1	1	1	0	0	0
Well # 23,	0	1	1	1	0	0	0
All other wells	0	0	0	0	0	0	0

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