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Detection of Enteric Viruses in East Tennessee Public Ground Water Systems

Trisha Baldwin Johnson
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Trisha Baldwin Johnson entitled "Detection of Enteric Viruses in East Tennessee Public Ground Water Systems." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Geology.

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We have read this thesis and recommend its acceptance:

Alice C. Layton, Edmund Perfect

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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DETECTION OF ENTERIC VIRUSES IN EAST TENNESSEE PUBLIC GROUND WATER SYSTEMS

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Trisha Baldwin Johnson
December 2005

DEDICATION

This thesis is dedicated to my husband and best friend, Brandon Johnson, my parents, Claude and Mary Ann Baldwin, and all of my dear family and friends. Thank you for your love, support, and constant encouragement over the past three years as I've strived to obtain my Master of Science Degree.

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ABSTRACT

A two-part study was conducted by University of Tennessee-Knoxville, the U.S. Geological Survey, and the U.S. Environmental Protection Agency-National Exposure Research Laboratory to (1) develop, validate, and test a real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) assay for enteroviruses in ground water samples and to (2) perform the first survey of enteric viral occurrence in the karst aquifers of East Tennessee. Karst aquifers are expected to have a high susceptibility to viral contamination because of the rapid flow (100's of m/day) and frequent occurrence of fecal indicator bacteria typically observed in these systems.

Real-time RT-PCR primers and probes specific for enteroviruses were developed and tested at the University of Tennessee's Center for Environmental Biotechnology (UTCEB). The real-time RT-PCR assay was validated using co-standards: attenuated poliovirus and a DNA plasmid constructed at UTCEB (cDNA to the attenuated poliovirus). The assay was confirmed to have good PCR efficiency, reproducibility, and sensitivity. The real-time RT-PCR assay was quantitative over 6 orders of magnitude and had low minimum detection limits (0.5 plaque forming units (PFU) of the attenuated poliovirus per reaction and 10 copies of the DNA plasmid per reaction).

In the field study, eight wells and springs used as raw water sources for East Tennessee public ground water systems were sampled between March and August of 2004. The wells and springs were sampled one to two times under baseflow conditions. The ground water samples were tested for enteroviruses and reoviruses by cell culture methods (total culturable viruses), enteroviruses and reoviruses by conventional RT-PCR, enteroviruses by the real-time RT-PCR assay developed at UTCEB, fecal indicator bacteria (*E. coli* and *Bacteroides*), total coliforms, and physical and chemical water-quality parameters. The wells and springs were chosen on the basis of prior monitoring of *E. coli* and geochemical parameters, their hydrogeologic settings, and the presence or absence of likely input sources of fecal contamination to the ground water supplies. Four sites were designated as "high risk" for fecal contamination and four sites were designated as "low risk" for fecal contamination. "High risk" sites were expected to have higher occurrences and concentrations of enteric viruses as well as other indicators of fecal contamination, such as *Bacteroides* and *E. coli*, than "low risk" sites.

The major results of the field study were: (1) 88% of the wells and springs sampled were positive for culturable viruses (concentrations ranged from 2 MPN/100 mL to 156 MPN/100 mL), (2) 75% of the wells and springs were positive for at least one of the indicator organisms, (3) None of the wells or springs were positive for enteric viruses using the conventional RT-PCR or real-time RT-PCR methods, and (4) "High risk" sites had more frequent detections of enteric viruses and indicator bacteria than "low risk" sites. However, only total coliform concentrations were statistically different (higher) between "high risk" and "low risk" sites. A statistically significant positive correlation was found between total culturable virus concentrations and total coliform concentrations. Of the fecal indicators, *Bacteroides* had the highest co-occurrence with enteric viruses.

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NOMENCLATURE

°C	degrees Celsius
cm	centimeters
CFU/100 mL	colony forming units per 100 milliliters of water
ft	feet
g	grams
in	inches
km	kilometers
km^2	square kilometers
L	liters
m	meters
mg	milligrams
mg/L	milligrams per liter
mi^2	square miles
mL	milliliters
mM	millimolar
nm	nanometers
µL	microliters
µM	micromolar
µS/cm	microsiemens per centimeter

Abbreviations

AWWARF	American Water Works Association Research Foundation
BGMK	Buffalo green monkey kidney
BSA	bovine serum albumin
$CaCO_3$	calcium carbonate
CFU	colony forming unit
CPE	cytopathic effect
C_T	threshold cycle
DNA	deoxyribonucleic acid
GPM	gallons per minute
GWR	Ground Water Rule
GWUDI	ground water under the direct influence of surface water
HAV	hepatitis A virus
HEV	hepatitis E virus
ICC	integrated cell culture
ICR	Information Collection Rule
LPM	liters per minute
MCL	maximum contaminant level
MDBK	Madin-Darby bovine kidney
MGD	millions of gallons per day
MLD	millions of liters per day
MOR	monthly operational reports

MPA	microscopic particulate analysis
MPN	most probable number
NTU	nephelometric turbidity unit
PBS	phosphate buffer saline
PBSAA	phosphate buffer saline + 0.2% BSA
PCR	polymerase chain reaction
PFU	plaque forming unit
PSI	pounds per square inch
QA/QC	quality assurance and quality control
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SARS	Severe Acute Respiratory Syndrome
TDEC	Tennessee Department of Environment and Conservation
TVA	Tennessee Valley Authority
USCDC	United States Centers for Disease Control and Prevention
USEPA	United States Environmental Protection Agency
USEPA-NERL	United States Environmental Protection Agency-National Exposure Research Laboratory
USGS	United States Geological Survey
UTCEB	The University of Tennessee's Center for Biotechnology
UTK	University of Tennessee in Knoxville
VOC	volatile organic compound

CHAPTER I
INTRODUCTION

Human Enteric Viruses

Human enteric viruses, pathogenic viruses that infect the gastrointestinal tracts of humans and can be transmitted by the fecal-oral route through contaminated water, are the most common waterborne pathogens associated with disease outbreaks in ground water (Rose and Yates 1998). Over 100 different types of enteric viruses have been identified (Metcalf et al. 1995) and include adenovirus, astrovirus, calicivirus, coronavirus, enterovirus, hepatitis A virus (HAV), hepatitis E virus (HEV), Norwalk virus, parvovirus, reovirus, and rotavirus (Environment Agency 2000). Although enteric viral infections begin in the gastrointestinal tract, they often spread to other parts of the body and cause an array of illnesses including acute gastrointestinal illness (fever, nausea, diarrhea and/or vomiting), conjunctivitis, skin rashes, cold and flu-like illnesses, hand-foot-and-mouth disease, pneumonia, muscle inflammation, arthritis, inflammation of the kidneys, hepatitis, paralysis, respiratory disease, meningitis, myocarditis, aseptic meningitis encephalitis, poliomyelitis, organ failure, and even death (Maier et al. 2000).

This study focused primarily on enteroviruses and reoviruses. Enteroviruses are human enteric viruses belonging to the family *Picornaviridae* ("Pico" means small; the viruses are about 30 nanometers in diameter). There are at least 65 enterovirus serotypes and the group includes poliovirus, coxsackievirus A and B, echovirus, and the ungrouped enteroviruses. Enteroviruses are well-characterized, non-enveloped, positive-strand single-stranded ribonucleic acid (RNA) viruses that have been used as enteric viral targets in many ground water studies (Abbaszadegan et al. 1999, Fout et al. 2003, Francy

et al. 2004). Enteroviruses are commonly found in human fecal-contaminated waters and are excreted in high numbers in feces of infected individuals ($\geq 10^6$ viruses/gram feces). It is estimated that enteroviruses cause 5-10 million symptomatic infections in the United States each year (Strikas et al. 1986).

Reoviruses are enteric viruses that infect both humans and animals and belong to the family *Reoviridae* (which also includes rotavirus and Colorado Tick Fever). Reoviruses are doubled stranded RNA viruses for which there are three known serotypes (Types 1, 2, and 3). They are not as well-studied as enteroviruses because they often cause asymptomatic infections, are endemic in most populations, and have not been well-documented in waterborne disease outbreaks. However, reoviruses are human pathogens that cause illnesses ranging from mild respiratory infections and gastroenteritis to more serious diseases such as meningitis and myocarditis (Tyler et al. 2004 and Spinner and Giovanni 2001). Reovirus infections have been shown to contribute to the susceptibility of immunocompromised individuals to serious respiratory bacterial infections and a recent study provided some evidence for reovirus co-infection in SARS (Duan et al. 2003).

Enteric Viruses and Ground Water-Related Disease Outbreaks

Enteric viral occurrence in drinking water supplies derived from ground water is a major public health concern. One recent study (Macler and Merkle 2000) estimated that 0.75 to 5.9 million illnesses per year occur in the U.S. due to ground water contaminated with microbial pathogens. In addition, ground water-related disease outbreaks are likely to be underreported, because of the scarcity of monitoring.

In the U.S., there were 647 waterborne disease outbreaks (with 566,403 cases) reported to the United States Centers for Disease Control and Prevention (USCDC) from 1971 through 1996. A majority of the outbreaks (371 outbreaks or 57%) were associated with ground water systems (USEPA 2000). Contaminated source water was the cause for 86% of the ground water-related outbreaks. Enteric viruses were determined to be the most frequent cause of the ground water-related outbreaks of known etiology, causing 34 outbreaks (9%). Bacterial pathogens, parasites, and chemical poisoning were identified as the causes of the other outbreaks of known etiology. Of the ground water-related outbreaks, there were 232 (63%) for which the etiological agent could not be determined. However, the United States Environmental Protection Agency (USEPA) suspects that most of the outbreaks of unknown etiology were virus-caused because it is more difficult to analyze for viruses than for bacteria and symptoms of viral infections are often mistaken for other illnesses (USEPA 2000).

In a more recent study (Lee et al. 2002), 39 drinking water-related waterborne outbreaks in the U.S. from 1999-2000 were evaluated. These outbreaks caused illness in an estimated 2,068 people, hospitalization of 122 people, and two deaths. Twenty-eight (72%) of these outbreaks were associated with ground water. Enteric viruses were determined to be the cause of 20% of the 22 outbreaks of known etiology. Bacterial pathogens, parasites, and chemical poisoning were identified as the causes of the other outbreaks of known etiology. The cause of 17 (44%) of the outbreaks was not identified. Again, enteric viruses were the suspected cause of these outbreaks.

Enteric Virus Occurrence in Public Ground Water Systems in the United States

In 1992 the USEPA began development of the Ground Water Disinfection Rule, now called the Ground Water Rule (GWR), with a goal to minimize the public health risk associated with the consumption of pathogens in ground water. They quickly realized the need for more studies of microbial contamination of ground water. Specific needs included studies on the extent to which ground water may be contaminated, on aquifer vulnerability with respect to different hydrogeological conditions and land use patterns, on the relationship between enteric virus occurrence and indicators of fecal contamination (viral, bacterial, chemical, etc.), and general studies of virus occurrence to aid in a national microbial risk assessment. This call for research motivated many recent studies that provide insight into the occurrence of enteric viruses in ground water supplies as well as factors relating to those occurrences. Some of these studies are summarized in the final proposed GWR (USEPA 2000). Another summary of the studies, including studies conducted after the final proposed GWR was published, is provided in a United States Geological Survey (USGS) report (Francy et al. 2004). Five recent studies of virus occurrence in public ground water systems in a variety of hydrogeologic settings are summarized in the following paragraphs. These studies varied in their experimental designs, in their use of different enteric viral analysis methods, and in their use of different laboratories to conduct those analyses.

The largest study (Abbaszadegan et al. 1999a) was conducted by the American Water Works Association Research Foundation (AWWARF) and sampled 448 public water supply wells in 35 states in different physical, chemical, and geological settings. Some wells were sampled more than once for a total of 539 samples. The wells were

tested for enteroviruses and reoviruses by cell culture (together called "total culturable viruses") and for HAV, rotaviruses, Norwalk viruses, enteroviruses, and reoviruses by reverse transcription-polymerase chain reaction (RT-PCR). Wells were also tested for indicator bacteria (total coliforms, enterococci, and *Clostridium perfringens*), somatic coliphage, and male-specific coliphage. Investigators found that 5% of the wells were positive for polioviruses by cell culture (Abbaszadegan et al. 1999b), suggesting possible false positives due to contamination with the laboratory control virus. By RT-PCR, 16% of the wells were positive for enteroviruses, 15% of the wells were positive for rotaviruses, 7% of the wells were positive for HAV, and 1% of the wells were positive for Norwalk viruses. About 19% of the wells were positive for indicator bacteria and 15% of the wells were positive for coliphage. In general, more samples were positive for viruses by RT-PCR than by cell culture. There was no statistically significant relationship found between the occurrence of enteric viruses and indicator bacteria or coliphage.

Another similar, two-phase study was conducted by the USEPA and targeted public water supply wells in aquifers determined to be vulnerable to human fecal contamination. Phase I (Lieberman et al. 2002) included selecting 94 public water supply wells in 22 states and two U.S. territories that were expected to be vulnerable to human fecal contamination because of historical indicator bacteria data, proximity of a potential contaminant source, or location in a sensitive hydrogeological setting (e.g. karst). The wells were sampled and tested for somatic coliphage, total coliforms, *E. coli*, enterococci, and *Clostridium perfringens*. Based on the results from Phase I, 30 public water supply wells (23 of the wells from Phase I and seven additional wells) were selected for one year

of monthly sampling in Phase II (Lieberman et al. 2002, Dahling 2002, and Fout et al. 2003). The wells, located in 17 states and two U.S. territories, were sampled and tested for total culturable viruses, enteroviruses, reoviruses, HAV, rotaviruses, and Norwalk viruses by RT-PCR, *Legionella*, enterococci, *E. coli*, *Clostridium perfringens*, total coliforms, somatic coliphage, male specific coliphage, and *Bacteroides* phages. Investigators found that 23% of the wells were positive for enteroviruses (coxsackievirus and echovirus) or reoviruses by cell culture but 38% of the wells were positive for enteroviruses by RT-PCR. In addition, by RT-PCR, 21% of the wells were positive for Norwalk viruses, 62% for reoviruses, and 14% for HAV. About 80% of the wells were positive for total coliforms, 50% for *E. coli*, 70% for enterococci, and 53% and 20% were positive for somatic coliphage and male-specific coliphage, respectively. The detection rates for viruses and indicators were higher in this study than for other studies because this study targeted vulnerable wells, had a high sampling frequency (each well was sampled 12 times), and sampled larger volumes of water. A statistically significant positive correlation was found between total culturable viruses and some of the indicators (total coliforms, *E. coli*, enterococci, somatic coliphage, and male-specific coliphage). Of the seven wells that tested positive for enteric viruses by cell culture, three of them were in karst aquifers and tested positive more than once throughout the study. The geological setting with the next highest number of wells positive for enteric viruses was non-karst fractured bedrock.

The USGS and the USEPA recently conducted a study (Francy et al. 2004) of small public water supply wells in sand and gravel aquifers of southeastern Michigan. Thirty-eight wells were sampled and tested for total culturable viruses and enteroviruses,

HAV, reoviruses, rotaviruses, and Norwalk viruses by RT-PCR. The wells were also sampled and tested for total coliforms, *E. coli*, enterococci, somatic coliphage, and male-specific coliphage. Each well was sampled one to five times. Culturable viruses were found in 6% of the wells, but none of those wells were also positive for viruses by RT-PCR. A separate 10% of the wells were positive for enteroviruses by RT-PCR and 13% of the wells were positive for HAV by RT-PCR. One or more of the indicators were found in 47% of the wells and at least one of the indicators was detected 44% of the time when viruses were present. However, no indicators were found in any samples from four out of the nine virus-positive wells. Total coliforms were detected in 34% of the wells, *E. coli* was detected in 10% of the wells, enterococci was detected in 16% of the wells, somatic coliphage was detected in 3% of the wells, and male-specific coliphage was detected in 6% of the wells. Wells served by septic systems had more virus detections than wells served by sewer lines.

A study of noncommunity public water systems in Pennsylvania (Lindsey et al. 2002) focused on carbonate and crystalline fractured bedrock aquifers. Fifty-nine wells were sampled and tested for total culturable viruses, *Helicobacter pylori* (a bacterial pathogen), total coliforms, *E. coli*, *Clostridium perfringens*, somatic coliphage, male-specific coliphage, and enterococci. Investigators found culturable viruses (poliovirus, echovirus, reovirus, and coxsackievirus) in 8% of the wells, *H. pylori* in 7% of the wells, *E. coli* in 12% of the wells, total coliform in 46% of the wells, *Clostridium perfringens* in 15% of the wells, somatic coliphage in 8% of the wells, male-specific coliphage in 5% of the wells, and enterococci in 14% of the wells. Detection rates for both the viruses and indicators were slightly higher in the carbonate aquifers than in the crystalline aquifers,

though not statistically different. The investigators did not find a correlation between land use and the presence of viruses or indicators. Of the indicators, the bacteriophages had the highest co-occurrence with viruses. They were detected 60% of the time when viruses were present. However, 20% of the virus-positive ground water samples tested negative for all of the indicators.

The USGS and the Missouri Department of Natural Resources conducted a two-phase study in the mostly rural Missouri Ozark Aquifer (Davis and Witt 2000 and Femmer 2000). To date, it is the only study that focused exclusively on enteric viruses in carbonate aquifers, some of which had karst features such as caves and sinkholes. In Phase I of the study (Davis and Witt 2000), 109 wells ≤ 15 years old were sampled and tested twice (during the summer and winter) for total culturable viruses, enteroviruses by RT-PCR, fecal coliforms, *E. coli*, fecal streptococci, somatic coliphage, and male-specific coliphage. They found that only about 1% of the wells tested positive for total culturable viruses (polioviruses only, suggesting possible contamination with the laboratory control virus) and 12% of the wells tested positive for enteroviruses by RT-PCR. Fecal coliforms were found in 1.8% of the wells and somatic and male specific coliphages were found in 1.8% and 12% of the wells, respectively. Phase II of the study involved sampling and testing 109 older wells (pre-1970) once (during the spring) for total culturable viruses, fecal coliforms, *E. coli*, fecal streptococci, somatic coliphage, and male-specific coliphage (enteroviruses were not analyzed by RT-PCR in Phase II). None of the wells tested positive for viruses by cell culture. *E. coli* were found in 8% of the wells, fecal coliforms were found in 7% of the wells, and somatic coliphage and male-specific coliphage were each found in about 3% of the wells. In contrast to what was

expected, most of the enteric virus contamination was observed in the non-karst aquifers of the study area.

Microbial Water Quality of the Karst Aquifers of East Tennessee

Karst aquifers are water-bearing formations that typically contain large fractures, conduits, or caves. They usually occur in soluble carbonate rocks such as limestone or dolomite and are formed by chemical dissolution of the bedrock. Karst regions are often, but not always, distinguished topographically by sinkholes and sinking streams (White 1988). Numerous studies have confirmed that karst aquifers are especially vulnerable to contamination by microbial pathogens such as enteric viruses (Wolfe et al. 1997, Kacaroglu 1999, Boyer 1999, Mahler et al. 2000, Hampson et al. 2000, Byl and Williams 2000, Johnson 2002). One of the largest waterborne disease outbreaks in the U.S., attributed to contamination of the source water of a ground water system, occurred in 1980 in a karst aquifer in Georgetown, TX. About 7,900 people became ill and coxsackievirus (an enterovirus) and HAV were detected in the raw well water (USEPA 2000). In the final proposed GWR (USEPA 2000), the USEPA lists karst aquifers as having a high risk of fecal contamination because of their capability to transport fecal contamination long distances over short time periods. In these aquifers, ground water preferentially flows through fractures and conduits (secondary porosity) at very high flow rates (10's to many 100's of meters per day), instead of filtrating through intergranular pores (primary porosity) (White 1988). Also, ground water flow is unpredictable in these hydrogeologically complex systems and is subject to seasonal and short-term changes due to direct connection with surface water and/or because of rapid recharge through the thin soils covering the aquifer. Due to this variability, regular water-quality monitoring

schedules may be insufficient to detect or fully describe contamination events (Mahler et al. 2000).

Approximately one fifth of the U.S. is underlain by karst bedrock and 40% of the ground water used for drinking in the U.S. comes from karst aquifers (Quinlan 1989). Karst aquifers are often capable of producing large amounts of water. East Tennessee derives most of its ground water used for drinking from karst aquifers (Webbers 2000). In this region, wells and springs are used for water supplies by small cities and towns, rural schools, churches, farms, and private homes. Sources of enteric viruses to these ground water supplies may include septic tanks and fields, leaking sewer lines, land application of wastes, animal feed lots, dairy farms, and other livestock operations, injection wells, landfill leaches, unintentional wastewater plant overflows, and inadequately treated wastewater effluent (Gantzer et al. 1998, Azadpour-Keeley et al. 2003, Environment Agency 2000).

Fractured and fine-grained subsoil derived from the weathering of carbonate-rich rocks, commonly referred to as saprolite or residuum, are widespread materials in the U.S. Saprolite often blankets the karst aquifers of East Tennessee and ongoing field studies in these materials (Wilson et al. 1993, McKay et al. 1997, Cumbie and McKay 1999, McKay et al. 2000, Johnson and Connell 2001, McKay et al. 2002) indicate that microorganisms can travel rapidly through fractured saprolite, thereby increasing the potential for contamination of the underlying karst aquifers. McKay and others (McKay et al. 2000) observed transport rates of 10's to 100's of meters per day in a field experiment in East Tennessee saprolite. Subsequent laboratory studies of these materials

showed that transport would occur rapidly with little retention under conditions similar to that of recent infiltration (i.e. high flow and low ionic strength).

Although the potential for enteric viral contamination of ground water in East Tennessee seems cause for public health concern, there have been no studies of enteric viral occurrence in the region. However, water-quality studies emphasizing indicator bacteria have been conducted to assess the level of microbial contamination. A study conducted by the USGS (Johnson 2002) surveyed 35 springs in the carbonate rocks of the Valley and Ridge Physiographic Province of the Upper Tennessee River Basin for indicator bacteria, nutrients, pesticides, and volatile organic compounds (VOC's) under baseflow conditions. An important finding of the study was that indicator bacteria (total coliforms and/or *E. coli*) were found in every spring sampled. In addition, *E. coli* concentrations in all of the sampled springs exceeded the drinking-water standard for public water systems of 1 colony forming unit per 100 milliliters of water (CFU/100 mL). Another similar USGS study (Hampson et al. 2000) was conducted from 1998 to 1999 in the same geographic area and under similar conditions. This study, however, examined water quality of domestic and public supply wells. The study found that while the wells had less microbial contamination than the springs, total coliforms (at concentrations exceeding the drinking-water standard of 4 CFU/100 mL) were found in 37% of the sampled wells and *E. coli* (at concentrations exceeding the drinking-water standard for public water systems) were found in 30% of the sampled wells. Although these studies increased the understanding of fecal contamination problems in East Tennessee ground water, they did not reveal the source of the fecal contamination (i.e. total coliforms and *E.*

coli can come from human or animal sources) or address the potential for enteric viral contamination.

Methods for Detection of Enteric Viruses in Ground Water

The conventional method for enteric virus detection in ground water samples is incubation of samples with animal cell cultures. Viral infection is monitored microscopically via cellular cytopathic effects (CPE). Cell culture assays are time-consuming, expensive, and only detect enteric viruses that replicate and produce CPE in the cultured cells. Unfortunately, no cell culture system alone can propagate all human enteric viruses and some viruses are not detectable in any cell line (Chapron et al. 2000). In addition, the filtration and concentration steps typically used in viral sampling of ground water may result in the concentration of compounds that are toxic to the cultured cells used for the assays (Reynolds et al. 1996). Although there are limitations to cell culture assays, the main benefit is that they detect only infectious viruses, which have the most public health significance.

In recent years, RT-PCR has become widely used as an alternative to cell culture methods for detection of enteric viruses in ground water samples. RT-PCR has enabled the detection of both the culturable and the difficult or impossible-to-culture enteric viruses in environmental water samples (Abbaszadegan et al. 1993, Kopecka et al. 1993, Tsai et al. 1993). Compared to conventional cell culture assays, this technique offers decreased costs and time, high sensitivity and specificity, and can detect enteric viruses that do not produce typical CPE, such as HAV (Reynolds et al. 1996) and noroviruses (Parshionikar et al. 2003). Disadvantages of RT-PCR assays compared to cell culture methods include (1) the inability to discriminate between infectious and noninfectious

viruses and (2) the inability to enumerate viruses. The assays are qualitative and samples are identified only as positive or negative for the target enteric viral genomes. In addition, sample volumes analyzed in RT-PCR assays (typically 5 microliters) are much smaller than sample volumes analyzed in cell culture assays (typically 20 milliliters). Therefore, ground water samples require more concentration before RT-PCR analysis than they do before cell culture analysis. This additional concentration leads to an increase in substances that inhibit the enzymes involved in RT-PCR reactions and may lead to false negative results (Ijzerman et al. 1997, Lewis et al. 2000, Shieh et al. 1995).

Integrated cell culture-RT-PCR procedures (ICC-RT-PCR) have been developed (Reynolds et al. 1996, Chapron et al. 2000) that allow for detection of enteric viruses, mostly infectious, within a shorter time frame than would be required for cell culture alone. Briefly, the method involves inoculation of the concentrated water sample onto the cells for at least 24 hours. Cell cultures are then lysed by freeze/thaw cycles followed by RT-PCR analysis of the cell culture lysate. This method allows the infectious enteric viruses to replicate for 24 hours, increasing the chance of their detection by RT-PCR. Also, by performing RT-PCR on the cell culture lysate, rather than directly on the water sample, inhibitory substances in the water sample that adversely affect RT-PCR enzymes are diluted. ICC-RT-PCR combines the main benefit of cell culture (determination of virus infectivity) with the main benefits of RT-PCR (specificity and speed of analysis). However, the greatest advantage of ICC-PCR (or ICC-RT-PCR) is that it detects viruses that do not produce CPE (Chapron et al. 2000).

A report by the American Academy of Microbiology (Rose and Grimes 2001) emphasized the need for development and validation of new molecular tools for detection

of pathogens in order to make pathogen monitoring faster, more efficient, cost-effective, and accessible in order to improve microbial risk assessments. The report challenges researchers in applied microbiology and environmental science to adapt molecular techniques used successfully in clinical medicine for environmental testing applications. One of those molecular techniques already widely used in clinical settings is real-time polymerase chain reaction (real-time PCR). Real-time PCR is a rapid, highly sensitive method for quantifying nucleic acid sequences using fluorescent signals. Real-time monitoring of florescent signals during PCR amplification eliminates the need for post-PCR sample processes such as gel electrophoresis (Harms et al. 2003). Reverse transcription can easily be added for a one-step real-time RT-PCR assay to detect RNA from enteric viruses. Real-time RT-PCR has the capabilities of conventional RT-PCR but can be quantitative (if viral RNA quantitation standards are used) and is much faster. Analysis times can be reduced from 6 hours for conventional RT-PCR (plus an additional 18 hours for a post RT-PCR hybridization assay) to < 1-2 hours for real-time RT-PCR. Recent studies have successfully applied real-time RT-PCR assays to detect, identify, and quantify enteroviruses in surface waters and sponge tissue in the Florida Keys (Donaldson et al. 2002) and to detect and quantify enteroviruses and astroviruses in sludge samples (Monpoeho et al. 2000, Monpoeho et al. 2004, and Le Cann et al. 2004). Studies where real-time RT-PCR was successfully used for detection and quantification of enteric viruses in actual ground water samples had not been published at the time this thesis was written.

Purpose and Scope of This Study

Studies of the spatial and temporal occurrence and concentration of enteric viruses in the source waters of East Tennessee ground water systems are needed to evaluate current treatment strategies, properly conduct microbial risk assessments, evaluate current regulations for developing Wellhead Protection Plans, and further our understanding of fate and transport of viruses in karst aquifers. Research is needed to determine whether there is any relationship between enteric virus occurrence and other measures of fecal contamination such as indicator bacteria, and physical and chemical characteristics of karst aquifers. In addition, development and validation of new molecular tools that make detection of enteric viruses in ground water easier, faster, and more cost-effective is needed in order to facilitate enteric virus monitoring programs.

This thesis describes a study funded primarily by the Tennessee Department of Environment and Conservation (TDEC) and conducted by the University of Tennessee in Knoxville (UTK), in cooperation with the USGS and the USEPA-Office of Research and Development, National Exposure Research Laboratory (NERL) in Cincinnati, Ohio. The two-part study was designed to meet some of the important research needs regarding occurrence of enteric viruses in public ground water systems. The main goals of the study were to (1) develop and validate a fast, efficient method for detecting and quantitatively measuring concentration of enteric viruses in ground water samples using real-time RT-PCR and (2) perform the first survey of enteric viral occurrence in the karst aquifers of East Tennessee. In this study, raw ground water sources from eight public water systems (four wells and four springs) in karst aquifers of East Tennessee were sampled one to two times and tested for total culturable viruses, enteroviruses and

reoviruses by RT-PCR, enteroviruses by real-time RT-PCR, total coliforms, *E. coli*, and *Bacteroides*. Each ground water sample was also analyzed for chloride, fluoride, nitrate, sulfate, and phosphate. Field water-quality measurements taken in association with each ground water sample included water temperature, turbidity, pH, specific conductance, alkalinity, and hardness.

Specific Objectives

The specific objectives of this study were to (1) develop, test, and evaluate a real-time RT-PCR method for enteric viruses in ground water samples, (2) measure enteric viruses in the wells and springs chosen for sampling, and (3) investigate the relationship between measured concentrations of enteric viruses and other indicators of human fecal contamination.

Hypotheses

This thesis contains one methodological hypothesis and three field-based hypotheses.

Methodological Hypothesis:

A real-time RT-PCR method can be developed that is quantitative and provides equivalent sensitivity to conventional RT-PCR.

Field-Based Hypotheses:

For wells and springs previously designated as "high risk" or "low risk" for fecal contamination (risk designation is explained in Chapter 2.), it is hypothesized that:

(1) Samples from "high risk" sites will have higher *E. coli* values than samples from "low risk" sites.

(2) Samples from "high risk" sites will have higher values of other indicators of fecal contamination (such as *Bacteroides*, chloride, and nitrate) than "low risk" sites.

(3) "High risk" sites will have higher occurrences and concentrations of enteric viruses as measured by cell culture-based or PCR-based methods than "low risk" sites.

If more than one of the field-based hypotheses is met, then the indicator bacteria and enteric virus concentrations will be compared statistically to determine whether there is a relationship between the level of indicators and enteric viruses.

Expected Benefits of This Study

Results of this study may provide useful information for the GWR and other federal regulations regarding microbial risk of ground water systems. This study may also supply information needed to assess the effectiveness of methods for determining aquifer vulnerability and state regulations for developing Wellhead Protection Plans in karst areas. On a local level, results of this study will help water resource managers of East Tennessee make informed decisions about protection, treatment, and use of source waters derived from karst aquifers. In general, this study will add to the pool of knowledge regarding enteric virus occurrence in ground water systems, relationships between enteric viruses and indicators of human fecal contamination, and methods for detection of enteric viruses in ground water samples.

Research Plan and Organization of Thesis

An overview of the research plan for this study is illustrated in a flow chart (Figure 1-1). The first tasks of the study were to select the wells and springs for sampling

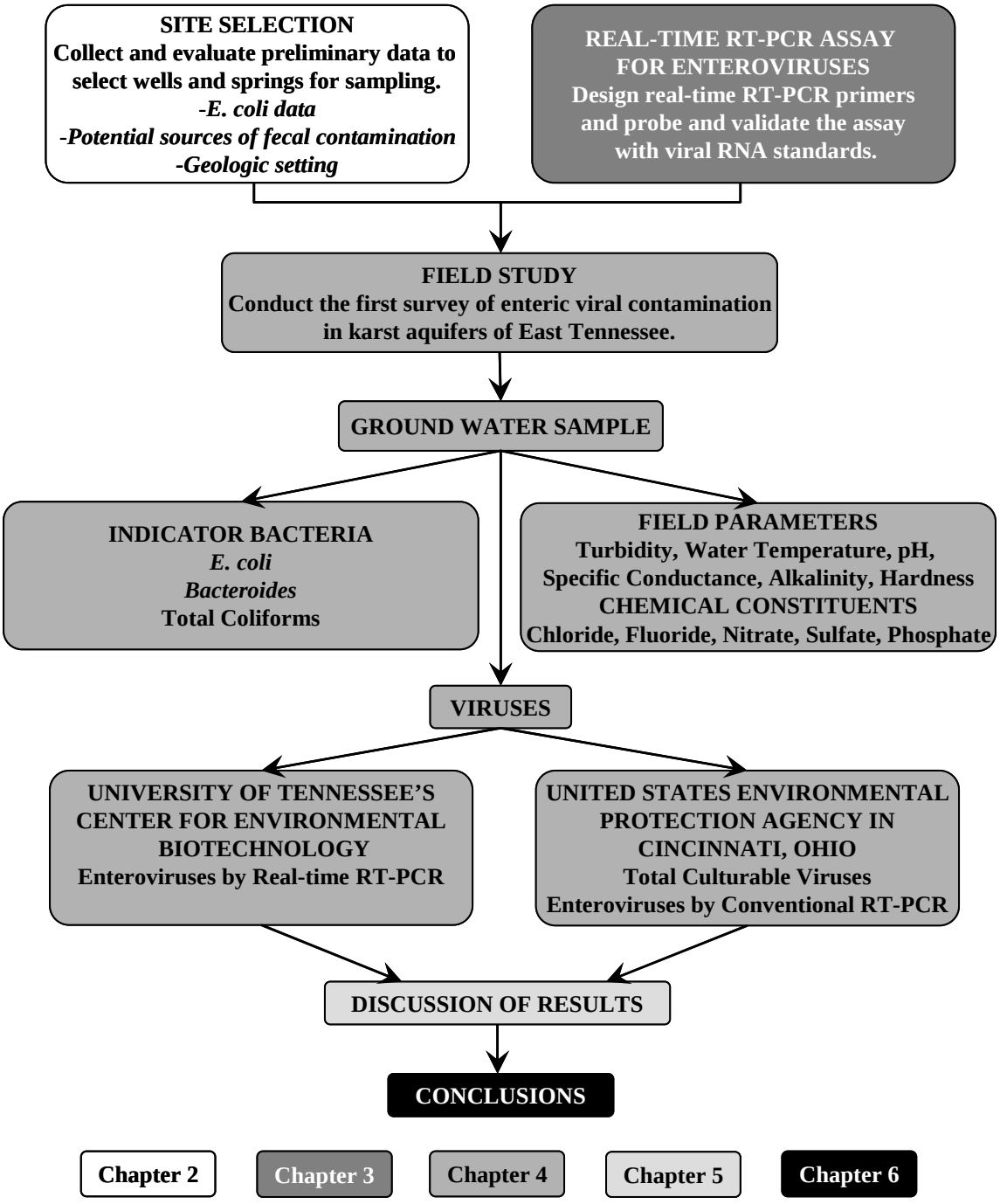

Figure 1-1. Flow chart of research plan and organization of thesis chapters.

and to develop the real-time RT-PCR assay for enterovirus. Site selection and characterization are discussed in Chapter 2 of this thesis. Development of the real-time RT-PCR assay is discussed in Chapter 3. Once the real-time RT-PCR assay was developed and the sampling sites were determined, the field study was initiated. Chapter 4 of this thesis describes the field sampling procedures and the different analyses performed on or in association with each ground water sample collected. Chapter 5 reviews and discusses the results of the study and Chapter 6 provides conclusions for the study and recommendations for future work.

CHAPTER II
SITE SELECTION AND CHARACTERIZATION

The purpose of this chapter is to review the hydrogeology of the study areas, to discuss the process for selecting the ground water sampling locations for this study, and to describe the characteristics of the chosen sampling locations. Eight wells and springs from public water systems in East Tennessee were chosen for enteric virus sampling between March and August of 2004. Due to the sensitive nature of pathogen monitoring as well as drinking-water source security concerns, the participating public water systems asked that their names and the exact locations of their raw water sources not be used in any final written publications stemming from this study. Therefore, arbitrary site ID's for the sampled wells (W-1 through W-4) and springs (S-1 through S-4) are used throughout this document.

Hydrogeology of the Valley and Ridge Physiographic Province of East Tennessee

The study area for this research was the Valley and Ridge physiographic province of East Tennessee as well as the transition zone between the Valley and Ridge and the Blue Ridge physiographic provinces of East Tennessee. The Valley and Ridge physiographic province lies west of the Blue Ridge physiographic province and east of the Cumberland Plateau (Figure 2-1). It encompasses an area of about 19,900 km^2 (7,690 mi^2) in East Tennessee (Johnson 2002) and is a long, narrow belt of Paleozoic-aged carbonates (limestone and dolomite), shales, sandstones, and conglomerates that were intensely folded and faulted during the Appalachian orogeny (DeBuchananne and Richardson 1956). Figure 2-2 is a generalized geologic map of the Valley and Ridge.

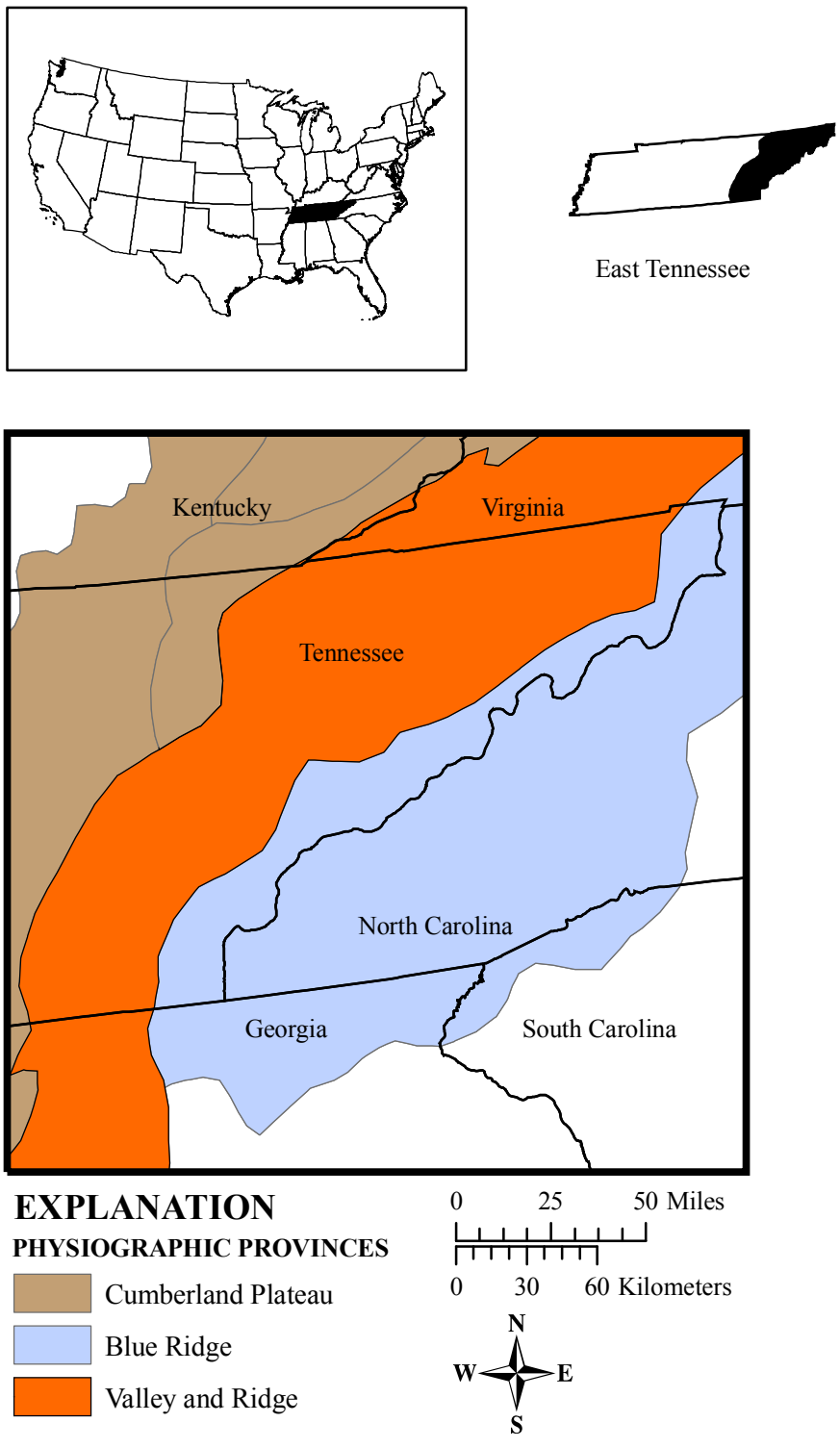

Figure 2-1. Physiographic map of East Tennessee.

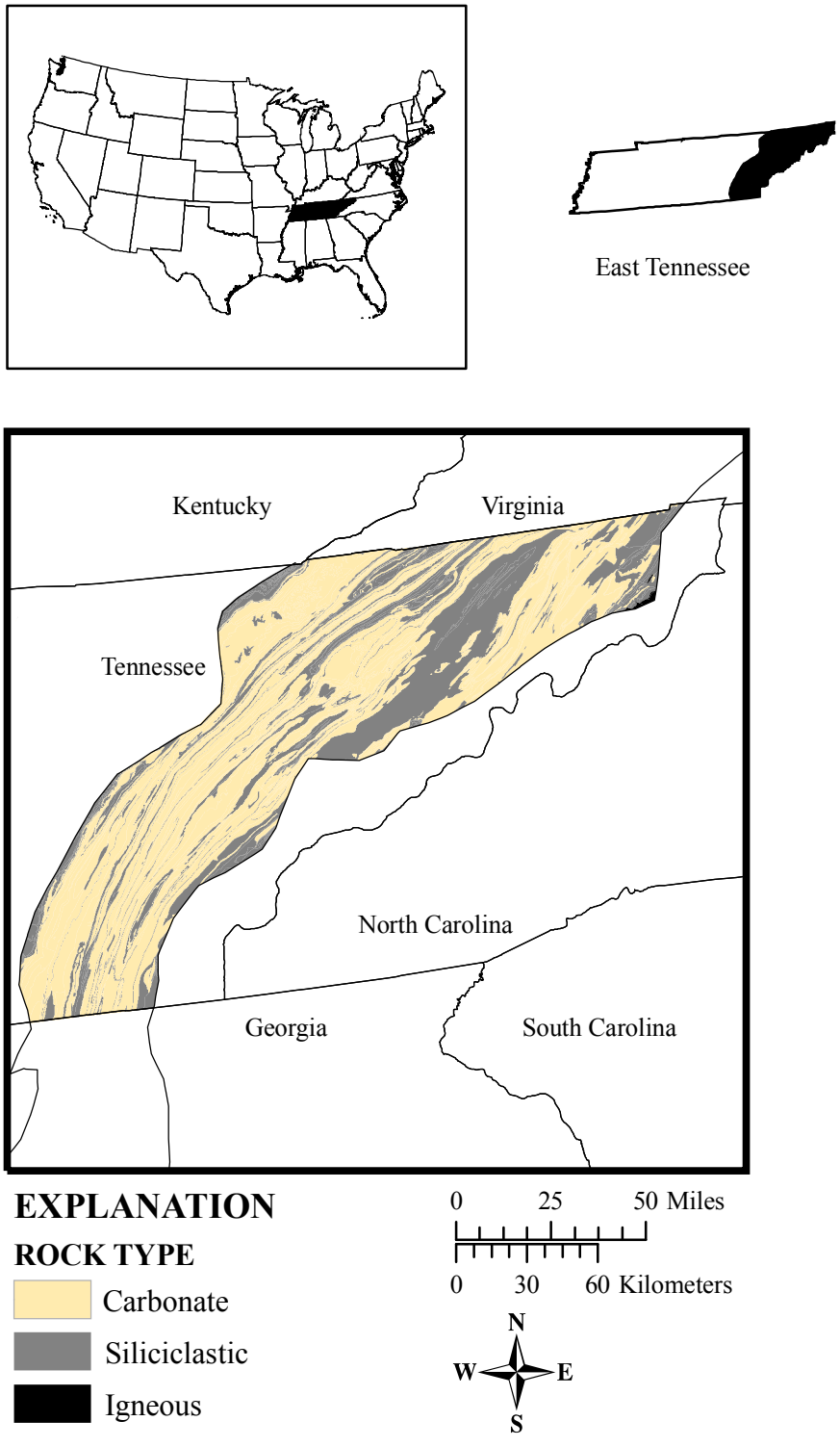

Figure 2-2. Generalized geologic map of the Valley and Ridge. (Base from Tennessee Division of Geology, 1966, Geologic Map of Tennessee, 1:250,000)

The region gets its name from the characteristic long sub-parallel NE-SW trending ridges and valleys in which the geologic units have been thrust several miles northwestward and dip steeply to the southeast. In general, ridges are mostly comprised of the less soluble carbonates and sandstones, while valleys are underlain by more soluble carbonates and shales (DeBuchananne and Richardson 1956). Due to the extensive thrust faulting of these formations, the Rome Formation, the Conasauga Group, and the Knox Group are found throughout the Valley and Ridge in repeating sequences (Figure 2-3) and form a series of compartmentalized aquifer systems (Brahana et al. 1986 and Seaber et al. 1988).

The transition zone between the Valley and Ridge and Blue Ridge physiographic provinces has the structural features of the Valley and Ridge but with rock formations more common to the western Blue Ridge including the Unicoi, Hampton, and Erwin Formations of the Cambrian-aged Chilhowee Group. These formations are comprised of shales, siltstones, feldspathic sandstones, and conglomerates. In this transition zone, ground water occurs mainly in the fractures, joints, and bedding planes of the deformed rocks in the first several hundred feet below ground surface (Brahana et al. 1986).
In the Valley and Ridge, ground water occurs in the dissolutionally-enlarged openings of carbonate rocks, in the fractures of sandstones and shales (especially calcareous shales), and along the bedding planes of the carbonates and shales (Brahana et al. 1986). The most productive aquifers in the region are carbonates, found in the limestones of the Chickamauga Group, limestone and dolomite of the Knox Group, Conasauga Group, and Rome formation, and Shady dolomite (Johnson 2002). Most of the permeability of these aquifers results from karst processes. In most cases they are described as unconfined karst aquifers, and are considered to be the most susceptible to contamination of all

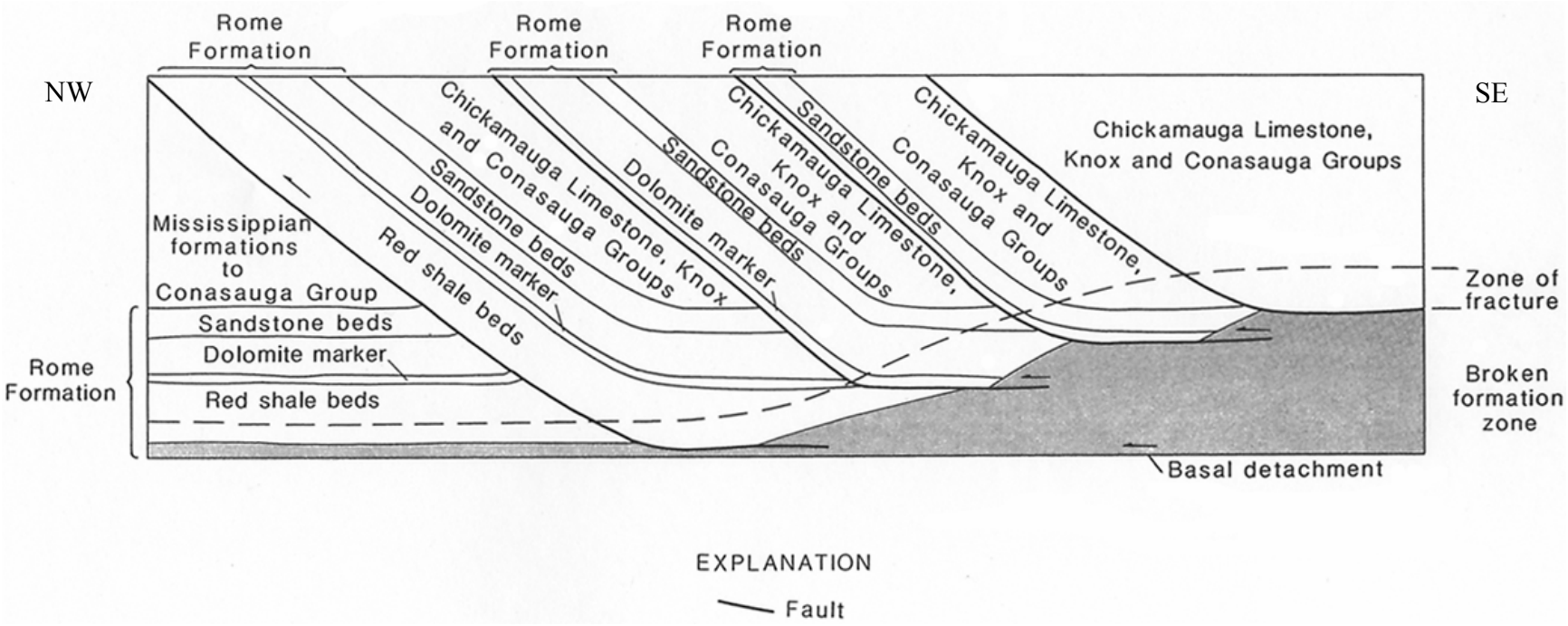

Figure 2-3. Generalized geologic cross section through the Valley and Ridge (Modified from Brahana et al. 1986).

aquifers in the region (Hampson et al. 2000). Figure 2-4 is a generalized hydrogeologic cross section through the Valley and Ridge. Recharge occurs primarily by infiltration of rainfall through the 1-10 meters of weathered residuum and thin soil that mantles the aquifers but also through sinkholes and sinking/losing streams. Natural discharges are numerous (and sometimes large) springs (Brahana et al. 1986).

Although the karst aquifers of the Valley and Ridge are vulnerable to contamination, they are productive and thus important ground water resources to the people of East Tennessee. About 17% of the population of the Valley and Ridge of East Tennessee relies on public supply wells and springs in these aquifers for drinking water. Another 21% of the population obtains their drinking water from domestic wells and springs (including roadside springs) in these aquifers (Johnson 2002). Typical well yields vary from 20 to 760 liters per minute (LPM) (5 to 200 gallons per minute (GPM)), but some wells, located in substantial dissolution features, yield as much as 7,570 LPM (2,000 GPM) (Webbers 2003). The majority of the surveyed springs in the area have discharges less than 760 LPM (200 GPM), but many, especially springs used as public water supplies, yield over 3,800 LPM (1,000 GPM) and several yield between 19,000 and 170,000 LPM (5,000 and 45,000 GPM) (Sun et al. 1963). In 2000, 170 million liters per day (MLD) (44 million gallons per day (MGD)) of ground water was withdrawn from wells in East Tennessee aquifers by 93 public water systems in 31 counties. Another 100 MLD (27 MGD) was provided by springs originating in the Cambrian-Ordovician aquifers of the Valley and Ridge (Webbers 2003).

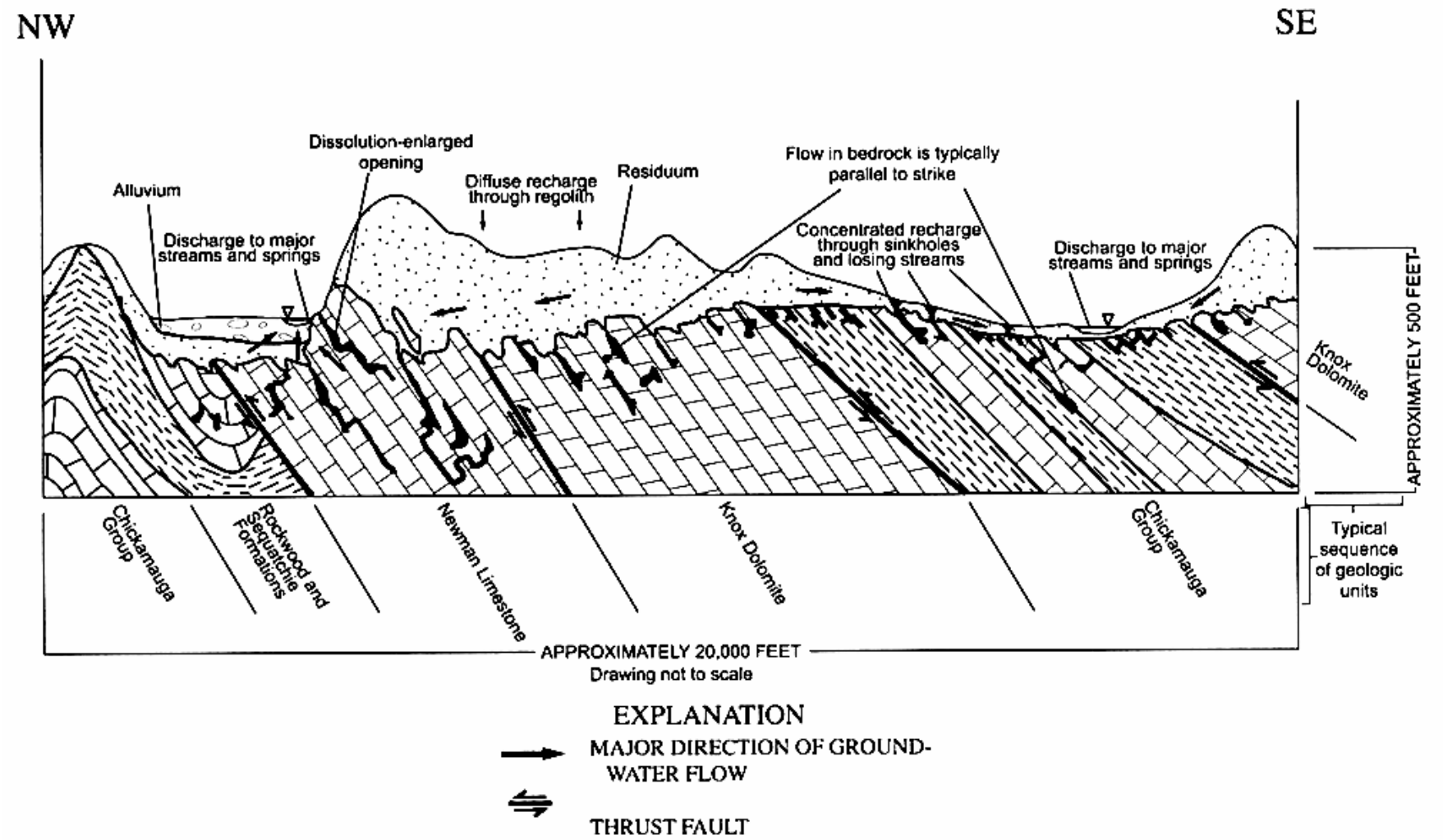

Figure 2-4. Generalized hydrogeologic cross section through the Valley and Ridge (from Johnson 2002).

Site Selection Process

The wells and springs chosen for this study were selected based on their expected risk of fecal contamination, with half of the sites designated as "high risk" for fecal contamination and the other half designated as "low risk" for fecal contamination. This designation was based on historical records of *E. coli* measurements carried out by water treatment plants, preliminary *E. coli* data collected by the research team (Appendix 2-1), the presence or absence of potential contamination sources at each site, and geologic factors such as the presence of thick layers of residuum or sediments which could hinder the movement of pathogens to the aquifer.

Wells or springs that were always positive for *E. coli*, had 100-1000 CFU/100 mL of *E. coli* in storms, had obvious potential sources of human fecal contamination nearby on the watershed, and had geologic factors likely to lead to vulnerability of the aquifer were designated as "high risk" for fecal contamination and thus vulnerable to viral contamination. Wells or springs that were usually to always negative for *E. coli*, always had less than 10 CFU/100 mL of *E. coli* in storms, had few or no known potential sources of human fecal contamination nearby on the watershed, and had geologic factors likely to provide protection to the aquifer were designated as "low risk" for fecal contamination and thus less vulnerable to viral contamination. For the "low risk" sites, every attempt was made to find the lowest risk public water systems in the region using any available TDEC public water system data. The "high/low risk" designation had two purposes. The first was to aid in data interpretation. The second was to, for this first survey of enteric viruses in East Tennessee ground water, target sites that would be the most likely to be

contaminated with viruses and also sites that would have a low likelihood of being contaminated with viruses.

Another important goal of the site selection process was to select wells and springs used as drinking water supplies for public water systems. A secondary goal was to select wells and springs representative of different sizes, geologic settings, and geographic locations of East Tennessee public water systems. Other selection criteria included the willingness of the public water system to participate in the study, the accessibility of the wellhead or spring box tap, and proximity of the site to UTK. After agreeing to participate in the study, all of the utilities managers and water treatment plant operators at the selected sampling locations received a letter (Appendix 2-2) and verbal communication thoroughly explaining the purpose of the research and the activities of the field crew before sampling commenced.

Site Characterization

As a result of the site selection process, a total of 8 sites (4 wells and 4 springs) from public water systems serving communities ranging from 750 to 55,000 people in East Tennessee were selected for viral sampling. Table 2-1 lists the site ID's for the selected wells and springs and gives the risk designation for each. The general locations of the sampling sites are given in Figure 2-5. Tables 2-2 and 2-3 list the physical and water-use characteristics for the selected wells and springs, respectively. Seven of the sites were located in karst aquifers of the Valley and Ridge and one site was located in a highly fractured rock aquifer in the transition zone between the Valley and Ridge and Blue Ridge physiographic provinces. Six of the eight sites were designated by the state (TDEC Division of Water Supply) as GWUDI (ground water under the direct influence

Table 2-1. Site ID's and risk designations for wells and springs sampled in this study.

"High Risk" Sites		"Low Risk" Sites	
Site ID	Site Type	Site ID	Site Type
S-1	Spring	W-1	Well
S-2	Spring	W-3	Well
S-4	Spring	W-4	Well
W-2	Well	S-3	Spring

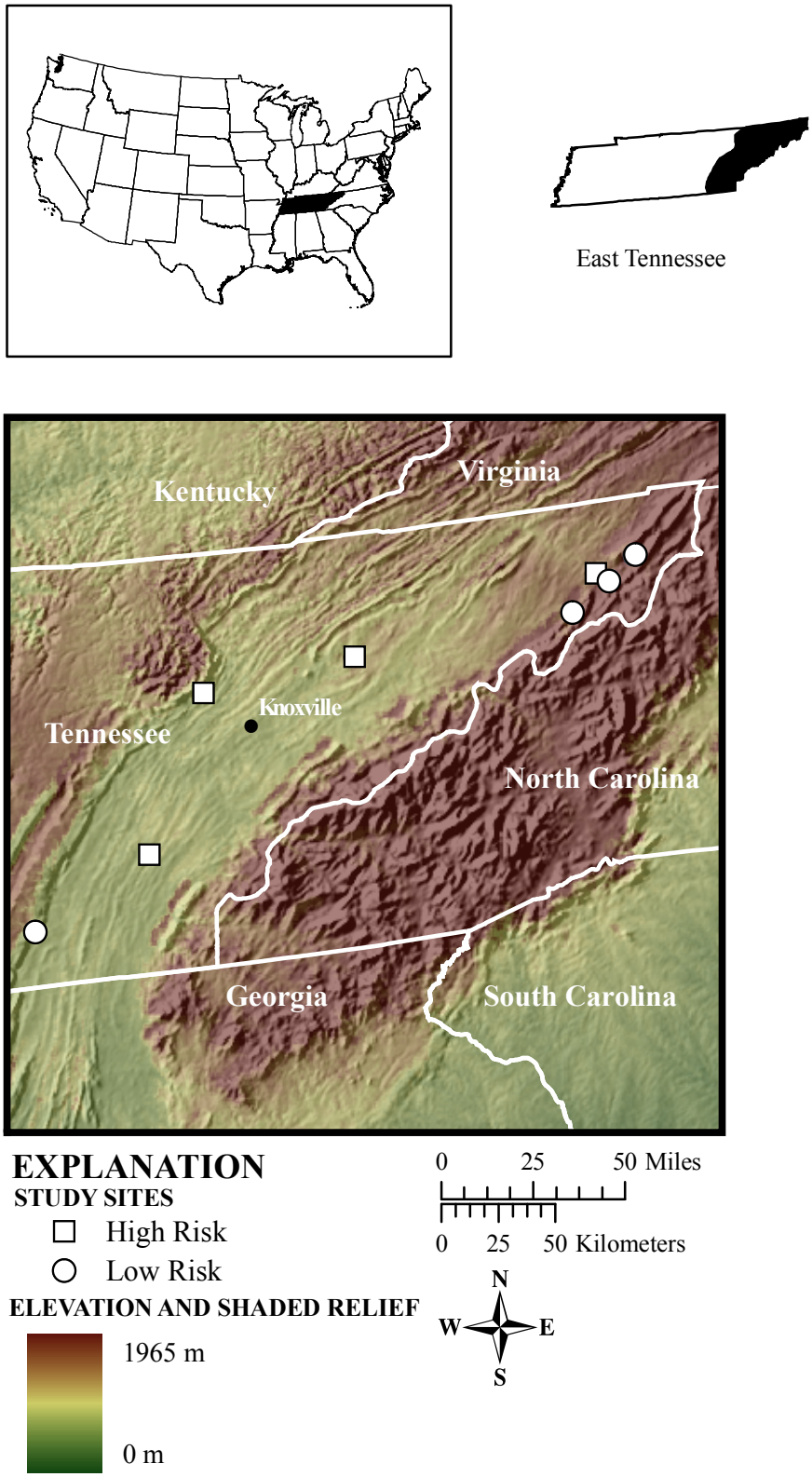

Figure 2-5. Sampling sites and shaded relief.

Table 2-2. Characteristics of wells sampled in this study.

Site ID	Dates Sampled	Risk for Fecal Contamination	Geologic Setting	Land-Use Classification of Watershed	Number of People on PWS	Year of Well Construction	Well Depth (m)	Well Yield (LPM)	Average Daily Pumpage (MLD)	Treatment	GWUDI
W-1	3/1/04 8/10/04	Low	Cambrian-aged Honaker Dolomite of the Conasauga Group	Near city limits/forest/ agriculture	11,600	1993	93	4,500	3.4	Disinfection, fluoride adjustment	Yes
W-2	4/12/04 8/16/04	High	Ordovician-aged Knox Group Carbonates undifferentiated	Inside city limits	17,000	2001	34	>3,800	1.5	Pre and post chlorination, coagulation, sedimentation, filtration, fluoride adjustment	Yes
W-3	8/3/04	Low	Cambrian-aged Unicoi Formation of the Chilhowee Group	National forest	750	1960's	64	190*	0.19	Filtration, disinfection	Yes
W-4	8/4/04	Low	Cambrian-aged Copper Ridge Dolomite of the Knox Group	Some development/ forest/ agriculture	55,000	1993	126	>7,600	7.6	Disinfection, fluoride adjustment	No

*Indicates pumping capacity because well yield data was unavailable

Abbreviations: PWS, public water system; m, meters; LPM, liters per minute; MLD, millions of liters per day; GWUDI, ground water under the direct influence of surface water

Table 2-3. Characteristics of springs sampled in this study.

Site ID	Dates Sampled	Risk for Fecal Contamination	Geologic Setting	Land-Use Classification of Watershed	Number of People on PWS	Average Discharge (LPM)	Average Daily Pumpage (MLD)	Treatment	GWUDI
S-1	3/22/04 8/8/04	High	Cambrian-aged Copper Ridge Dolomite of the Knox Group and Maynardville Limestone of the Conasauga Group	Some development/ forest/ agriculture	5,300	6,800	2.6	Pre and post chlorination, coagulation, filtration, fluoride adjustment	Yes
S-2	3/29/04 8/14/04	High	Ordovician-aged Knox Group Carbonates undifferentiated	Inside city limits	28,750	12,100	4.9	Pre and post chlorination, coagulation, filtration, fluoride adjustment	Yes
S-3	5/19/04 8/9/04	Low	Cambrian-aged Rome Formation (dolomitic sandstone, siltstone, and shale, dolomite, and limestone) of the Conasauga Group	Inside city limits	28,750	16,000	12.0	Disinfection, fluoride adjustment	No
S-4*	6/7/04 8/15/04	High	Ordovician-aged Mascot Dolomite of the Knox Group	Inside city limits	8,000	66,000	N/A	N/A	Yes

*S-4 serves only as a back up water source for a public water system, and the water receives full treatment as surface water if/when it is used.
Abbreviations: N/A, not applicable; PWS, public water system; LPM, liters per minute; MLD, millions of liters per day; GWUDI, ground water under the direct influence of surface water

of surface water). Under the Surface Water Treatment Rule (USEPA 1989), all public drinking water systems were required to determine whether their raw ground water sources were directly influenced by surface water. This determination was made for wells and springs in the state of Tennessee using a combination of hydrogeologic setting information, sanitary surveys, well construction logs and other infrastructure information, monitoring for fluctuations in chemical and physical parameters, and most importantly, MPA (Microscopic Particulate Analysis) tests. MPA tests were performed twice for each suspect well or spring following significant rainfall events. The tests involved filtration of the raw ground water source followed by microscopic analysis of the "bioindicators" such as *Giardia*, algae, insects, and plant debris that were attached to the filter. When a ground water source tested positive for these bioindicators, the source was designated as GWUDI (Draughon 1991).

Four of the sites were designated as "high risk" for fecal contamination and four of the sites were designated as "low risk" for fecal contamination. Most of the "high risk" sites (three out of four) were springs and most of the "low risk" sites (three out of four) were wells. This was expected because in general, the largest springs in the Valley and Ridge (which are more likely to be used as public water supplies) are located in well-developed karst terrain with thin soils, abundant sinkholes, and sinking streams, thereby increasing the potential for fecal contamination from the surface or shallow subsurface. Many wells in the Valley and Ridge, however, are drilled in alluvial valleys where the overlying sediments and weathered residuum provide the aquifer with increased protection from surface or shallow subsurface sources of fecal contamination. In addition, previous studies of microbial contamination in the Valley and Ridge have

confirmed that wells are less susceptible to fecal contamination than are springs (Johnson 2002 and Hampson et al. 2000).

The following sections are brief descriptions of each site (in the order in which they are listed in Table 2-1), with only non-sensitive information discussed. The amount and type of site information available for each sampling location varied. This information was compiled from sources such as TDEC, the USGS, and individual utilities managers and operators, often through verbal or written communication only. Other sources of site information included Wellhead Protection Plans, TDEC Division of Water Supply Source Water Assessments, TDEC Public Water System Data Sheets, and water treatment plant Monthly Operational Reports (MOR's) for the individual public water systems. There were also some publications used to compile the site descriptions that cannot be referenced in association with a specific site at the utilities' request, but are useful in providing background information on the ground water resources of the study areas. They are listed below:

Bradfield, A.D. 1992. Hydrology of the Cave Springs area near Chattanooga, Hamilton County, Tennessee: U.S. Geological Survey Water Resources Investigations Report 92-4018, 28 p.

Bradfield, A.D. 1994. Erwin Utilities develops cost-effective ground-water supplies, Tennessee Chapter, American Public Works Association Tennessee Public Works, Volume 11, Number 7, p. 24-26.

Bradfield, A.D. 1996. Ground water: Tennessee's liquid gold, Tennessee Chapter, American Public Works Association Tennessee Public Works, Volume 14, Number 5, p. 19-20.

Brahana, J.V., D. Mulderink, J.A. Macy, and M.W. Bradley. 1986. Preliminary delineation and description of the regional aquifers of Tennessee – The East Tennessee Aquifer System: U.S. Geological Survey Water Resources Investigations Report 82-4091, 30 p.

DeBuchananne, G.D. and R.M. Richardson. 1956. Ground-water resources of East Tennessee: Tennessee Division of Geology Bulletin 58, Part 1, 393 p.

Hollyday, E.F. and M.A. Smith. 1990. Large springs in the Valley and Ridge Province in Tennessee: U.S. Geological Survey Water Resources Investigations Report 89-4205, 9 p.

Ogden, A.E., K.G. Hamilton, and T.L. Brown. 1990. Delineation of "Wellhead Protection Areas" for municipal used springs of eastern Tennessee, Research Project Technical Completion Report #124 submitted to the Tennessee Water Resources Research Center, The University of Tennessee, Knoxville, Tennessee, 128 p.

Ogden, A.E. and S.C. Kimbro. 1997. Exploratory drilling in Hixson, Tennessee discovers "new" aquifer overlying the karstic Knox Dolomite, Proceedings of the Karst-Water Environment Symposium, p. 80-91.

Sun, P-C.P., J.H. Criner, and J.L. Poole. 1963. Large springs of East Tennessee: U.S. Geological Survey Water-Supply Paper 1755, 52 p.

Webbers, A. 2003. Public water-supply systems and associated water use in Tennessee, 2000: U.S. Geological Survey Water Resources Investigations Report 03-4264, 96 p.

Site S-1

Site S-1 is a spring that discharges at the base of an amphitheater-shaped carbonate bluff. The spring issues from a cave at the contact between the Cambrian-aged lowest member of the Knox Group, the Copper Ridge Dolomite, and the Cambrian-aged upper member of the Conasauga Group, the Maynardville Limestone. The spring discharges as it contacts a relatively impermeable shale member of the Upper Cambrian part of the Conasauga Group (probably the Nolichucky Shale). The spring has an average discharge of about 6,800 LPM (1800 GPM), but flows have been observed as low as 1,500 LPM (400 GPM) and as high as 22,000 LPM (5,800 GPM).

Site S-1 has served as a raw water source for a public water system for a nearby town and its surrounding areas since the late 1940's. Today the public water system

serves about 5,300 people. Average daily pumpage from the spring is about 2.6 MLD (0.7 MGD). The utility also purchases 7.6 million liters (2 million gallons) per month from a neighboring utility. Treatment at the site consists of prechlorination, coagulation, filtration, disinfection with chlorine, and fluoride adjustment. The watershed area for the spring contains some large sinkholes and the spring is designated by the state as GWUDI. During sampling, the field crew inspected the watershed and observed some large sinkholes draining a few home construction sites.

This public water system was given a susceptibility score of "moderate" in the TDEC Source Water Assessment because the raw water source is derived from a fractured/karst rock aquifer, is under the influence of surface water, and has cemeteries, homes with septic tanks, highways, railroads, and a few small businesses within either Zone 1 or 2 of its Wellhead Protection Area. The TDEC Source Water Assessment used a host of factors, including the geologic setting of the aquifer (7 points for a karst/fractured rock aquifer) and the source's GWUDI designation (14 points if the source was GWUDI) to determine the risk of contamination (microbial or otherwise) to the ground water source. Each factor was assigned a point value out of 100 possible points. Sources with a total score of less than 20 points were assigned a low susceptibility rating, sources with a total score of 20 to 40 points were assigned a moderate susceptibility rating, and sources with a total score of greater than 40 points were assigned a high susceptibility rating. More information about the way susceptibility was scored in the TDEC Source Water Assessments is available at http://www.state.tn.us/environment/dws.

Various water-quality parameters for the raw spring water are collected on a daily basis by the water treatment plant. Table 2-4 gives the monthly averages, maximums, and minimums for water temperature, turbidity, pH, alkalinity, and pumping rates measured at the water treatment plant for site S-1 from January 2003 through December 2004.

For this virus study, site S-1 was assumed to have a "high risk" of fecal contamination. The spring was sampled on March 22, 2004 and August 8, 2004. Samples for viruses were collected from a faucet in the spring pump house, prior to any water treatment. Water for other analyses was collected directly from the spring as it discharged out of the rock.

Site S-2

Site S-2 is a spring located within a hilly, well-developed sinkhole plain underlain by a broad northeast-trending syncline of Cambrian and Ordovician-aged rocks (Knox and Conasauga Groups). The spring issues from crevices beneath a bluff formed by interbedded cherty dolomite and limestone of either the lower Knox Group or the upper Conasauga Group. The spring has an average discharge of about 12,000 LPM (3,200 GPM), but flows have been observed as low as 2,400 LPM (630 GPM) and as high as 33,000 LPM (8,660 GPM). Site S-2 is one of three springs used by a public water system that serves about 28,750 people. The spring has been a water source for the city since 1981. The spring is pumped at an average daily rate of about 5 MLD (1.3 MGD) and makes up about 23% of the total public water system production of 23 MLD (6 MGD).

Site S-2 is designated by the state as GWUDI and turbidity values spike (sharply increase, then decrease) suddenly during storm events, indicating rapid recharge. Treatment consists of prechlorination, coagulation with poly-aluminum chloride,

Table 2-4. Raw water-quality parameters measured by the treatment plant for site S-1 from January 2003 to December 2004.

Month/ Year	Daily Pumping Rate (MLD)			Temperature (°C)			Turbidity (NTU)			pH (standard units)			Alkalinity (mg/L as $CaCO_3$)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	2.7	3.3	1.6	14	15	13	0.71	1.71	0.34	7.4	7.5	7.3	156	160	154
Feb/03	2.5	3.3	1.4	14	14	14	9.84	48.0	0.53	7.3	7.5	7.2	155	160	146
Mar/03	2.4	3.1	1.0	15	15	14	2.79	8.21	0.67	7.2	7.3	7.1	150	160	144
Apr/03	2.4	3.0	1.7	15	15	15	4.68	26.5	0.98	7.2	7.4	7.1	145	150	140
May/03	2.5	3.1	1.1	15	15	15	2.55	20.1	0.32	7.3	7.4	7.3	145	150	140
Jun/03	2.6	3.2	1.1	15	16	15	2.15	11.3	0.36	7.2	7.4	7.0	145	150	140
Jul/03	2.6	3.3	0.4	16	16	16	3.18	9.92	0.70	7.1	7.2	7.1	149	154	146
Aug/03	2.7	3.2	1.7	16	16	16	1.27	5.84	0.32	7.2	7.2	7.1	154	158	150
Sep/03	2.7	3.2	2.2	16	16	15	1.65	4.59	0.56	7.1	7.2	7.1	155	158	148
Oct/03	2.5	3.1	1.9	15	15	15	1.15	2.63	0.64	7.1	7.2	7.0	156	158	154
Nov/03	2.4	3.0	1.8	15	15	14	1.85	10.3	0.55	7.2	7.3	7.1	154	159	150
Dec/03	2.5	3.1	1.8	15	15	14	1.18	9.87	0.53	7.3	7.4	7.1	155	156	152
Jan/04	2.6	3.2	1.5	14	15	14	1.34	6.26	0.34	7.4	7.4	7.3	151	158	144
Feb/04	2.6	3.2	1.2	15	15	14	3.31	29.1	0.42	7.3	7.5	7.1	149	154	138
Mar/04	2.5	3.3	0.2	15	15	15	3.17	13.6	0.35	7.2	7.2	7.1	145	152	140
Apr/04	2.7	3.2	2.0	15	15	15	0.88	4.21	0.36	7.2	7.3	7.1	146	150	142
May/04	2.7	3.3	1.8	15	15	15	1.00	6.83	0.48	7.2	7.3	7.1	145	150	140
Jun/04	2.8	3.3	2.0	15	15	15	3.84	20.5	0.33	7.2	7.3	7.1	152	156	148
Jul/04	2.5	3.3	0.8	15	15	15	25.0	51.7	8.56	7.2	7.3	7.1	150	156	146
Aug/04	2.6	3.3	1.4	15	15	15	2.30	31.9	0.53	7.2	7.2	7.1	153	156	150
Sep/04	2.8	3.3	1.9	15	15	15	2.25	23.3	0.55	7.1	7.3	7.0	155	158	152
Oct/04	2.8	3.3	2.0	15	15	15	1.03	3.51	0.51	7.1	7.2	7.1	156	158	154
Nov/04	2.6	3.4	1.1	15	15	15	2.40	10.2	0.46	7.1	7.3	7.0	155	160	150
Dec/04	2.6	3.4	0.6	15	15	14	5.11	46.6	0.66	7.2	7.3	7.0	154	156	150

Abbreviations: MLD, million of liters per day; °C, degrees Celsius; NTU, nephelometric turbidity units; mg/L as $CaCO_3$, milligrams per liter as calcium carbonate

filtration through two high-pressure filters (anthracite, sand, and rock), phosphate for corrosion control, fluoride adjustment, and disinfection with chlorine. Various water quality parameters for the raw spring water are collected on a daily basis by the water treatment plant. Table 2-5 gives the monthly averages, maximums, and minimums for water temperature, turbidity, pH, and pumping rates measured at the water treatment plant for site S-2 from selected months of 2003 and 2004 (UTK was not able to obtain data for all of 2003 and 2004). The water treatment plant for site S-2 also tests the raw water daily for the presence of total coliforms and *E. coli* using the IDEXX Colilert® Test, and the spring tested positive for both total coliforms and *E. coli* every day of 2004. This public water system was given a susceptibility score of "high" in the TDEC Source Water Assessment because the water is derived from a fractured/karst rock aquifer under the influence of surface water, and there are many potential contaminant sources in Zones 1 or 2 of its Wellhead Protection Area. These sources include septic systems, agricultural activities, livestock production, junk/trash in sinkholes, and abandoned wells.

For this virus study, site S-2 was assumed to have a "high risk" of fecal contamination. The spring was sampled on March 29, 2004 and August 14, 2004. Samples were collected directly from the spring as it discharged from underneath a concrete enclosure which houses the pump and protects the spring orifice from surface runoff.

Site S-4

Site S-4 is a spring that upwells from a large water-filled cavern in the underlying folded and faulted carbonate rocks. The spring issues from the Early Ordovician-aged Mascot Dolomite, a siliceous dolomite member of the Knox Group. During high flow, a

Table 2-5. Raw water-quality parameters measured by the treatment plant for site S-2 from selected months of 2003 and 2004.

Month/ Year	Daily Pumping Rate (MLD)			Temperature (°C)			Turbidity (NTU)			pH (standard units)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	4.92	6.09	2.54	14	15	13	0.98	6.82	0.39	7.18	7.23	7.11
Feb/03	3.63	5.68	0.57	14	15	12	11.71	53.30	0.88	7.18	7.31	7.00
Mar/03	NT	NT	NT	15	15	14	3.83	16.20	0.80	7.36	7.49	7.28
Apr/03	5.07	6.51	3.44	15	15	15	6.69	51.20	1.09	7.31	7.43	7.06
May/03	5.45	7.00	2.88	15	15	15	4.05	27.34	0.88	7.60	7.81	7.41
Jun/03	5.15	5.90	4.16	15	17	15	4.00	13.12	0.97	7.67	7.84	7.50
Aug/03	5.22	6.25	3.86	16	17	16	15.34	97.80	1.43	7.41	7.49	7.30
Sep/03	5.19	5.87	3.75	16	16	15	1.50	5.05	0.59	7.39	7.54	7.16
Oct/03	4.73	5.90	3.48	15	16	15	1.53	2.83	1.00	7.09	7.33	6.92
Nov/03	4.88	5.41	4.24	16	17	15	37.77	784	1.01	7.03	7.28	6.60
Dec/03	4.84	5.75	3.10	15	15	15	2.59	9.10	1.29	7.13	7.20	7.04
Jan/04	5.11	5.87	4.28	15	16	14	1.30	4.91	0.62	7.18	8.90	6.93
Mar/04	4.92	5.64	3.18	15	16	15	2.01	12.70	0.73	7.21	7.70	6.95
Aug/04	5.30	6.85	3.07	17	18	16	0.61	3.80	0.03	7.64	8.10	7.44

Abbreviations: NT, not tested; MLD, million of liters per day; °C, degrees Celsius; NTU, nephelometric turbidity units

large boil is visible but usually the spring looks like a large circular pond that outlets to a stream which eventually flows into a Tennessee Valley Authority (TVA) lake. The area surrounding the spring has well-developed karst topography with abundant sinkholes. The spring is large and has an average discharge of about 66,000 LPM (14,450 GPM), but flows have been observed as low as 12,000 LPM (2,700 GPM) and as high as 182,000 LPM (40,400 GPM).

Site S-4 was used as the primary water source for a public water system from 1941 to 1976 and during that time was pumped at an average daily rate of about 11 MLD (3 MGD). However, the water quality of the spring was highly variable and the water had to be treated at a surface water plant. In 1976, the utility drilled a well 610 m (2,000 ft) northwest of the spring into a mine shaft of an abandoned and flooded zinc mine. Since that time the well has been used as the primary water source for the city (current population is about 8,000) and the spring now serves only as the backup source. The well is pumped at an average daily rate of 11 MLD (3 MGD). The utility pumps the spring for a few days at the beginning of each quarter to assess the water quality. The spring is chemically similar to the well but has higher and more variable turbidity values. Table 2-6 gives some raw water-quality data collected by the water treatment plant for site S-4 during pumping of the spring on the first few days of each quarter of 2004. This public water system was given a susceptibility score of "high" in the TDEC Source Water Assessment because the state considers the well GWUDI and the spring a surface water source, and there are potential contamination sources located within Zones 1 or 2 of the Wellhead Protection Area such as highways, railroads, cemeteries, small farms that practice sludge application, and hazardous waste and superfund facilities.

Table 2-6. Raw water-quality parameters measured by the water treatment plant for site S-4 from days the spring was pumped in 2004.

Date	Daily Pumping Rate (MLD)	Temperature (°C)	Turbidity (NTU)	Alkalinity (mg/L as $CaCO_3$)	pH (standard units)	Hardness (mg/L as $CaCO_3$)
1/1/04	11.5	15	3	255	7.4	200
1/2/04	11.9	15	2	250	7.3	210
1/3/04	11.1	15	2	250	7.4	200
1/4/04	11.5	16	4	255	7.4	200
4/1/04	11.8	16	7	255	7.0	210
4/2/04	10.5	15	7	245	7.0	210
4/3/04	10.5	15	11	250	7.1	210
4/4/04	11.1	15	11	245	7.1	210
7/1/04	13.0	16	4	250	7.0	220
7/2/04	13.1	17	4	250	7.1	210
7/3/04	12.6	17	3	260	7.1	200
7/4/04	11.5	17	3	255	7.1	200
10/1/04	10.1	17	4	250	7.4	220
10/2/04	9.7	16	5	245	7.2	210
10/3/04	9.5	16	4	245	7.2	210
10/4/04	12.1	16	3	240	7.3	210

Abbreviations: MLD, million of liters per day; °C, degrees Celsius; NTU, nephelometric turbidity units; mg/L as $CaCO_3$, milligrams per liter as calcium carbonate

For this virus study, site S-4 was assumed to have a "high risk" of fecal contamination. The spring was sampled on June 7, 2004 and August 15, 2004. Samples were collected from the spring pool, a few feet from the edge.

Site W-2

Site W-2 is a well that was completed in 2001. The well field for site W-2 is in a creek basin underlain by Ordovician-aged Knox Group formations. The Knox Group consists of formations that are dominated by siliceous dolomite, interbedded limestone (thick in some places), and some sandstone beds. The bedrock is fractured and karst and is overlain by about 9 m (31 ft) (in the immediate vicinity of the well) of the weathered clay residuum that typically blankets the Knox Group throughout the Valley and Ridge.

The well is 34 m (111 ft) deep (see Drillers Report in Appendix 2-3). Because of an extensive fractured zone from about 14 m to 25 m (47 to 83 ft) deep, drillers installed perforated or slotted steel pipe from 17 m to 25 m (55 to 83 ft). The borehole is open from 25 m to 34 m (83 to 111 ft). The estimated well yield is $3{,}800^{+}$ LPM ($1{,}000^{+}$ GPM). The well is pumped at an average daily rate of about 1.5 MLD (0.4 MGD). This well is one of four raw water sources (three wells and one spring) for a public water system that serves about 17,000 people. Average daily production for all four sources combined is about 9.5 MLD (2.5 MGD) and site W-2 accounts for about 16% of that production. The utility also purchases about 2.3 MLD (0.6 MGD) from a neighboring utility. The three wells are pumped into the discharge pool of the spring where they mix together before being pumped to the water treatment plant. All four raw water sources are designated by the state as GWUDI and are treated as surface water. Treatment includes prechlorination, coagulation with ferric sulfate, pH adjustment with lime, sedimentation, flow through

sand filters, disinfection with chlorine, fluoride adjustment, and zinc orthophosphate addition for pipe corrosion inhibition.

Various water quality parameters for the raw water sources are collected on a daily basis by the water treatment plant. Table 2-7 gives the monthly averages, maximums, and minimums for water temperature, turbidity, pH, alkalinity, hardness, total coliforms, and pumping rates measured at the water treatment plant from January 2003 through December 2004. It is important to note that these data were collected after all four raw water sources had mixed. Routine raw water-quality measurements are not collected for site W-2 before it mixes with the other three sources. This public water system was given a susceptibility score of "high" in the TDEC Source Water Assessment because the ground water is derived from a fractured/karst rock aquifer under the influence of surface water, and there are many potential contaminant sources in Zones 1 or 2 of its Wellhead Protection Area. These sources include highways, railroads, sewer lines, commercial industries, and the nearby creek (which has tested positive for *Cryptosporidium* in the past).

For this virus study, site W-2 was assumed to have a "high risk" of fecal contamination. The well was sampled on April 12, 2004 and August 16, 2004. Samples were collected from a faucet in the well pump house, before the water mixed with the other sources or received any treatment. There was too much air coming from the faucet to connect the intake hose for the virus sampling apparatus directly to it. Instead, water was allowed to flow from the faucet into a sterile bucket and was then pumped through the virus sampling apparatus using a sterile electric pump.

Table 2-7. Raw water-quality parameters measured by the treatment plant for site W-2 from January 2003 to December 2004. The measurements were taken after all four raw water sources for the utility mixed prior to treatment.

Month/ Year	Daily Pumping Rate (MLD)			Temperature (°C)			Turbidity (NTU)			pH (standard units)			Alkalinity (mg/L as $CaCO_3$)			Hardness (mg/L as $CaCO_3$)			Total Coliforms (CFU/100 mL)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	9.6	12.2	5.0	14	15	13	5.1	15.8	2.8	7.50	7.58	7.42	135	142	130	143	152	138	113	200	25
Feb/03	9.4	11.8	4.8	14	15	14	5.7	22.3	2.3	7.47	7.54	7.32	131	143	112	142	154	120	354	1080	40
Mar/03	9.0	12.1	4.2	15	16	14	3.0	4.4	2.4	7.45	7.55	7.33	126	139	115	132	148	124	165	520	30
Apr/03	9.3	11.5	4.8	15	16	15	6.2	71.6	2.4	7.45	7.52	7.38	130	138	123	139	142	130	801	4600	70
May/03	9.2	11.8	6.1	16	17	15	7.9	11.9	2.0	7.35	7.47	7.23	119	135	104	126	144	110	1233	7400	280
Jun/03	9.4	12.1	5.3	16	17	16	4.2	16.5	2.5	7.43	7.59	7.34	130	138	121	135	142	124	943	6200	200
Jul/03	9.7	11.8	5.0	17	17	16	3.6	8.8	2.2	7.40	7.54	7.31	135	138	131	138	144	130	1619	7900	400
Aug/03	9.3	11.0	5.5	17	17	16	3.0	3.9	2.4	7.42	7.49	7.34	142	146	138	149	154	142	1019	2550	300
Sep/03	9.2	10.8	4.7	17	17	16	4.1	11.0	2.6	7.44	7.52	7.31	146	150	142	153	158	148	1204	4900	200
Oct/03	8.6	10.5	4.5	16	16	15	3.1	5.8	2.4	7.45	7.53	7.33	149	152	147	155	160	150	509	1200	200
Nov/03	7.8	10.1	3.7	15	16	15	3.4	8.7	2.4	7.49	7.57	7.36	148	151	144	151	154	148	1222	6400	200
Dec/03	8.4	10.7	4.2	14	15	14	3.3	9.2	2.1	7.43	7.34	7.52	145	150	138	147	152	140	791	5100	200
Jan/04	8.9	10.4	4.5	14	16	13	3.4	10.3	2.0	7.45	7.51	7.38	138	145	133	143	146	138	967	6800	150
Feb/04	8.9	11.1	4.5	14	15	13	3.8	35.4	1.6	7.41	7.50	7.35	131	138	125	137	146	130	514	1300	100
Mar/04	9.2	11.8	4.6	15	15	14	2.7	5.7	2.0	7.46	7.52	7.38	133	138	130	137	142	134	925	1800	400
Apr/04	8.9	10.9	4.5	15	16	14	2.7	5.4	1.6	7.47	7.53	7.38	137	140	132	139	142	132	979	5300	500
May/04	7.9	10.8	2.5	16	17	14	3.0	4.4	2.3	7.54	7.60	7.45	140	145	135	144	148	138	701	2500	300
Jun/04	8.5	9.7	2.8	16	16	15	3.1	6.7	1.7	7.55	7.61	7.46	142	144	140	144	150	142	597	2200	150
Jul/04	6.5	8.6	2.7	16	17	16	2.3	3.7	1.8	7.52	7.62	7.38	145	153	144	148	158	142	645	1900	250
Aug/04	7.0	9.5	2.8	16	16	15	2.1	3.4	1.6	7.54	7.62	7.48	145	150	142	147	150	142	618	1200	300
Sep/04	7.2	10.2	2.8	15	16	15	3.1	30.0	1.5	7.53	7.61	7.40	141	148	134	147	152	140	807	6000	300
Oct/04	9.2	13.6	2.6	15	16	14	2.8	4.1	2.1	7.51	7.59	7.35	145	152	137	156	160	144	298	600	100
Nov/04	8.9	11.5	5.2	15	15	14	3.8	26.0	1.7	7.49	7.55	7.34	142	151	134	154	160	134	619	2500	200
Dec/04	8.8	10.2	4.7	14	15	13	3.2	18.6	1.2	7.40	7.48	7.33	124	132	117	131	140	122	713	4200	200

Abbreviations: MLD, million of liters per day; °C, degrees Celsius; NTU, nephelometric turbidity units; mg/L as $CaCO_3$, milligrams per liter as calcium carbonate; CFU/100 mL, colony forming units per 100 milliliters of water

Site W-1

Site W-1 is a well that was completed in 1993. The well field for site W-1 is located in a valley underlain by fractured and karst Cambrian-aged rocks. The well is drilled into the Honaker Formation (also known as the Honaker Dolomite) of the Cambrian-aged Conasauga Group. The Honaker Formation consists of dolomite, limestone, and shale with interbedded layers of gravel. The driller's log for this well (Appendix 2-4) indicated about 35 m (115 ft) of weathered-clay residuum and alluvial materials (clay, gravel, sand, and cobbles) overlying fractured or karst interbedded limestone and shale with gravel seams.

The well is 93 m (305 ft) deep, encased in 20.3-cm (8-inch) steel to 68 m (223 ft), and is an open borehole from 68 m to 93 m (223 to 305 ft). From the driller's report, the total well yield was 4,500 LPM (1,200 GPM). The well is pumped at an average daily rate of about 3.4 MLD (0.9 MGD). This well is one of four raw water sources (3 wells and 1 spring) for a public water system that serves about 11,600 people. Average daily production for all four sources combined is about 7.2 MLD (1.9 MGD). Almost half of the water produced from site W-1 is sold to a neighboring town. The rest of the water produced from site W-1 provides about 27% of the total water supply for the public water system.

Site W-1 has been designated in the TDEC Division of Water Supply Source Water Assessment as GWUDI and was given a susceptibility score of "high" because the raw water source is derived from a fractured/karst rock aquifer, is under the influence of surface water, and has highways, cemeteries, facilities with underground injection permits, and hazardous waste and superfund facilities within Zones 1 or 2 of the

Wellhead Protection Area. Filtration, however, is not required at site W-1 and the average daily turbidity of the raw water measured at the treatment plant ranges from only about 0.04 to 0.2 nephelometric turbidity units (NTU). Treatment at this site consists of disinfection with chlorine, zinc orthophosphate for control of pipe corrosion, and fluoride adjustment. The raw water is tested by the utility for total coliforms once per week and during the two-year period from January 2003 to December 2004, the water only tested positive for total coliform bacteria on two days (at a concentration of 1 CFU/100 mL). Neither total coliforms nor *E. coli* were detected at this site during preliminary sampling by researchers at UTK (Appendix 2-1). Various water quality parameters for the raw well water are collected on a daily basis by the water treatment plant. Table 2-8 gives the monthly averages, maximums, and minimums for raw water temperature, turbidity, pH, and pumping rates measured at the water treatment plant for site W-1 from January 2003 through November 2004.

For this virus study, site W-1 was assumed to have a "low risk" of fecal contamination. The well was sampled on March 1, 2004 and August 10, 2004. Samples were collected from a faucet in the well pump house, before the water received any treatment.

Site W-3

Site W-3 is a well that was constructed sometime during the 1960's. Due to the loss of records (including drill logs) during changes in management of the utility, there is little information (including the date of well completion) available about the construction of this well. It is known that the well is about 64 m (210 ft) deep with a 15.2-cm (6-inch) steel casing. The well is drilled on a mountain side into the upper Unicoi Formation of

Table 2-8. Raw water-quality parameters measured by the treatment plant for site W-1 from January 2003 to November 2004.

Month/ Year	Daily Pumping Rate (MLD)			Temperature (°C)			Turbidity (NTU)			pH (standard units)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	3.1	3.3	3.0	12	12	12	0.04	0.06	0.04	7.8	7.8	7.8
Feb/03	3.2	3.5	3.0	12	12	12	0.05	0.08	0.04	7.8	7.8	7.7
Mar/03	3.4	3.6	3.3	12	12	12	0.04	0.06	0.04	7.8	7.8	7.7
Apr/03	3.4	3.7	3.1	12	12	12	0.08	0.09	0.04	7.8	7.8	7.7
May/03	3.4	3.6	3.3	12	12	12	0.09	0.11	0.09	7.8	7.9	7.8
Jun/03	3.4	3.7	3.2	12	12	12	0.09	0.09	0.09	7.9	7.9	7.8
Jul/03	3.4	3.6	3.3	12	12	12	0.10	0.11	0.09	7.7	7.8	7.7
Aug/03	3.4	3.6	3.3	12	12	12	0.11	0.13	0.11	7.8	7.8	7.6
Sep/03	3.4	3.5	3.3	12	12	12	0.11	0.19	0.11	7.8	7.8	7.7
Oct/03	3.4	3.5	3.1	12	12	12	0.12	0.12	0.11	7.8	7.8	7.7
Nov/03	3.4	3.5	3.1	12	12	12	0.17	0.23	0.11	7.7	7.8	7.7
Dec/03	3.4	3.5	3.3	12	12	12	0.19	0.28	0.18	7.8	7.9	7.7
Jan/04	3.4	3.5	3.3	12	12	12	0.19	0.19	0.18	7.8	7.8	7.7
Feb/04	3.4	3.7	2.8	12	12	12	0.16	0.19	0.15	7.7	7.8	7.7
Mar/04	3.4	3.6	3.3	12	12	12	0.15	0.16	0.15	7.8	7.8	7.7
Apr/04	3.4	3.7	3.1	12	12	12	0.16	0.19	0.16	7.7	7.8	7.7
May/04	3.4	3.6	2.9	12	12	12	0.16	0.17	0.14	7.7	7.7	7.7
Jun/04	3.4	3.5	3.3	14	14	14	0.15	0.15	0.15	7.7	7.8	7.7
Jul/04	3.4	3.5	3.3	14	14	14	0.15	0.15	0.15	7.8	7.8	7.8
Aug/04	3.4	3.5	3.3	14	14	14	0.11	0.16	0.09	7.8	7.8	7.7
Sep/04	3.4	3.6	3.3	14	14	14	0.09	0.10	0.09	7.8	7.8	7.7
Oct/04	3.3	3.5	2.6	14	14	14	0.10	0.19	0.09	7.8	7.8	7.7
Nov/04	3.4	3.5	3.3	14	14	14	0.09	0.10	0.09	7.9	7.9	7.8

Abbreviations: MLD, millions of liters per day; °C, degrees Celsius; NTU, nephelometric turbidity units

the Cambrian-aged Chilhowee Group. The upper Unicoi Formation in this location consists of highly fractured feldspathic sandstones and conglomerates. Less than 30 m (100 ft) north of the well is a nearly vertical normal fault which, along with the extensive rock fractures, probably contributes to the productivity of the well.

Site W-3 is one of two wells in the same well field for a small public water system that serves about 750 people. The raw water pump capacity for each well is 190 LPM (50 GPM) and each well is pumped at an average daily rate of about 190,000 liters per day (50,000 gallons per day). However, only one well is pumped at a time, they are rotated every few days, and do not always run every day. The storage tank for the water treatment plant holds 303,000 liters (80,000 gallons) of treated water. About 76,000 to 95,000 liters (20,000 to 25,000 gallons) of finished water are pumped to the system in the winter and about 114,000 to 132,000 liters (30,000 to 35,000 gallons) in the summer. Treatment at this site consists of gravity filtration through two slow sand filters each 76 m^2 (822 ft^2), disinfection with sodium hypochlorite, pH adjustment with sodium hydroxide, and addition of a corrosion inhibitor. According to the utility managers, neither the raw water nor water in the distribution system has tested positive for total coliform bacteria for at least seven years (since the last change in utility management). This public water system was given a susceptibility score of "moderate" in the TDEC Source Water Assessment because the wells are drilled in a fractured rock aquifer and are considered by the state as GWUDI.

The water treatment plant for site W-3 does very little monitoring of its raw water. Table 2-9 gives the monthly averages, maximums, and minimums for raw water turbidity and raw water temperature measured at the water treatment plant for site W-3

Table 2-9. Raw water-quality parameters measured by the water treatment plant for site W-3 from January 2003 to December 2004.

Month/ Year	Finished Water Pumped to System (MLD)			Temperature (°C)			Turbidity (NTU)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	0.07	0.17	0.05	9	10	8	0.04	0.05	0.03
Feb/03	0.07	0.20	0.04	9	10	8	0.04	0.07	0.04
Mar/03	0.06	0.12	0.04	11	12	9	0.05	0.06	0.04
Apr/03	0.06	0.07	0.04	14	16	12	0.04	0.06	0.03
May/03	0.10	0.19	0.05	14	15	14	0.04	0.08	0.03
Jun/03	0.09	0.13	0.05	18	19	16	0.04	0.08	0.03
Jul/03	0.09	0.20	0.05	21	22	19	0.03	0.05	0.03
Aug/03	0.12	0.30	0.04	21	22	20	0.03	0.07	0.03
Sep/03	0.14	0.34	0.03	19	22	18	0.07	0.15	0.04
Oct/03	0.05	0.14	0.01	16	18	16	0.05	0.08	0.03
Nov/03	0.08	0.19	0.03	16	17	14	NT	NT	NT
Dec/03	0.08	0.15	0.05	15	16	14	NT	NT	NT
Jan/04	0.09	0.14	0.05	15	16	14	NT	NT	NT
Feb/04	0.09	0.14	0.06	14	16	11	NT	NT	NT
Mar/04	0.10	0.17	0.04	13	15	11	NT	NT	NT
Apr/04	0.08	0.17	0.03	13	14	12	NT	NT	NT
May/04	0.07	0.21	0.02	15	16	14	NT	NT	NT
Jun/04	0.06	0.10	0.02	17	19	15	NT	NT	NT
Jul/04	0.11	0.23	0.04	20	20	19	NT	NT	NT
Aug/04	0.22	1.01	0.01	19	20	18	NT	NT	NT
Sep/04	0.16	0.52	0.03	16	17	15	NT	NT	NT
Oct/04	NT	NT	NT	14	16	13	NT	NT	NT
Nov/04	0.18	0.26	0.09	13	14	12	NT	NT	NT
Dec/04	0.23	0.90	0.05	11	12	9	NT	NT	NT

Abbreviations: NT, not tested; MLD, millions of liters per day; °C, degrees Celsius; NTU, nephelometric turbidity units

from January 2003 through December 2004. The table includes both wells and the treatment plant did not record on the MOR which well was being pumped on a given day. The water quality from the two wells is very similar except for higher iron content in the well that was not sampled during this study. To give an indication of water usage for the utility, the amount of finished water pumped to the system from the storage tanks is also given in the table. The utility did not record the pumping rates of the wells for the years of data given in the table.

For this virus study, site W-3 was assumed to have a very "low risk" of fecal contamination especially since the entire Wellhead Protection Area/recharge area for the well is located within National Forest. The well was only sampled once during the study. Samples were collected on August 3, 2004 from a tap near where the well water discharges into the sand filters, prior to receiving any treatment. On the day of sampling, site W-3 was the only well in the well field being pumped.

Site W-4

Site W-4 is a well that was completed in 1993. The well field for site W-4 is located within a broad northeast-trending syncline of the Knox Group. The bedrock is fractured and karst, and is overlain by about 30 to 46 m (100 to 150 ft) of chert-rich weathered clay residuum which provides substantial protection from surficial contaminants. The contact between the Cambrian-aged Copper Ridge Dolomite and Ordovician-aged Chepultepec Formation intersects the well field. The Copper Ridge Dolomite is the lowest formation of the Knox Group and, in this location, is characterized by massive beds of dolomitic sandstone. The lower Chepultepec Formation consists of chert-rich, sandy dolomite and thin beds of sandstone and cherty limestone. The well is

drilled into these formations and much of its productivity comes from a relatively clean chert gravel aquifer at the top of the Copper Ridge Dolomite.

The well is 126 m (415 feet) deep and is open borehole from 78 to 126 m (257 ft to 415 ft) and screened from 67 to 78 m (221 to 257 ft) (Appendix 2-5). The estimated well yield is $7{,}600^{+}$ LPM ($2{,}000^{+}$ GPM). The well is pumped at an average daily rate of about 7.6 MLD (2 MGD). This well is one of six wells (two wells in the well field for site W-4 and four wells in a separate well field) for a public water system that serves about 55,000 people. Average daily production for all six wells combined is about 25 MLD (6.5 MGD) but can range between 19 and 32 MLD (5 and 8.5 MGD). Site W-4 is usually rotated on a monthly to several-month basis with a second well in the same well field except for a few days each summer when both wells are pumped simultaneously.

Unlike many wells and springs in the Valley and Ridge of East Tennessee, the water derived from site W-4 has been designated by TDEC as "true groundwater", and is not considered under the influence of surface water. The Division of Water Supply considered multiple factors in the assignment of this designation. First, the bedrock in this area is largely composed of sandy dolostones and dolomitic sandstones and is therefore less prone to solution enlargement of fractures than are dolomites of the Knox Group typically used as aquifers. Second, the aquifer is protected from surface water influences as well as shallow subsurface contaminant sources by the overlying 46 m (150 ft) of clay residuum. Raw water turbidity for site W-4 is very stable and low and generally ranges from about 0.03 to 0.04 NTU, thereby exempting the utility from filtration. In addition, results of a dye trace in a nearby ephemeral stream in August of 1996 and two MPA (Microscopic Particulate Analysis) tests in 1995 (conducted after

heavy rains) confirmed that there was virtually no influence of local surface water infiltration on the ground water. Lastly, the well has never tested positive for *E. coli* and preliminary sampling by researchers at UTK found only very low levels of total coliform bacteria when present (Appendix 2-1). Table 2-10 gives the monthly averages, maximums, and minimums for raw water turbidity, pH, and pumping rates measured by the water treatment plant for site W-4 from January 2003 through December 2004. Treatment at this site consists of disinfection with chlorine, zinc orthophosphate for control of pipe corrosion, and fluoride adjustment. Interestingly, this public water system was given a susceptibility score of "high" in the TDEC Source Water Assessment because of potential contamination sources located within Zones 1 and 2 of its Wellhead Protection Area such as highways, cemeteries, septic systems, and light industry.

For this virus study, site W-4 was assumed to have a very "low risk" of fecal contamination. It was sampled only once during the study. Samples were collected on August 4, 2004 from a well tap in a concrete pit, as the water was being pumped from the well to the treatment building, prior to receiving any treatment.

Site S-3

Site S-3 is a spring that discharges through alluvium but appears to be sourced primarily from dissolutionally formed flowpaths in the underlying carbonate rock. The spring, which upwells at several points within an area of a few hundred square meters (few thousand square feet), is covered by a cinder block and steel building which houses the pump. The remaining spring water overflows into a reservoir pond. The spring issues from the Cambrian-aged Rome Formation which is highly fractured and faulted in this location. The Rome Formation consists of sandstone, siltstone, shale (often

Table 2-10. Raw water-quality parameters measured by the water treatment plant for site W-4 from January 2003 to December 2004.

Month/ Year	Daily Pumping Rate (MLD)			Turbidity (NTU)			pH (standard units)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	8.10	8.33	6.40	0.04	0.06	0.03	7.25	7.34	7.00
Feb/03*	7.19	8.21	4.39	0.03	0.04	0.03	7.28	7.33	7.14
Mar/03	7.80	8.74	7.15	0.03	0.06	0.01	7.26	7.33	7.12
Apr/03	8.63	12.8	5.60	0.05	0.11	0.01	7.27	7.34	7.20
May/03	7.76	8.33	6.06	0.04	0.06	0.03	7.28	7.34	7.25
Jun/03	7.87	8.93	4.39	0.04	0.06	0.03	7.26	7.30	7.20
Jul/03	7.83	8.10	7.31	0.03	0.04	0.01	7.23	7.28	7.19
Aug/03	7.76	8.02	5.79	0.04	0.04	0.03	7.23	7.29	7.19
Sep/03	8.14	15.0	6.96	0.03	0.04	0.01	7.25	7.33	7.17
Oct/03*	7.49	7.61	7.15	0.03	0.08	0.01	7.27	7.33	7.12
Nov/03*	7.12	7.61	3.29	0.03	0.06	0.01	7.21	7.30	7.00
Dec/03*	7.23	7.57	6.96	0.01	0.03	0.01	7.22	7.30	7.00
Jan/04	7.76	8.44	6.96	0.02	0.04	0.01	7.21	7.30	7.00
Feb/04*	7.46	9.42	7.12	0.01	0.03	0.01	7.23	7.80	7.00
Mar/04	8.44	8.63	8.06	0.05	0.11	0.03	7.22	7.27	7.16
Apr/04*	7.57	8.48	6.81	0.03	0.04	0.01	7.27	7.37	7.21
May/04	8.59	13.1	5.75	0.03	0.04	0.01	7.28	7.32	7.24
Jun/04	10.5	15.3	7.12	0.03	0.08	0.01	7.34	7.50	7.24
Jul/04*	7.38	8.63	6.85	0.03	0.06	0.01	7.50	7.57	7.43
Aug/04*	7.12	7.38	6.78	0.03	0.04	0.01	7.42	7.67	7.20
Sep/04*	7.08	11.0	4.73	0.04	0.11	0.03	7.36	7.76	7.25
Oct/04*	6.93	7.38	6.13	0.03	0.03	0.01	7.48	7.75	7.27
Nov/04*	6.51	7.19	5.56	0.02	0.03	0.01	7.32	7.70	7.11
Dec/04*	6.74	7.46	4.05	0.02	0.04	0.01	7.46	7.92	7.26

*Indicates months during which W-4 is being pumped. During the rest of the months, the other well in the well field is being pumped. Raw water quality for both wells is similar because they are in close proximity to each other and draw water from the same aquifer.
Abbreviations: MLD, millions of liters per day; NTU, nephelometric turbidity units

dolomitic), dolomite, and limestone. The thickness of alluvial deposits at the site is unknown. The spring has an average discharge of about 16,000 LPM (3,500 GPM) and does not exhibit great variation in flow. Site S-3 is one of three springs for a public water system that serves about 28,750 people. It has been a water source for the utility since 1958. The spring is pumped at an average daily rate of about 12 MLD (3.1 MGD) and makes up about 52% of the total public water system production of 23 MLD (6 MGD).

Site S-3 is not considered GWUDI by the state. Turbidity is stable during most storms and rarely exceeds 0.5 NTU. Filtration of the spring is not necessary. Treatment consists of disinfection with chlorine, phosphate for corrosion control, and fluoride adjustment. Various water quality parameters for the raw water are collected on a daily basis by the water treatment plant. Table 2-11 gives the monthly averages, maximums, and minimums for water temperature, pH, and pumping rates measured at the water treatment plant for site S-3 from selected months of 2003 and 2004 (UTK was not able to obtain data for all of 2003 and 2004). The water treatment plant for site S-3 also tests the raw water daily for the presence of total coliforms and *E. coli* using the IDEXX Colilert® Test. The spring was only positive for both total coliforms and *E. coli* on 49 days of 2004. Most days the spring was positive for total coliforms but not *E. coli* and on eight days of 2004 the spring was negative for both total coliforms and *E. coli*. This public water system was given a susceptibility score of "high" in the TDEC Source Water Assessment because of potential contamination sources located within Zones 1 and 2 of its Wellhead Protection Area such as highways, cemeteries, numerous septic systems, livestock, abandoned wells, and facilities with underground injection permits.

Table 2-11. Raw water-quality parameters measured by the treatment plant for site S-3 from selected months of 2003 and 2004.

Month/ Year	Daily Pumping Rate (MLD)			Temperature (°C)			pH (standard units)		
	AVG	MAX	MIN	AVG	MAX	MIN	AVG	MAX	MIN
Jan/03	11.6	12.9	10.8	14	14	14	7.38	7.46	7.27
Apr/03	11.8	12.9	11.1	14	14	14	7.31	7.38	7.18
May/03	11.7	12.6	10.7	14	14	14	7.39	7.56	7.21
Jun/03	11.7	12.3	11.3	14	14	14	7.31	7.40	7.17
Aug/03	12.0	13.4	10.9	14	14	14	7.08	7.23	6.92
Sep/03	11.6	13.1	10.1	14	14	14	7.23	7.36	7.03
Oct/03	11.7	13.2	10.1	14	14	14	7.42	7.49	7.33
Nov/03	11.9	12.9	11.0	14	14	14	7.38	7.53	7.14
Dec/03	11.7	14.0	11.0	14	14	14	7.32	7.44	7.14
Jan/04	11.7	13.2	10.3	14	14	14	7.37	7.54	7.24
Mar/04	11.6	12.7	10.4	14	14	14	7.62	7.70	7.55
May/04	11.5	12.7	5.3	14	14	14	7.59	7.65	7.40
Jun/04	11.8	15.1	10.5	14	14	14	7.54	7.65	7.45
Aug/04	11.7	14.5	8.1	16	16	16	7.57	7.90	7.40
Sep/04	11.4	11.8	10.7	15	16	14	7.60	7.70	7.46

Abbreviations: MLD, millions of liters per day; °C, degrees Celsius

For this virus study, site S-3 was assumed to have a "low risk" of fecal contamination. The spring was sampled on May 19, 2004 and August 9, 2004. Samples were collected from the overflow pipes as the spring water entered the reservoir pond.

CHAPTER III
DEVELOPMENT OF A REAL-TIME RT-PCR ASSAY FOR ENTEROVIRUSES IN GROUND WATER SAMPLES

The purpose of this chapter is to describe the development of a real-time RT-PCR assay for detecting enteroviruses in ground water samples. The assay was developed and validated at the University of Tennessee's Center for Environmental Biotechnology in Knoxville, TN (UTCEB). Each ground water sample collected during this study was analyzed using this real-time RT-PCR assay. Protocol for ground water sample analysis using this assay is discussed in Chapter 4 of this thesis.

Brief Introduction to Real-Time RT-PCR

Real-time RT-PCR, also known as quantitative RT-PCR or qRT-PCR, is a rapid, highly sensitive method for detection and quantitation of nucleic acid sequences (Wong and Medrano 2005). As in conventional RT-PCR, specific primers are used to amplify the target nucleic acid sequence during cycles of PCR following reverse transcription of viral RNA. In real-time PCR using TaqMan probes, the "real-time" or quantitative step derives from a fluorogenic probe added to the PCR reaction which is specific to the target sequence. The probe has a fluorescent reporter dye at its 5' end and quencher dye at it 3' end. As long as the reporter and quencher molecules are in close proximity to one another, no fluorescence is emitted by the probe. During the elongation step of each PCR amplification cycle, the Taq polymerase cleaves the quencher from the probe and the reporter begins to fluoresce. Therefore, the amount of overall fluorescence increases as the PCR amplification cycles progress and accumulates more quickly if there is more of the target sequence present in the PCR reaction initially. Fluorescence emitted by the

probe is measured in real time (once every PCR amplification cycle) by a fluorometer in the real-time PCR machine. Based on the PCR amplification cycle (or fractional cycle) during which fluorescence is emitted above a designated threshold value (threshold cycle or C_T), the original number of target sequences in the reaction can be calculated relative to a standard. This real-time monitoring of fluorescent signals during PCR amplification eliminates the need for post-PCR sample processing such as electrophoresis and hybridization (Harms et al. 2003), which saves time and reduces lab contamination with PCR products.

Primer and Probe Design

A real-time RT-PCR assay was developed at UTCEB to quantify enteroviruses in ground water samples. Primers and a fluorogenic probe were designed to amplify 143 bases of the highly conserved 5' untranslated region of enterovirus (De Leon et al. 1990, Schwab et al. 1995 and Rotbart 1990) and are listed in Table 3-1. The primers and probe were designed according to the guidelines provided by Applied Biosystems (http://home.appliedbiosystems.com; Applied Biosystems; Foster City, CA) as well as design guidelines routinely used by UTCEB (summarized in Appendix 3-1). The primer and probe sequences were analyzed in GenBank® (Benson et al. 2003) using nucleotide-nucleotide BLAST (blastn) (Altschul et al. 1997) to confirm that they were pan-specific for most human enterovirus strains in the database at the time of assay design and that they did not amplify unwanted RNA or DNA sequences ("Enterovirus Taxonomy Report" in Appendix 3-2). These sequences were also chosen so that they would bind within the packaged Armored RNA®-Enterovirus (Ambion Diagnostics; Austin, TX)

Table 3-1. Primers and probe designed for the enterovirus real-time RT-PCR assay.

Target	Primer/Probe Name	Sequence (5'-3')	T_M (°C)
5' untranslated region of Enterovirus RNA genome	EvUTR24fv3	CCCCTGAATGCGGCTAATC	60
	EvUTR145rv3	GTCACCATAAGCAGCCACAATA	60
	EvUTRprobe112rv3	AAGGAAACACGGACACCCAAAGTAGTC	68

Abbreviations: T_M, melting temperature; °C, degrees Celsius.

sequence, the viral RNA standard initially considered for validation of this assay (Figure 3-1). The primers and the BHQ™ probe (FAM fluorescing dye on the 5' end and Black Hole Quencher™ on the 3'end) were synthesized by Biosearch Technologies (Biosearch Technologies Incorporated; Novato, CA).

Comparison of Standards for the Enterovirus Real-time RT-PCR Assay

In this study, three different standards were evaluated for use in the enterovirus real-time RT-PCR assay. These standards differed in molecular types and were obtained from different sources (Table 3-2). The three standards were chosen based on the perceived advantages of each one.

Descriptions of Evaluated Standards

The Armored RNA® is a noninfectious commercially available standard. Armored RNA® was developed by Ambion, Inc. and Cenetron Diagnostics, LLC for use as a RNA control and standard in clinical and diagnostic settings (Pasloske et al. 1998). It is completely characterized viral RNA packaged in bacteriophage coat proteins to help protect the RNA from ribonucleases and stabilize the RNA for long-term storage. The use of Armored RNA® as a viral RNA standard for a real-time RT-PCR assay for enteroviruses has many possible advantages. First, Armored RNA® is not infectious and therefore does not pose any harm to lab workers. Second, use of Armored RNA® does not require maintenance of cell cultures. Third, the bacteriophages packaging the RNA are similar in size (~26 nm) and shape to enterovirus particles. Therefore, it was hoped that the Armored RNA® could be used in this study for seeded experiments, treated as actual virus particles, in order to accurately measure virus recovery efficiencies in ground water filtration, filter elution, and concentration and inhibition removal procedures.

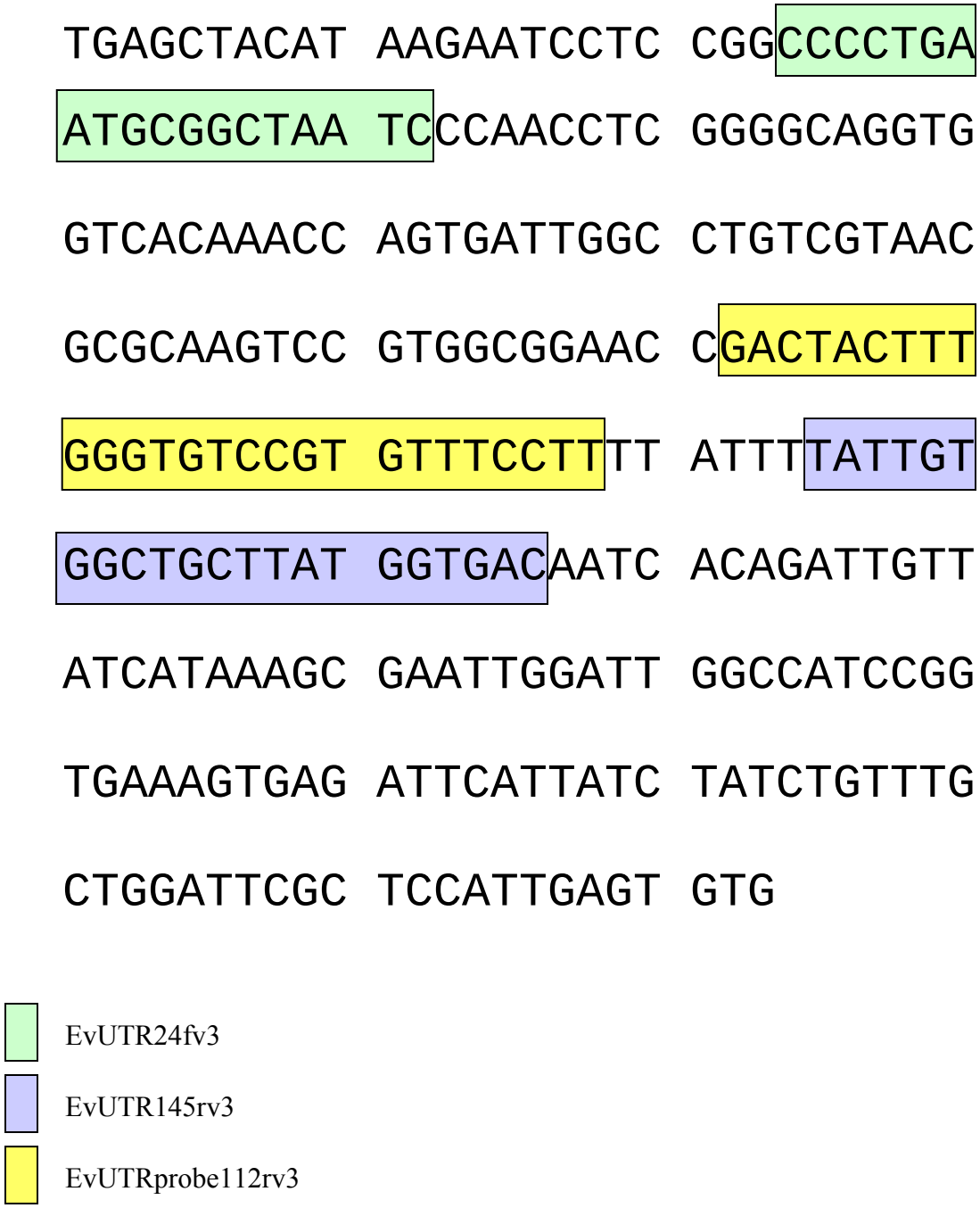

Figure 3-1. Locations of the binding sites for the real-time RT-PCR primers and probe within the packaged Armored RNA®-Enterovirus sequence.

Table 3-2. Standards evaluated for the enterovirus real-time RT-PCR assay.

Standard Name	Molecule Type	Source
Armored RNA®-Enterovirus	Bacteriophage encapsulated RNA	Ambion Diagnostics
Poliovirus Type I Chat Strain (ATCC® # VR-1562)	Live attenuated poliovirus (viral protein encapsulated RNA)	USEPA-NERL
P29 DNA Plasmid	DNA in plasmid (cDNA to poliovirus Chat strain)	UTCEB

The next standard examined was the CHAT strain of type 1 attenuated poliomyelitis virus (poliovirus) (ATCC® # VR-1562), a live-attenuated oral polio vaccine used to immunize millions of individuals in several countries from the late 1950's until about 1970, when Sabin strains replaced it as the dominant vaccine (Martin and Minor 2002). The poliovirus is infectious but is very well-studied and is often used, along with other attenuated poliovirus strains, in seed and recovery experiments by the USEPA to develop and validate virus collection, concentration, and detection protocols (e.g. Fout et al. 2003).

The plasmid DNA standard was developed as a noninfectious alternative to the poliovirus. The advantage of DNA is that it is stable and easy to use without special precautions. It also allows for a separate evaluation of the PCR phase of the RT-PCR assay. To construct the plasmid DNA standard, 4 µL of the poliovirus at a concentration of 10 PFU (plaque forming units)/µL was reverse transcribed and amplified using Ready-to-Go™ RT-PCR Beads (Amersham Biosciences; Piscataway, NJ). Table 3-3 describes the PCR primers used to amplify the target sequence for cloning as well as the PCR protocol. The resulting PCR product was cloned using a TOPO TA Cloning® Kit (Invitrogen Corporation; Carlsbad, CA). The colonies were transferred from LB-KAN agar to LB-KAN broth (LB or Luria-Bertani agar is comprised of 1% Bacto-Tryptone (10 g), 0.5% Yeast extract (5 g), 5 g NaCl, and 15 g Bacto Agar; the pH is adjusted to 7.0 with NaOH and the volume is brought up to 1 L; to make LB-KAN agar, the antibiotic Kanamycin is added to the agar at a concentration of 50 mg/mL after autoclaving). The samples were checked for inserts and a sample with an insert was grown overnight in LB-

Table 3-3. Primers and PCR protocol used to amplify a PCR product for construction of the DNA plasmid.

Primer Name	Sequence (5'-3')	PCR Protocol
EvUTR25fv3	CCCCTGAATGCGGCTAATC	1) 94°C for 5 min
EV-R614	ACCGGATGGCCAATCCAA	2) 94°C for 15 sec
		3) 65°C for 45 sec
		-1 °C per cycle
		4) 72°C for 1 min
		5) Go to 2) 9 times
		6) 94°C for 15 sec
		7) 55°C for 45 sec
		8) 72°C for 1 min
		9) Go to 6) 29 times
		10) 72°C for 10 min
		11) 4°C forever

KAN broth at 37°C. The plasmid DNA was then isolated using a Wizard® *Plus* Midipreps DNA Purification System (Promega Corporation; Madison, WI). The DNA concentration was measured using a fluorometer and this concentration was converted to number of copies based on the molecular weight of the DNA. A dilution of the DNA plasmid (named P29) was then made to produce a working stock solution of 10^7 copies of P29/ μL.

Methods for Testing Standards

For each standard, 1:10 serial dilutions were performed and each concentration was assayed in triplicate using the developed enterovirus primers and probe. The starting concentrations for each standard were as follows: Armored RNA®-Enterovirus – ~1.05 X 10^5 copies/uL (different lots used in 2003 and 2004 had the same estimated starting concentration), attenuated poliovirus – 1.0 X 10^4 PFU/μL, and P29 DNA plasmid – 1.0 X 10^7 copies/μL. Real-time RT-PCR was performed on a DNA Engine Opticon® Continuous Fluorescence Detection System (MJ Research Incorporated; Waltham, MA) using a QuantiTect Probe RT-PCR Kit (Qiagen Incorporated; Valencia, CA) designed for single tube, one-step real-time RT-PCR. Experiments were conducted to optimize the annealing temperature, the primer and probe concentrations, the $MgCl_2$ concentration, and the amount of template added to each real-time RT-PCR reaction.

Results

In real-time PCR, the fluorescence is measured at the end of each amplification cycle. The initial output data is shown as a graph of fluorescence versus amplification cycle (Figure 3-2). A minimum fluorescence threshold is calculated as five times the standard deviation of the background fluorescence measured in the first five to 10

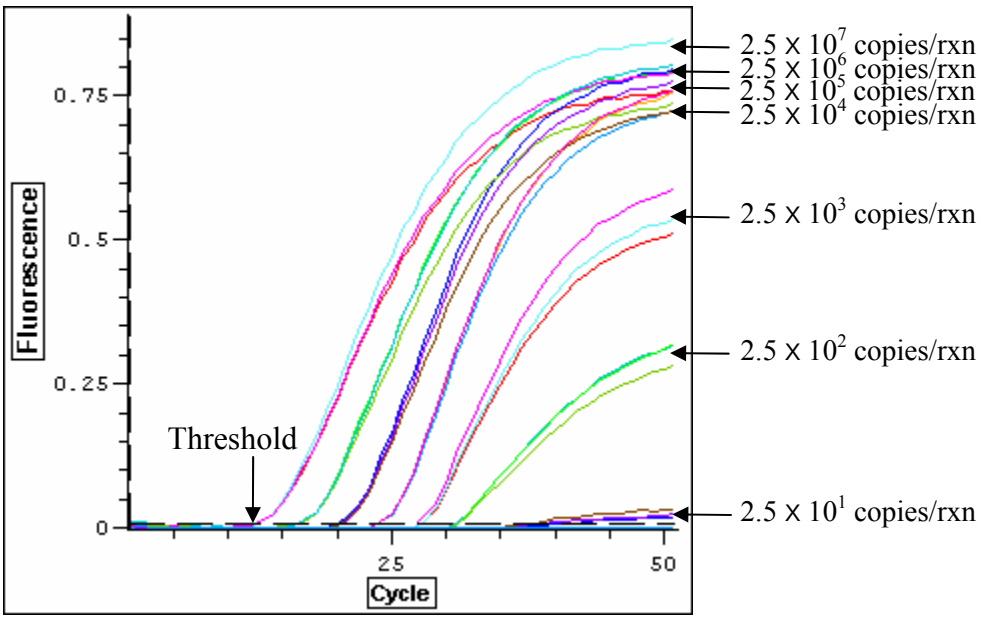

Figure 3-2. Amplification plot from a real-time RT-PCR run of the P29 DNA plasmid on 1/3/05. Each serial dilution was run in triplicate. Abbreviations: rxn, real-time RT-PCR reaction.

amplification cycles. The threshold cycle (C_T) is calculated for each PCR reaction as the calculated amplification cycle in which the fluorescence exceeds the threshold. A standard curve is generated by graphing the log of the starting concentration versus the calculated threshold cycle (C_T). The linear fit of the log concentration versus the C_T can be used to determine the efficiency and variability of the PCR reaction. The ideal slope representing perfect PCR efficiency is 0.30, although it is rarely obtained (values between 0.25 and 0.33 are generally acceptable).

The three main criteria in interpreting the performance of the different standards in the enterovirus real-time RT-PCR assay were (1) PCR efficiency, (2) reproducibility (variability between runs), and (3) sensitivity (minimum detection limits). The results for the 3 standards (4 representative runs for each standard) are provided in Figure 3-3 (Armored RNA®-Enterovirus), Figure 3-4 (attenuated poliovirus), and Figure 3-5 (P29 DNA plasmid). In the plots, the points represent the average C_T for the triplicate runs of each serial dilution. Figure 3-3 is also labeled to indicate the PCR efficiency and reproducibility. The PCR efficiency for each real-time RT-PCR run is represented by the slope of the equation for each regression line. The reproducibility between runs for each standard is represented by the R^2 value for the black dashed line in each figure.

Armored RNA®-Enterovirus

PCR efficiency for the Armored RNA®-Enterovirus was very low for most runs. The average PCR efficiency for the four representative runs presented in Figure 3-3 was 0.20 ± 0.07, below the acceptable range. There were only two runs during the entire study when the Armored RNA®-Enterovirus had acceptable PCR efficiency values (0.237 on 7/10/03 and 0.282 on 6/15/04).

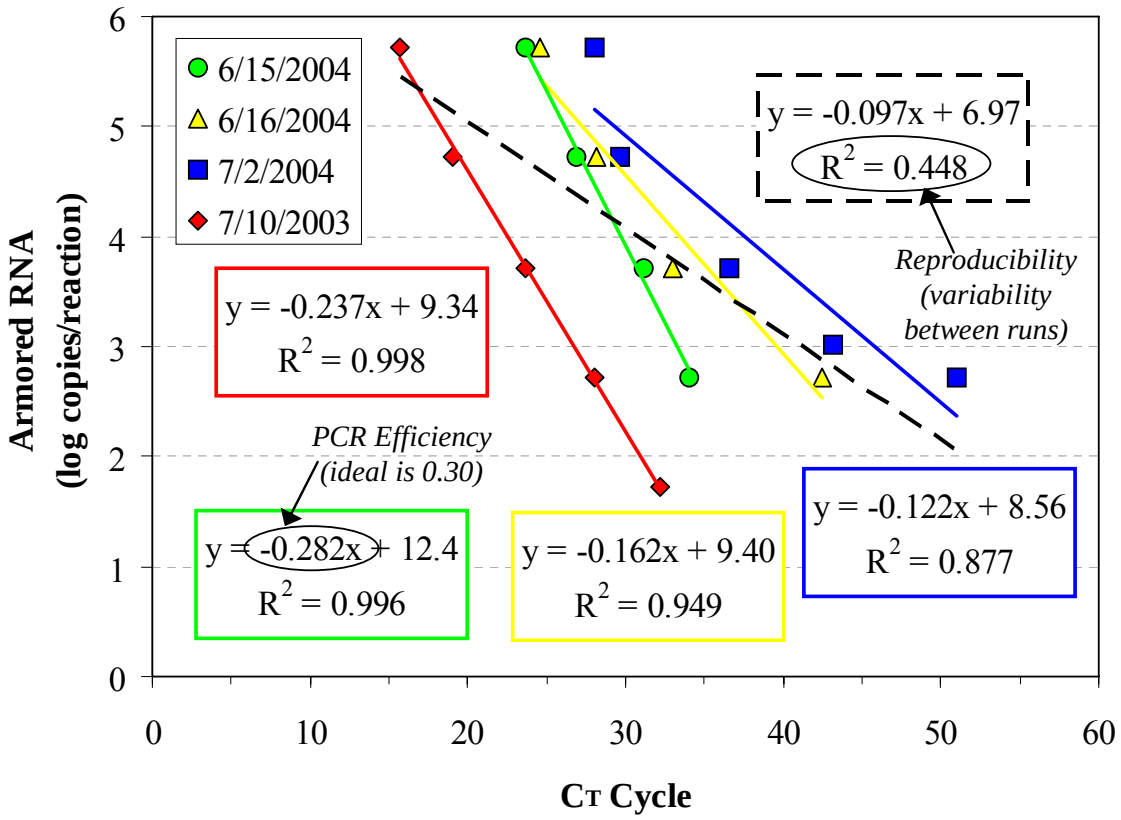

Figure 3-3. Real-time RT-PCR runs of Armored RNA®-Enterovirus from 7/10/03, 6/15/04, 6/16/04, and 7/2/04. The black dashed line represents the C_T's for all four runs.

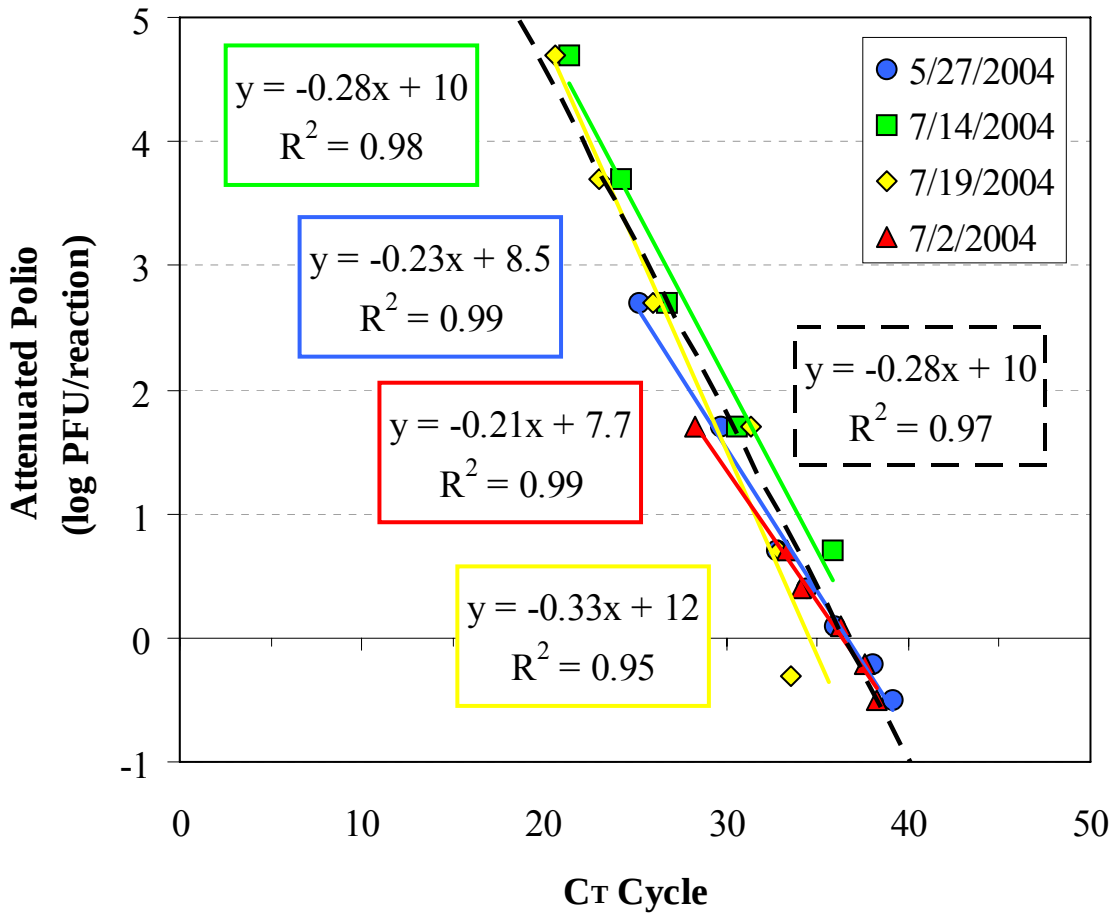

Figure 3-4. Real-time RT-PCR runs of attenuated poliovirus from 5/27/04, 7/2/04, 7/14/04, and 7/19/04. The black dashed line represents the C_T's for all four runs.

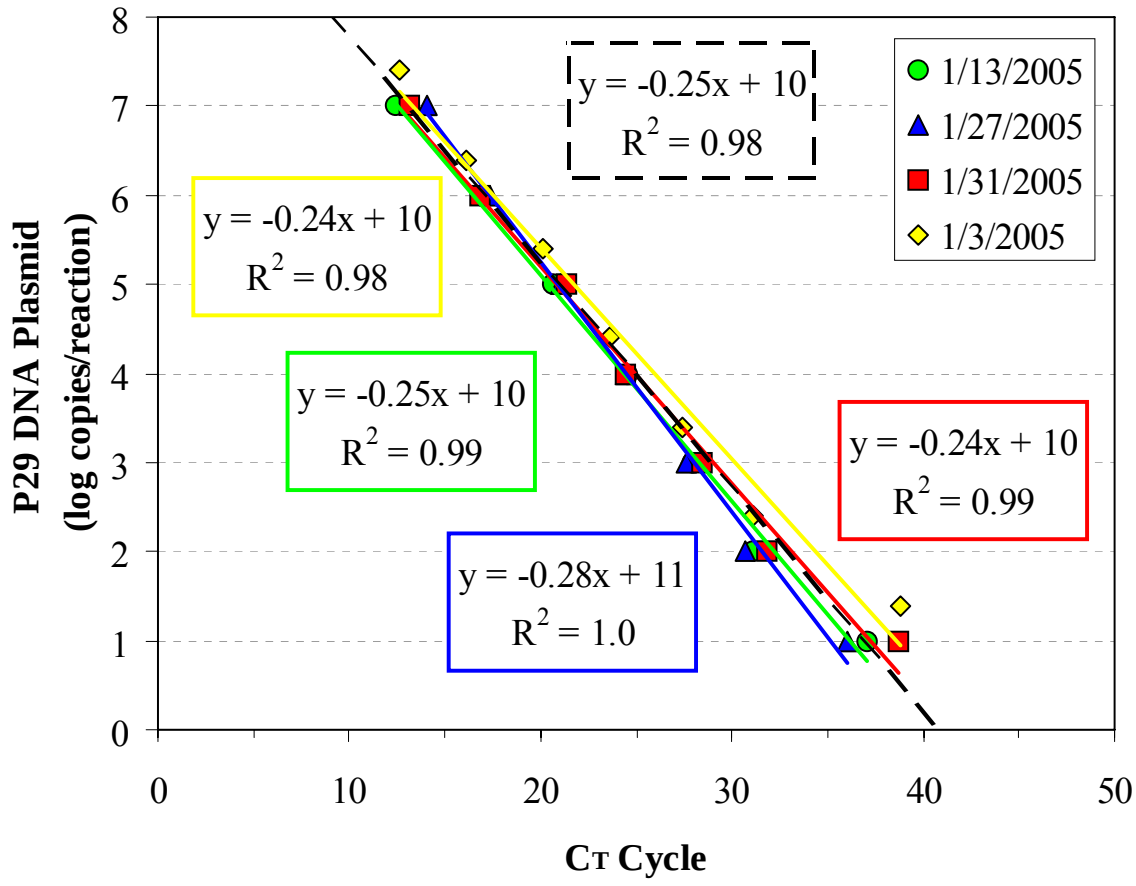

Figure 3-5. Real-time RT-PCR runs of P29 DNA plasmid from 1/3/05, 1/13/05, 1/27/05, and 1/31/05. The black dashed line represents the C_T's for all four runs.

Variability in the PCR reaction was low on the first run of the Armored RNA®-Enterovirus as indicated by the R^2 value of 0.998 on 7/10/03. However, over time this variability increased as indicated by lower R^2 values for subsequent runs. The Armored RNA®-Enterovirus had the lowest reproducibility (i.e. highest variability) between runs (R^2=0.448 for the four runs presented in Figure 3-3) of the three candidate standards.

The lowest value of Armored RNA®-Enterovirus routinely detected was 525 copies per PCR reaction. A detection range of four orders of magnitude (525,000 to 52.5 copies per PCR reaction) was achieved on the first run with the standard on 7/10/03 but was never repeated during the study.

The performance of the Armored RNA®-Enterovirus was highly variable and appeared to deteriorate over the course of the study. Some of the problems associated with the runs were probably attributed to improper handling of the Armored RNA®, which may have resulted in degradation of the standards. The original purchased stock had been frozen upon receipt in the lab at -80°C for several days when it should have been stored between 2°C and 8°C. Also, the Armored RNA® particles were mixed with nuclease-free water to make serial dilutions. In some cases, the dilutions were used immediately, but in other cases they were stored for up to two months before amplification. It was later learned that the Armored RNA® particles should only be diluted into TSM III buffer (a combination of Tris, NaCl, $MgCl_2$, microcide, and gelatin) and dilutions should be made fresh for each run. New Armored RNA®-Enterovirus standards were ordered to determine if better handling and storage procedures could improve the assays, but some problems persisted (data not shown).

Attenuated Poliovirus

PCR efficiency for the poliovirus was well within the acceptable range. The average PCR efficiency for the four runs presented in Figure 3-4 was 0.26 ± 0.05. PCR efficiency increased over the course of the study, probably due to better handling and sample preparation techniques such as heating the standard before making serial dilutions and making fresh dilutions for each real-time RT-PCR run.

Variability in the PCR reaction was low for most runs of the poliovirus as indicated by the R^2 values ranging from 0.95 to 0.99 for the runs presented in Figure 3-4. The attenuated poliovirus had higher reproducibility (lower variability) between runs than the Armored RNA®-Enterovirus but slightly lower reproducibility (higher variability) between runs than the DNA plasmid, as represented by the R^2 value of 0.97 for the four runs presented in Figure 3-4.

The lowest value of poliovirus routinely detected was 0.5 PFU per PCR reaction, although 0.16 to 0.3 PFU per PCR reaction was detected in 25% of the runs during the study. The linear detection range for the attenuated poliovirus was five orders of magnitude, from 50,000 to 0.5 PFU per PCR reaction. Detection limits were lowest in the beginning of the study and increased over time. One possible reason for this decrease in sensitivity is that the poliovirus stock may have been degraded by too many freeze/thaws and starting concentrations were actually lower than expected as the study progressed.

P29 DNA Plasmid

PCR efficiency for P29 was within the acceptable range and was similar to but slightly less variable than that of the poliovirus. The average PCR efficiency for the 4 runs presented in Figure 3-5 was 0.26 ± 0.02.

Variability in the PCR reaction was consistently very low for P29 as indicated by the R^2 values ranging from 0.98 to 1.0 for the runs presented in Figure 3-5. The DNA plasmid had slightly higher reproducibility between runs (lower variability) than the poliovirus as indicated by the R^2 value of 0.98 for the runs presented in Figure 3-5.

The lowest value of P29 routinely detected was 10 copies per PCR reaction. Detection limits were consistent throughout the study. The linear detection range for the DNA plasmid was six orders of magnitude, from 1×10^7 to 10 copies per reaction.

Viral RNA Copies in 1 PFU Poliovirus

When the C_T's from all of the runs of P29 and the attenuated poliovirus were averaged (including runs where the standards were run separately and runs where the standards were run simultaneously), the relationship between the DNA plasmid and the attenuated poliovirus was 27 ± 53 copies DNA plasmid = 1 PFU attenuated poliovirus (i.e. 1 PFU attenuated poliovirus and 27 ± 53 copies DNA plasmid gave the same average C_T value). When the DNA plasmid standard curve was used to calculate the copies of attenuated poliovirus RNA, the relationship was 0.86 ± 0.61 copies of DNA plasmid to 1 copy of attenuated poliovirus RNA (very close to the expected 1:1 ratio). Assuming a reverse transcription efficiency of 100%, a ratio of 23 ± 59 copies of the poliovirus RNA genome to 1 PFU attenuated poliovirus was calculated by dividing the number of copies

of attenuated poliovirus RNA (calculated when the plasmid was used as the standard curve) in a reaction by the PFU of poliovirus added to the reaction.

Comparison of Attenuated Poliovirus to P29 DNA Plasmid

Both the attenuated poliovirus and the DNA plasmid standards ran consistently (good reproducibility) and could be detected at low levels (high sensitivity). Direct comparisons between the performance of the two standards was the most valid when the standards were run in the same real-time RT-PCR reaction. Figure 3-6 is a plot of the average C_T's from only the real-time RT-PCR runs where the attenuated poliovirus and the DNA plasmid were analyzed simultaneously. Note that the poliovirus data is shifted to the left of the plasmid data because 1 PFU attenuated poliovirus > 1 copy DNA plasmid and therefore the poliovirus dilutions fluoresced at an earlier amplification cycle. Although the PCR efficiency for both the plasmid and the poliovirus was less than the ideal value of 0.30, the value of 0.24 was the same for both the plasmid and the poliovirus. The DNA plasmid had a slightly higher R^2 value indicating slightly higher reproducibility between runs.

Discussion

Based on the results of the above experiments, the three standards were ranked by their performance with regards to their PCR efficiency, reproducibility, and sensitivity. These rankings are provided in Table 3-4 and were used to determine the best standards for the real-time RT-PCR assay for enterovirus. The following is a brief discussion of the experimental results and some of the advantages and disadvantages of each standard.

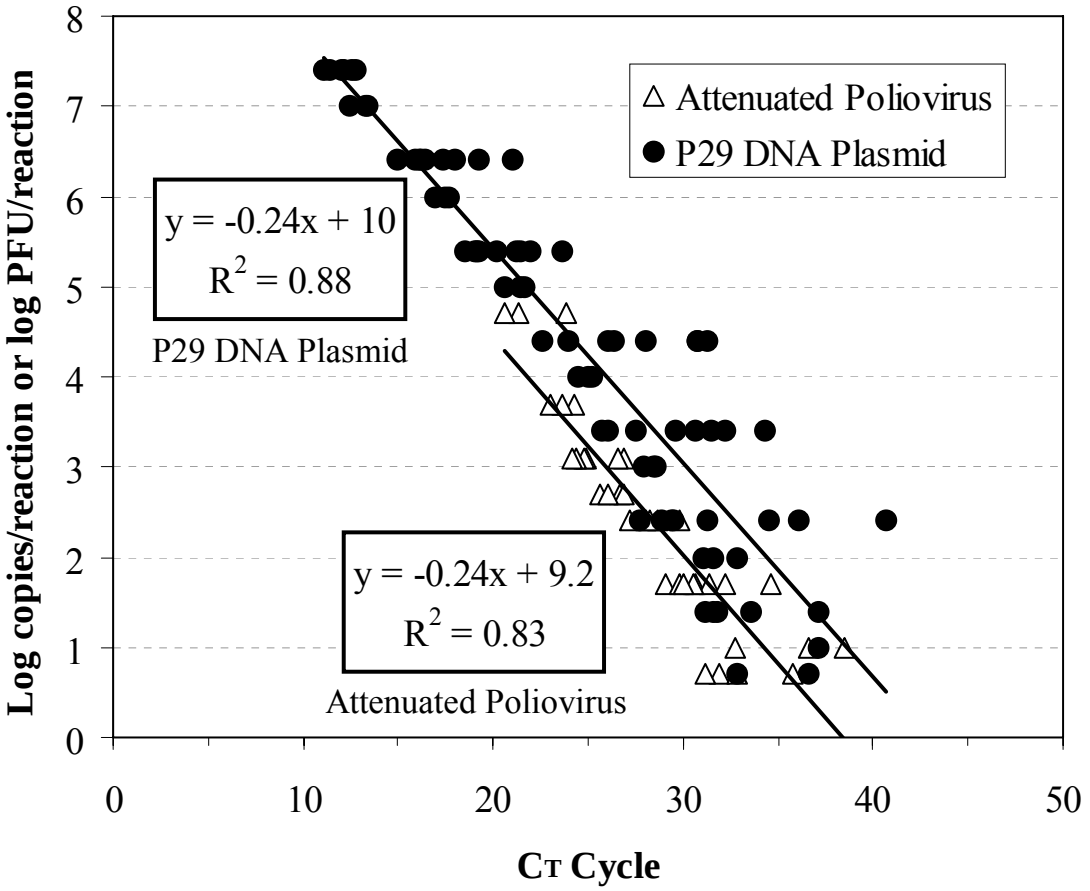

Figure 3-6. Comparison of the C_T values for simultaneous real-time RT-PCR runs of the P29 DNA plasmid and attenuated poliovirus.

Table 3-4. Standards ranked from 1 (best) to 3 (worst) with regards to PCR efficiency, reproducibility, and sensitivity.

Standard Name	PCR Efficiency*	Reproducibility*	Sensitivity*
Armored RNA®-Enterovirus	2	2	2
Poliovirus Type I Chat Strain (ATCC® # VR-1562)	1	1	1
P29 DNA Plasmid	1	1	1

*The poliovirus and DNA plasmid were virtually tied with regards to PCR efficiency, reproducibility, and sensitivity.

Armored RNA®-Enterovirus

The Armored RNA®-Enterovirus, which had the lowest PCR efficiency, reproducibility, and sensitivity of the three standards, was abandoned as the viral RNA standard because it did not meet the needs of this particular study. In preliminary experimentation (data not shown), the Armored RNA®-Enterovirus did not appear to bind to the 1MDS cartridge filters used for collecting viruses from ground water. It also degraded over time in environmental ground water samples. In addition, the Armored RNA®-Enterovirus could not be stored until analysis like other ground water samples (at -80°C) because it degrades at low temperatures. Therefore, the Armored RNA®-Enterovirus was not suitable for seeding ground water samples. The exact concentration of RNA in each lot of Armored RNA®-Enterovirus was unknown and therefore the Armored RNA®-Enterovirus was not an optimal standard for quantification. Essentially, the Armored RNA®-Enterovirus was designed for use as a RNA control and standard for conventional RT-PCR in clinical settings, not for real-time RT-PCR or environmental applications. However, the performance of the Armored RNA®-Enterovirus strictly as a standard for real-time RT-PCR assays may have been improved upon by carefully following the storage and handling conditions recommended by the manufacturer. In addition, the stock lot of Armored RNA®-Enterovirus could have been aliquoted into small volumes immediately upon receipt from the manufacturer so that one aliquot (used to make up fresh serial dilutions in TSM III Buffer) would be used up completely with each real-time RT-PCR run. Ambion Diagnostics is beginning to offer a quantitative version (exact starting concentration is known) of some types of Armored RNA® known

as Armored RNA® Quant Technology. If Armored RNA®-Enterovirus was converted to this quantitative format, the Armored RNA®-Enterovirus may be worth retrying as a standard for real-time RT-PCR of enterovirus because of the benefit of having a noninfectious, quantitative viral RNA standard.

Viral RNA Copies in 1 PFU Poliovirus

When the stock of CHAT used in this study was made (Fout el al. 2003), the final preparation contained 8.8×10^8 particles/μL and 6.5×10^6 PFU/μL, giving a ratio of 136 virus particles to 1 PFU. The number of viral RNA copies to PFU of attenuated poliovirus, as calculated from the real-time RT-PCR runs, was approximately six times lower than expected, assuming 100% efficiency in the reverse transcription step and assuming one virus particle equals one copy of viral RNA. There were a few possibilities for why, based on the results of the real-time RT-PCR runs, this ratio appeared to have been reduced. First, the diluted poliovirus stock (6.5×10^7 PFU/mL) may have been degraded during the course of the study due to too many freeze/thaws. There was some evidence for this in the decrease in sensitivity of the attenuated poliovirus over time. Second, it is possible that the ratio was skewed because the concentration of the DNA plasmid may have been overestimated. Third, the difference may simply represent the sum of multiple measurement variability in pipetting of serial dilutions, the efficiency of the RT step, day to day performance of the real-time PCR machine, the PCR efficiency of the assay, etc.

Attenuated Poliovirus and P29 DNA Plasmid as Co-Standards

It was difficult to find the perfect standard for both absolute quantitation of enterovirus RNA in ground water samples by real-time RT-PCR and seeding of ground

water samples to determine virus recovery efficiency in ground water filtration, filter elution, and concentration and inhibition removal procedures. Neither the attenuated poliovirus nor the DNA plasmid fulfilled both of these requirements.

Although the virus titer (given as PFU/volume) as well as the virus particle count (given as virus particles/volume) of the attenuated poliovirus was known (Fout et al. 2003), neither of these were measured by real-time RT-PCR. The virus titer was determined by the quantity of infectious viruses from a plaque assay and the virus particle count was determined by optical density of highly purified virus (Rueckert and Pallansch 1981). The real-time RT-PCR assay detected viral nucleic acid sequences in the concentrated ground water samples, which may or may not have been equivalent to the amount of infectious viruses or virus particles. Therefore, the attenuated poliovirus was useful for seeding ground water samples and determining recovery efficiencies as well as evaluating the RT step of the real-time RT-PCR assay. However, it was not ideal as a standard for quantitative measurement of viral nucleic acid sequences in ground water samples by real-time RT-PCR because the exact concentration of nucleic acid sequences in the attenuated poliovirus stock was not certain.

The constructed DNA plasmid was a better standard for quantitative measurement of viral nucleic acid sequences in ground water samples by real-time RT-PCR than the poliovirus because the concentration of nucleic acid sequences was known for the plasmid. An absolute quantitation real-time RT-PCR assay is a method of calculating the quantities of unknown samples by interpolating their quantity from a standard curve of standards whose absolute quantities are known by some independent means (http://www.appliedbiosystems.com/support/tutorials/pdf/

essentials_of_real_time_pcr.pdf). In the case of this assay, the standard curve was the DNA plasmid. However, in general, DNA cannot be used for absolute quantitation of RNA in real-time RT-PCR because the efficiency of the reverse transcription step cannot be known. In the future, in vitro transcribed RNA made from the P29 DNA plasmid may or may not provide a better quantitative viral RNA standard than the DNA plasmid.

The advantages of the attenuated poliovirus (useful as a seeded control and a measure of performance of the RT step) and the DNA plasmid (useful as a quantitative standard for measuring viral nucleic acid sequences) can be combined if both standards are used together for the real-time RT-PCR assay. Multiple standards allow for determination of where a problem in an assay is. For example, if the DNA plasmid worked but the poliovirus did not, then the failure of the assay was likely in the heating of the virus or in the reverse transcription step. However, if the DNA plasmid did not work (chances are the poliovirus did not work either), then the problem was likely in the PCR step.

Conclusions

The conclusion of this study was that both the attenuated poliovirus and the P29 DNA plasmid performed well with regards to PCR efficiency, reproducibility, and sensitivity and should be used as co-standards for the real-time RT-PCR assay for enteroviruses in ground water samples. Using both the attenuated poliovirus and the plasmid DNA standards in conjunction with one another was useful and gave different information about the performance of the real-time RT-PCR assay. One disadvantage to this approach was that the use of an infectious standard (attenuated poliovirus) was not eliminated.

Table 3-5 provides the final optimized RT-PCR mix and conditions for the real-time RT-PCR assay for enteroviruses in ground water samples developed by UTCEB. The PCR mix, with a total volume of 25 µL per reaction, contained 12.5 µL of the QuantiTect Probe RT-PCR Master Mix, 0.25 µL of the QuantiTect RT Mix, 0.75 µL (0.68 µM) of the forward primer (EvUTR24fv3), 0.75 µL (0.68 µM) of the reverse primer (EvUTR145rv3), 0.625 µL (0.25 µM) of the fluorogenic probe (EvUTRprobe112rv3), 7.625 µL of nuclease-free water, and 2.5 µL of the template (viral standard or ground water sample). The PCR program was 30 minutes at 50°C, 15 minutes at 95°C, and 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

The next necessary step after determination of the proper standards was validation of the real-time RT-PCR assay for enteroviruses on actual ground water samples, which represented a challenge because even after concentration and inhibitor removal procedures, concentrated ground water samples may contain RT-PCR enzyme inhibitors. The protocol for the real-time RT-PCR analysis of the ground water samples is described in Chapter 4 and the results of those analyses are presented in Chapter 5 of this thesis.

Table 3-5. Final RT-PCR mix and conditions for the enterovirus real-time RT-PCR assay.

RT-PCR Mix (25µL total volume per reaction)	PCR Conditions
12.5 µL QuantiTect Probe RT-PCR Master Mix	1) 50°C for 30 min
0.25 µL QuantiTect RT Mix	2) 95°C for 15 min
0.75 µL forward primer (EvUTR24fv3)	3) 95°C for 15 sec
0.75 µL reverse primer (EvUTR145rv3)	4) 60°C for 60 sec
0.625 µL probe (EvUTRprobe112rv3)	5) Plate read
7.625 µL of nuclease-free water	6) Go to 3) and repeat 50 times
2.5 µL template	7) End

CHAPTER IV
MATERIALS AND METHODS FOR FIELD SURVEY

Following the site selection process (Chapter 2) and the development of the real-time RT-PCR assay for detection of enteroviruses in ground water samples (Chapter 3), the survey of enteric virus occurrence in East Tennessee was conducted. The purpose of this chapter is to describe the materials and methods for the collection of ground water samples for this study and the field and laboratory analyses performed on those ground water samples. Figure 4-1 is a flow chart describing the analyses performed on each ground water sample as well as where they were performed. A total of four wells and four springs were sampled for enteric viruses, indicator bacteria, field water-quality parameters, and chemical constituents between March 2004 and August 2004. Sites S-1, S-2, S-3, S-4, W-1, and W-2 were sampled twice during the study while sites W-3 and W-4 were only sampled once. On nine of the 14 sampling dates, two ground water samples were collected for viruses simultaneously, yielding a total of 23 ground water samples collected and analyzed for viruses over the course of the study. Field water-quality parameters, indicator bacteria, and chemical constituents were measured once for each of the 14 sampling days. Ground water samples were collected under stable hydrologic conditions.

Field Sampling Procedures

A virus sampling apparatus was designed by UTK following the guidelines set forth by the USEPA (USEPA 1995 and USEPA 2001). The apparatus (Figure 4-2) consisted of an intake hose, a pressure regulator and gauge, a cartridge housing

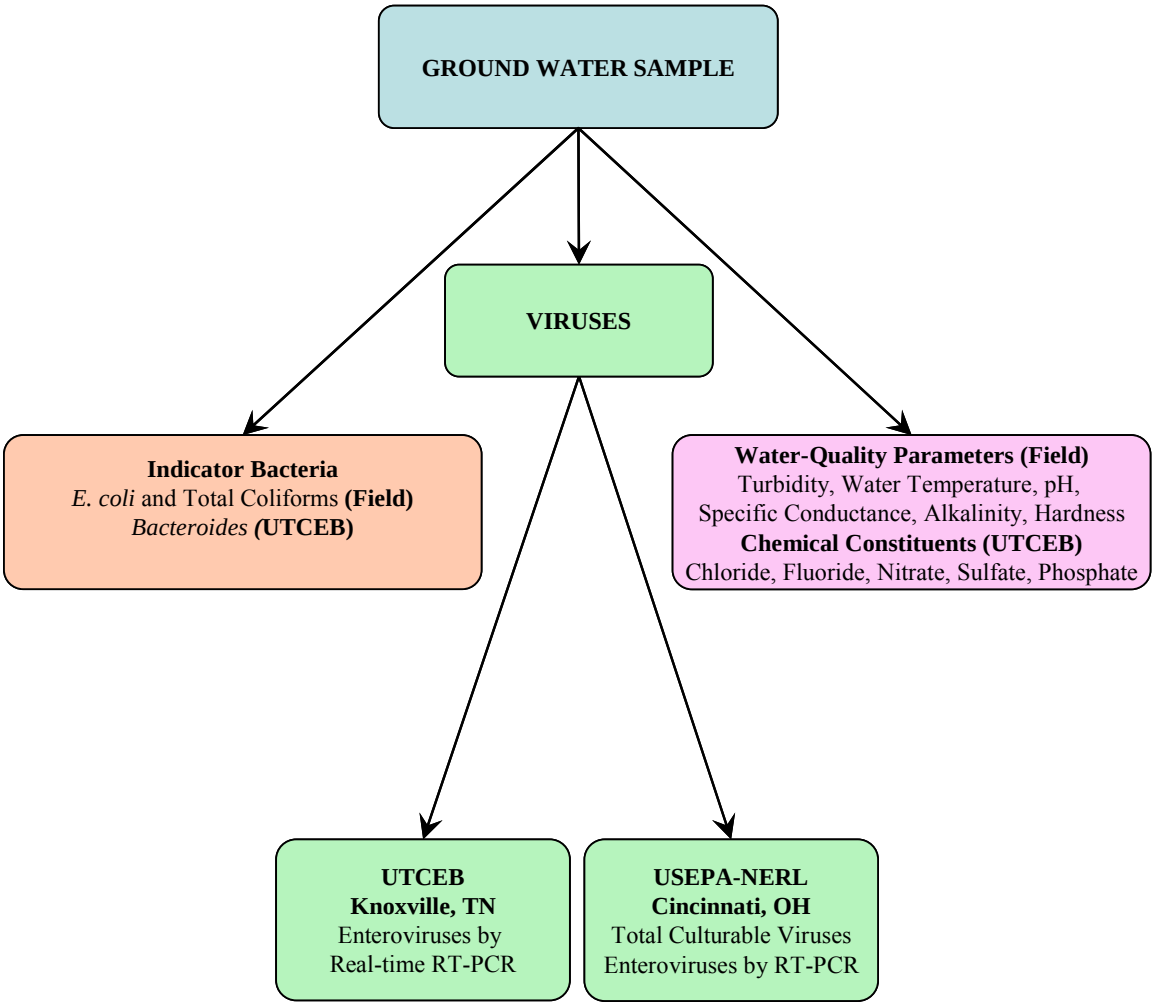

Figure 4-1. Flow chart showing analyses performed on each ground water sample collected for this study.

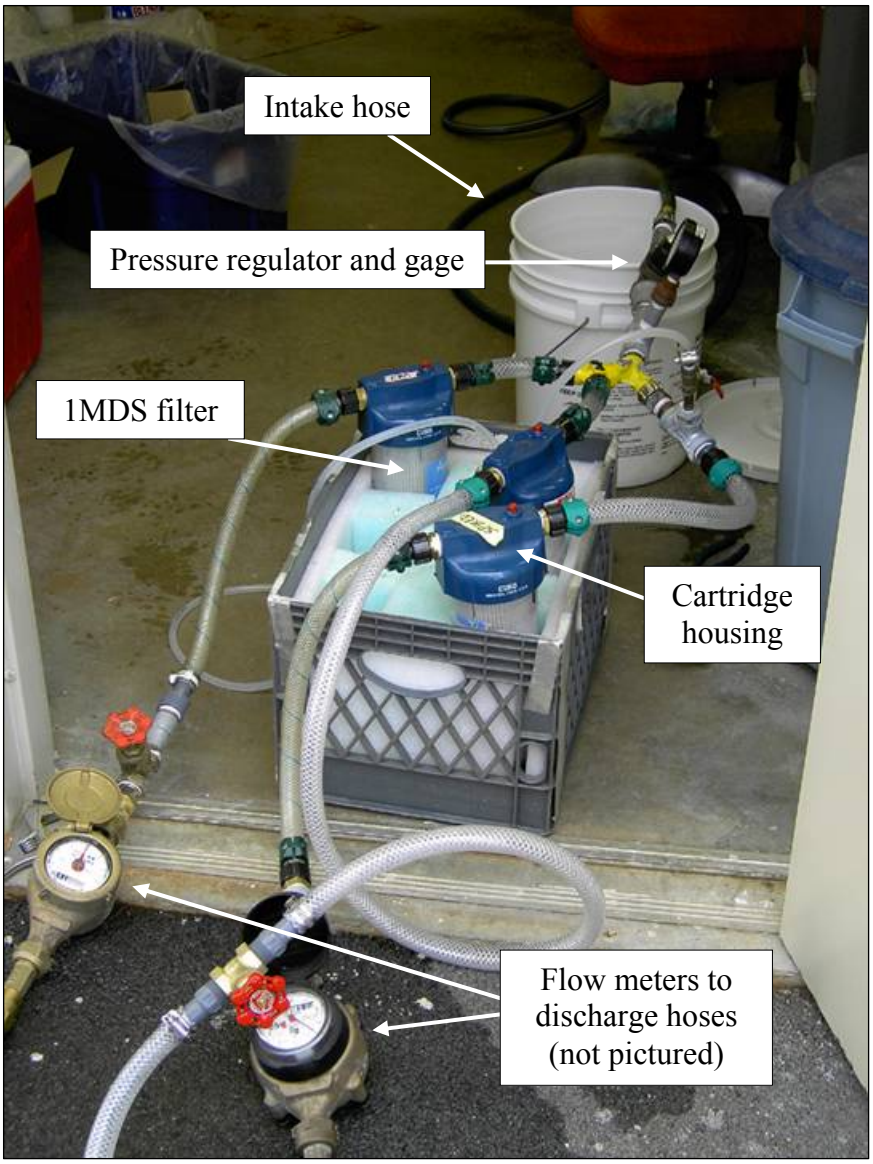

Figure 4-2. Virus sampling apparatus assembled by UTK.

containing a ZetaPor Virosorb 1MDS positively-charged cartridge filter (CUNO Incorporated; Meriden, Connecticut), a flow meter, and a discharge hose. When more than one filter was collected at the same time, "Y" splitter ball valves (Figure 4-3) were used downstream of the pressure regulator to split the flow to each filter (which had its own flow meter and discharge hose).

Before each sampling trip, the entire sampling apparatus was assembled, washed, and sterilized. Soapy water, tap water, and deionized water were circulated through the system using an electric pump. Next, a 10% chlorine bleach solution was circulated through the sampling apparatus for 30 minutes. The apparatus was then dechlorinated by circulating a 0.2% sodium thiosulfate solution through the sampling apparatus for a few minutes. A final rinse of sterile deionized water followed. Any open tube ends, connectors, or fittings were covered with parafilm and the apparatus was double bagged until ready for use. Cartridge filters were wrapped in aluminum foil and autoclaved for 30 minutes at 121°C. Glass bottles for collection of ground water for microbiological and chemical analyses were also thoroughly washed and autoclaved.

The specific sampling procedure varied for each field site. The field crew had to adapt to the public water supply plumbing at each spring or wellhead. In some cases, the sampling apparatus was connected directly to a faucet in a well pump house (Figure 4-4), pit, or inside a spring pump house (Figure 4-5). In these situations, water pressure through the sampling apparatus was controlled with the pressure regulator on the sampling apparatus or by simply turning the well or spring faucet handle. Other times, there was no threading on the faucet to connect the sampling apparatus or there was too

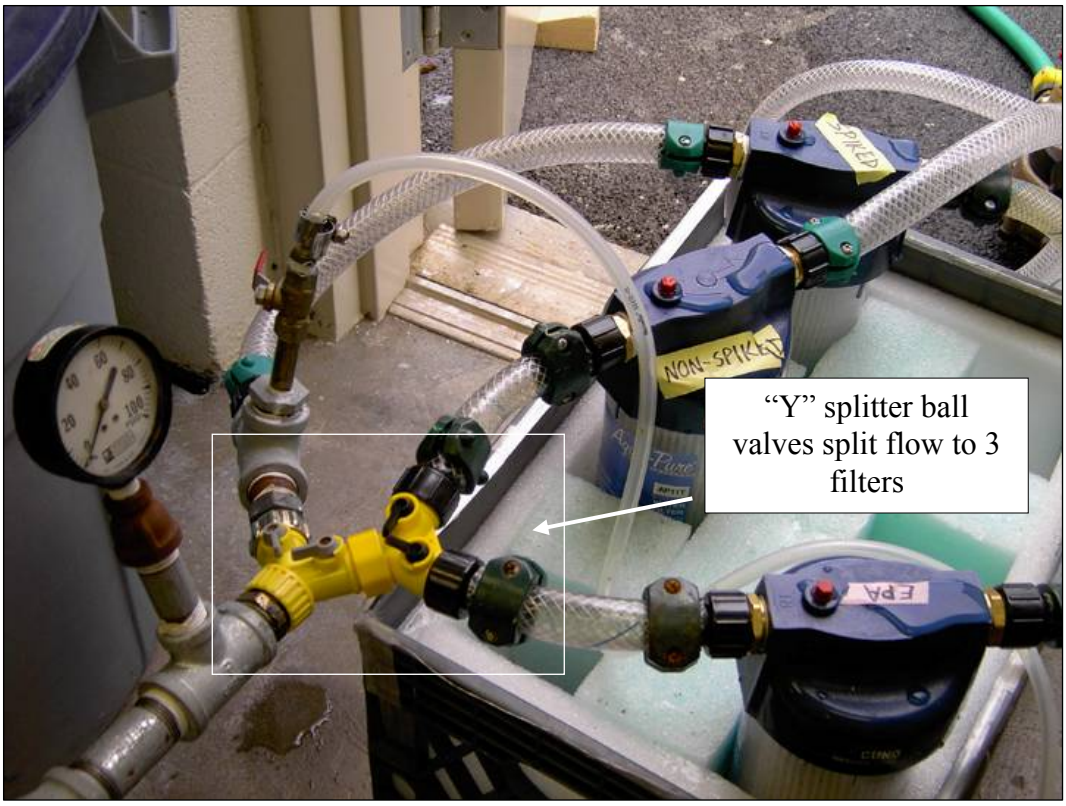

Figure 4-3. "Y" splitter ball valves were used downstream of the pressure regulator to split the flow to each filter.

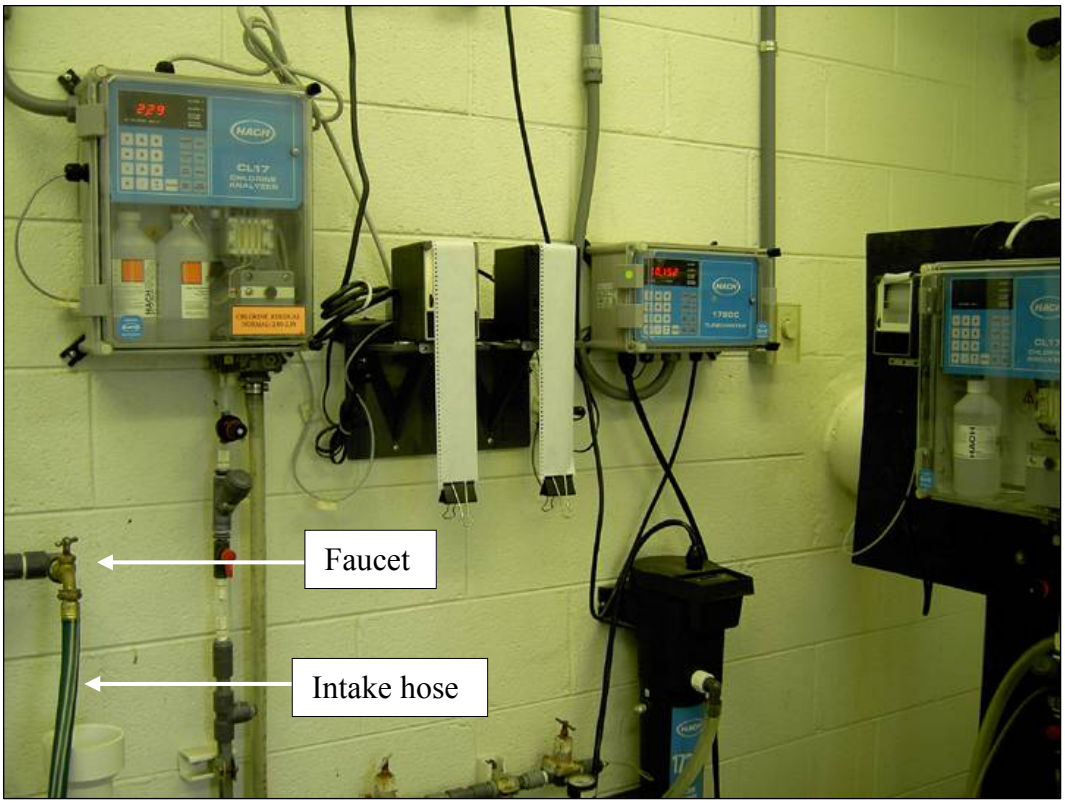

Figure 4-4. Intake hose for virus sampling apparatus connected to a faucet in a well pump house.

Figure 4-5. Intake hose for virus sampling apparatus connected to a faucet in a spring pump house.

much air coming out of the faucet. In these cases, water was allowed to flow from the faucet into a sterile bucket and was then pumped with a sterile electric pump through the sampling apparatus. At three of the four springs sampled, the intake hose for the sampling apparatus was put directly into the stream (as the spring water came out of the rock) or spring pool (Figure 4-6) and a sterile electric pump was used to push the water through the apparatus. In all cases, sterile techniques were used and ground water was sampled before receiving any treatment by the water treatment facility.

When the field crew first arrived at the field site, the virus sampling apparatus (without the cartridge filter) was connected to the water source and flushed. Water from the discharge hose was collected in a bucket and was measured for temperature, conductance, and pH (Figure 4-7). When these parameters stabilized, the water was cut off. This flush of the sampling apparatus never took longer than 10 minutes. Next, the field crew recorded the initial reading of the flow meters as well as the time of day. The cartridge filters were placed into the housings using gloved hands and, using a wench, the housing lids were screwed on tight. The water was turned back on and the vent release buttons on the cartridge housing lids (Figure 4-8) were pushed to release any air trapped in the apparatus. Every effort was made to minimize air bubbles on the cartridge filters during sampling.

Filtering rates and total sample volumes collected at each field site depended on multiple factors. The most influential factor was turbidity of the ground water. The more turbid the ground water, the faster the filter clogged and fewer liters could be filtered before the water pressure reached 30 PSI (maximum pressure capacity of the cartridge

Figure 4-6. At site S-4, the intake hose for the virus sampling apparatus was put directly into the large spring pool.

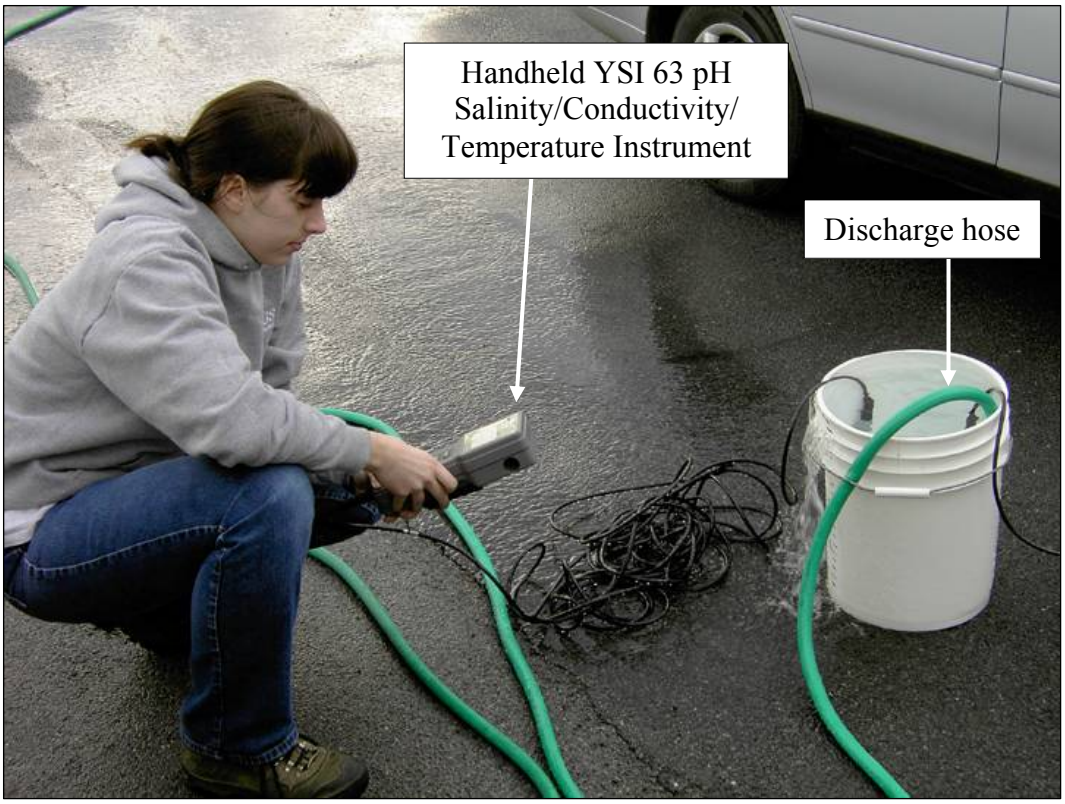

Figure 4-7. Water temperature, pH, and specific conductance of the ground water were measured during virus sampling.

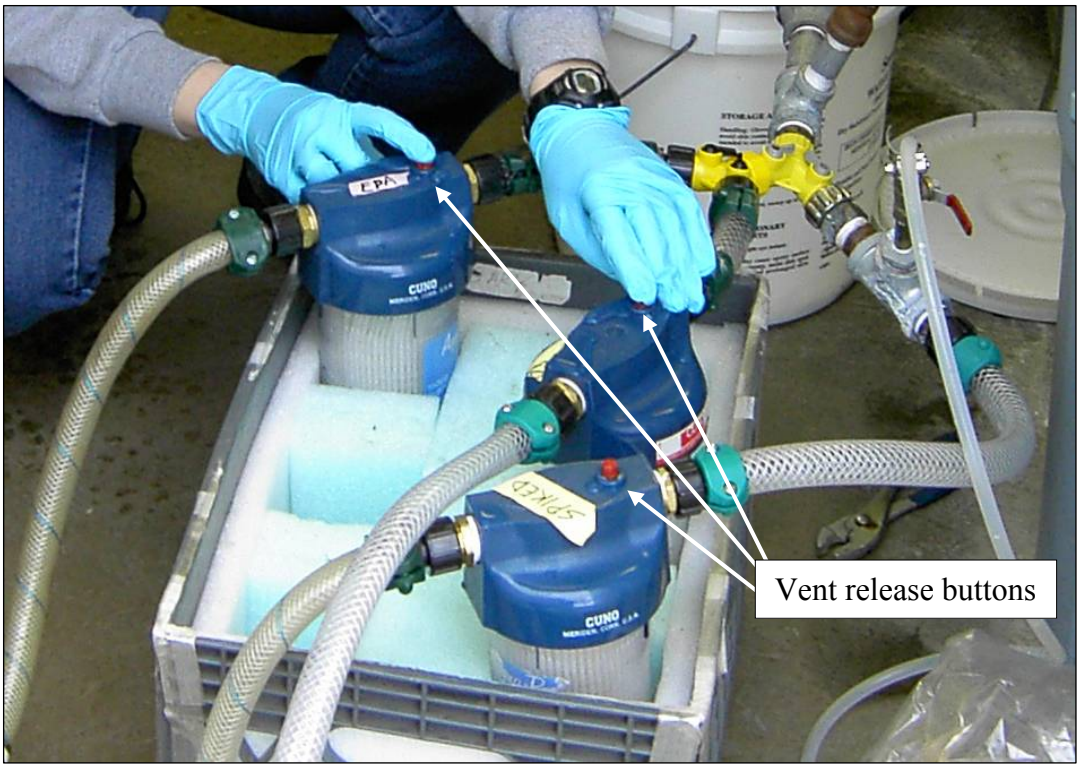

Figure 4-8. Vent release buttons on the cartridge housing lids were pushed to release air bubbles trapped in the virus sampling apparatus.

filters). Other factors included the pumping capacity of the electric pump, if used, and the water pressure of the ground water leaving the faucet, if used. In some cases, pumping time had to be limited because the field site was almost three hours away from UTK. Because this study was the first survey of virus occurrence in East Tennessee aquifers, there were no expectations for viral concentrations. Therefore, when possible, the maximum volume of ground water recommended in the USEPA ICR Rule (1,500 to 2,000 liters) (USEPA 1995), was filtered to maximize the likelihood of detecting viruses.

Field parameters such as water temperature, pH, and specific conductance were continuously monitored during virus sampling. About half-way through collection of the virus sample, ground water was collected for alkalinity, hardness, and turbidity measurements, indicator bacteria analyses, and chemical analyses. When there was only one faucet available to access the raw ground water from a well, the water was shut off, and the viral sampling apparatus disconnected in order to collect water for the various analyses. Other times, a short discharge port with an on-off valve would be connected to the viral sampling apparatus upstream of the cartridge housings (Figure 4-9) so that water for non-viral analyses could be collected without stopping the flow through the sampling apparatus. When springs were sampled, grab samples for non-viral analyses were collected from the spring pool or the stream. After filtration was complete, the final sample volumes for each filter, along with ancillary data, were recorded on USEPA sample data sheets (Appendix 4-1). The cartridge housings were disconnected from the sampling apparatus and the excess water was poured out. The open ends of the housings were wrapped in parafilm and the housings were double bagged individually and placed

Figure 4-9. A short discharge port with an on-off valve was connected to the viral sampling apparatus.

upright in a cooler of ice. The cooler was then either shipped or driven to the USEPA virology lab in Cincinnati, Ohio. Water for indicator bacteria was processed at the field site and analyzed later at UTCEB. Water for chemical constituents was put on ice and taken back to UTK for analysis. The virus sampling apparatus, along with the electric pump if used, was cleaned and sterilized immediately in the field as previously described and stored until the next sampling trip.

Quality-control samples were also collected during the field activities. A total of three field blanks, also known as equipment blanks, were collected throughout the study. A field blank was sterile deionized water used to determine if the sampling equipment was properly cleaned and sterilized, and if any contamination occurred during handling, processing, or analysis of the samples. To collect a field blank, 10 liters of sterile deionized water in a sterile carboy was transported to the field site and then pumped through the virus sampling apparatus after the equipment had been cleaned and sterilized. The cartridge filter was then handled like a regular sample, processed alongside the regular samples, and analyzed for enteric viruses by cell culture, conventional RT-PCR, and real-time RT-PCR. Matrix replicate spikes were also collected in the field. One matrix replicate spike was collected from each field site for a total of eight samples throughout the study. Matrix replicate spikes were used to assess virus recovery efficiency during sample collection and processing, to compare the effect of different water matrices on the recovery efficiencies, and to determine the reproducibility of the field and laboratory methods used. To collect a matrix replicate spike, an additional filter was collected at the same time as the regular sample/s by splitting the water flowing into the virus sampling apparatus (Figure 4-10). An additional 10 liters of ground water was

Figure 4-10. The flow into the virus sampling apparatus was split three ways to collect the matrix replicate spike and two regular samples.

also collected in a sterile carboy. Both the filter and the carboy were shipped or driven to the USEPA virology lab in Cincinnati, Ohio. The next day, the carboy was spiked with 100 µL of type 1 attenuated poliovirus (CHAT strain) at a concentration of 10^4 PFU/µL. The spiked water was then pushed through the virus filter using a peristaltic pump. The filter was processed as a regular sample and analyzed for enteric viruses by cell culture, RT-PCR, and real-time RT-PCR.

Field Measurements

In the USGS National Field Manual for the Collection of Water-Quality Data (Wilde and Radtke 2005), field measurements, also called field water-quality parameters, are defined as "determinations of physical or chemical properties that are measured onsite, as close as possible in time and space to the media being sampled". Field measurements collected in association with each viral sample collected in this study included water temperature, pH, specific conductance, turbidity, alkalinity, and hardness.

Water temperature, pH, and specific conductance were monitored constantly during collection of the ground water samples using a handheld YSI 63 pH Salinity/Conductivity/Temperature Instrument (YSI Incorporated; Yellow Springs, Ohio). When springs were sampled, the YSI 63 probe was placed directly in the spring pool or stream. When wells were sampled, the probe was placed in a bucket along with the discharge hoses from the virus sampling apparatus. The YSI 63 was calibrated for pH in the lab or field in conjunction with each sampling trip.

Turbidity was measured before each ground water sample was collected, midway through sampling, and at the end of sampling using a Hach 2100P portable turbidimeter (Hach Company; Loveland, Colorado). Water for turbidity measurements was collected

from the spring pool or stream when springs were sampled and from the faucet or extra discharge port when wells were sampled. Turbidity was always measured upstream of the virus filters. The calibration of the turbidimeter was checked with the included Gelex standards before every sampling trip.

Alkalinity, total hardness, and calcium hardness were measured in the field using Hach titration-based colorimetric test kits (Figure 4-11). The ground water for these measurements was collected midway through viral sampling from the spring pool or stream when springs were sampled or from the faucet or extra discharge port when wells were sampled.

Tennessee public water systems send monthly reports to TDEC called Monthly Operating Reports, or MOR's. These MOR's are filled out by the water treatment plant operators and often contain daily field measurements of the raw ground water. Whenever available, MOR raw ground water data for the day of sampling was compared to the field parameters measured by the UTK field crew for additional quality assurance.

Chemical Analyses

Ground water samples were collected in 1-liter sterile, clean glass bottles for measurement of nutrients and major ion constituents. The ground water was collected directly from the spring pool or stream when springs were sampled and from the faucet or extra discharge port when wells were sampled. The samples were put on ice and then into a 4°C refrigerator at UTK until they were analyzed. The nutrient analyses consisted of nitrate and reactive phosphate and the major ion analyses included chloride, fluoride, and sulfate. Samples were analyzed using a Hach DR/4000 UV-VIS Laboratory Spectrophotometer (Hach Company, Loveland, CO). These chemical constituents were

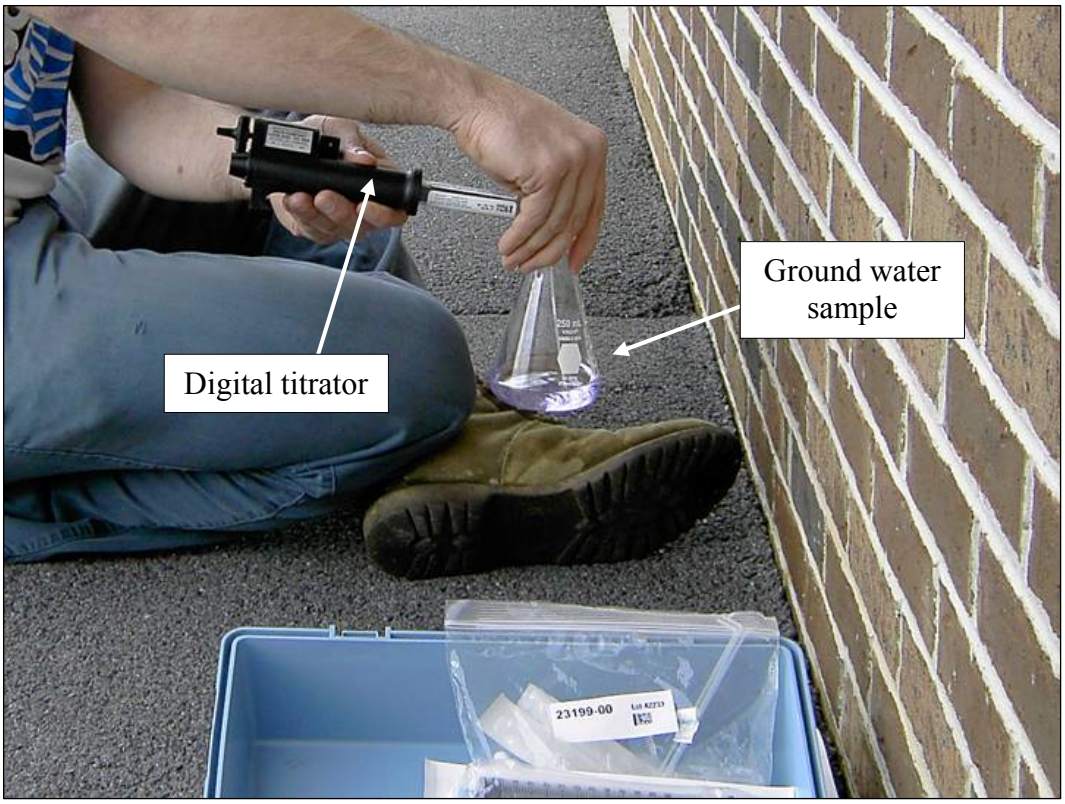

Figure 4-11. The alkalinity of a ground water sample is tested using a Hach titration-based test kit.

measured to provide information about the dominate bedrock type as well as the influence of anthropogenic activities on the ground water source.

Indicator Bacteria

E. coli and Total Coliforms

Ground water samples were collected and analyzed for *E. coli* and total coliforms using the Hach ColiBlue24™ Test assay (MEL/MF Total Coliform Laboratory, HACH Company, Loveland, CO). This membrane filtration method is approved by the USEPA as a presence/absence method for *E. coli* and total coliforms. Ground water samples were collected in sterile glass bottles midway through virus filtration either from the well faucet, extra discharge port on the sampling apparatus, or from the spring pool or stream. Samples were immediately filtered using suction through a membrane filter in a sterile filter funnel (Figure 4-12). The sides of the funnel were washed down with sterile phosphate buffer saline (PBS). The membrane filter was placed in a petri dish on top of an absorbent pad that had been soaked with an m-ColiBlue24®1 Broth ampule. All assays were performed in triplicate and quality-control filter blanks (sterile buffer filtered before the ground water sample) and procedure blanks (sterile buffer filtered after the ground water sample) were also performed. The petri dishes were incubated at 35°C for 24 hours and then enumerated. The blue colonies represented *E. coli* and the red colonies plus the blue colonies represented total coliforms.

Bacteroides

Ground water was collected and analyzed for *Bacteroides*, a host-specific fecal anaerobe found in high concentrations (10^{10} cells per gram of feces) (Matsuki et al. 2004), using real-time PCR assays developed at UTCEB (Layton et al. *in revision*).

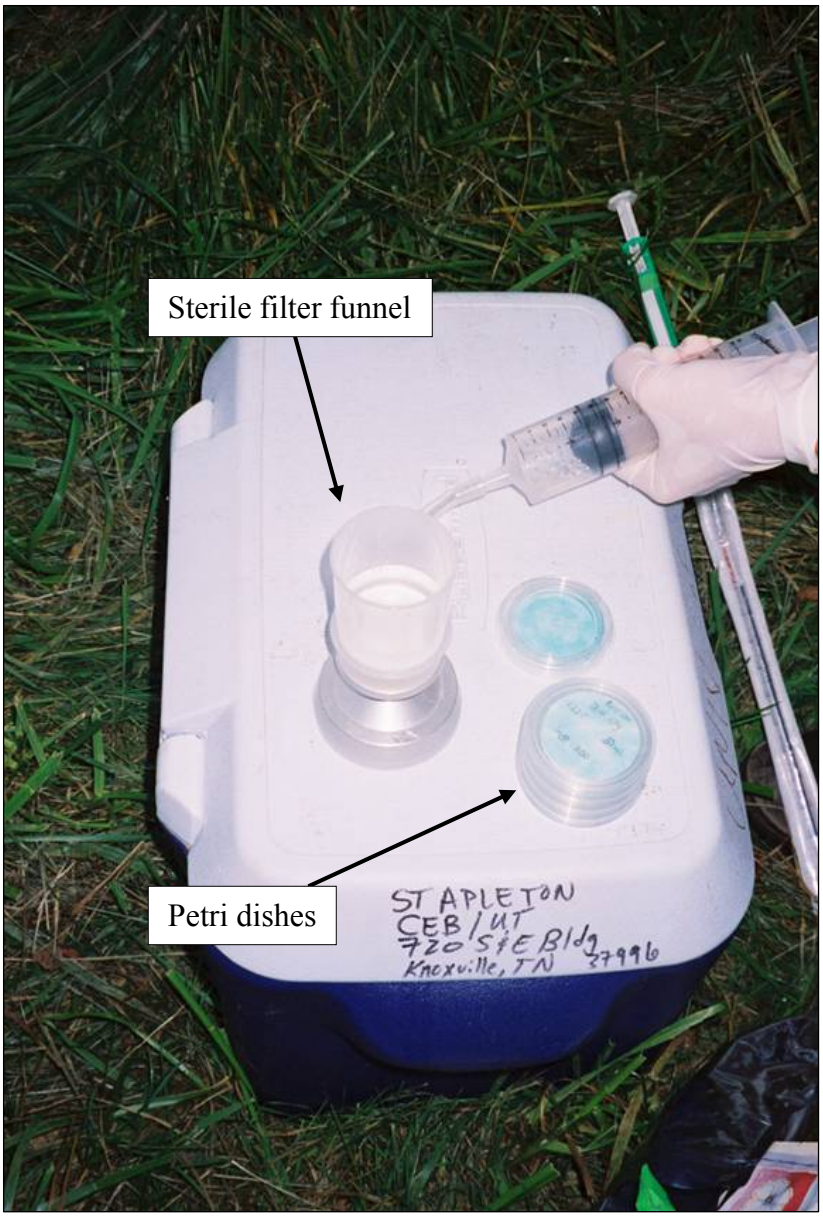

Figure 4-12. A UTK field crew member processes a ground water sample for *E. coli* and total coliforms using the Hach ColiBlue24™ Test.

These real-time PCR assays amplify the 16S rRNA gene of *Bacteroides*. By targeting different sequences within the gene, the assays can quantify (as milligrams of feces per liter of water) the amount of fecal contamination in a ground water sample as well as differentiate between total *Bacteroides*, human *Bacteroides* and bovine *Bacteroides*. Ground water samples were collected in sterile glass bottles midway through virus filtration either from the well faucet, extra discharge port on the sampling apparatus, or from the spring pool or stream. A 100-mL aliquot of sample was poured into a sterile plastic syringe and pushed through a 0.45-µm syringe disc filter. The filter was then inverted and backwashed with 1 mL of Tris buffer into a sterile 1.5 mL tube. The tube was put on ice and brought back to UTCEB and frozen at -80°C until real-time PCR of the sample could be performed. Table 4-1 lists the primers and probes for the total (AllBac), human (HuBac), and bovine (BoBac) *Bacteroides* real-time PCR assays. Table 4-2 gives the PCR protocols for each assay. The real-time PCR assays were run in triplicate on an Opticon™ real-time PCR detector (DNA Engine Opticon® Continuous Fluorescence Detection System, MJ Research Incorporated; Waltham, MA). Reactions were setup with reagents from the QuantiTect Probe PCR Kit (Qiagen Incorporated; Valencia, CA) according to the manufacturer's instructions and 2.5 µL of sample was analyzed per reaction. Standard quality-control samples run with each *Bacteroides* real-time PCR assay included PCR negative controls (nuclease-free water blanks) and, for quality assurance of quantitation, serial dilutions of DNA standards in triplicate.

Table 4-1. Primers and probes for *Bacteroides* real-time PCR assays.

Assay	Probe/Primer Name	Sequence (5'-3')
AllBac	ABac296f	GAGAGGAAGGTCCCCCAC
	ABac412r	CGCTACTTGGCTGGTTCAG
	ABac375Bhqr	(FAM)-CCATTGACCAATATTCCTCACTGCTGCCT-(BHQ-1)
BoBac	CBac367f	GAAG(G/A)CTGAACCAGCCAAGTA
	CBac467r	GCTTATTCATACGGTACATACAAG
	CBac402Bhqf	(FAM)-TGAAGGATGAAGGTTCTATGGATTGTAAACTT-(BHQ-1)
HuBac	Hubac566f	GGGTTTAAAGGGAGCGTAGG
	Hubac692r	CTACACCACGAATTCCGCCT
	Hubac594Bhqf	(FAM)-TAAGTCAGTTGTGAAAGTTTGCGGCTC-(BHQ-1)

Table 4-2. PCR protocols for *Bacteroides* real-time PCR assays.

Assay	PCR Protocol
AllBac	1) 50°C for 2 min 2) 95°C for 10 min 3) 95°C for 30 sec 4) 60°C for 45 sec 5) Plate read 6) Go to 3) and repeat 44 times 7) End
BoBac	1) 50°C for 2 min 2) 95°C for 5 min 3) 95°C for 30 sec 4) 57°C for 45 sec 5) Plate read 6) Go to 3) and repeat 44 times 7) End
HuBac	1) 50°C for 2 min 2) 95°C for 10 min 3) 95°C for 30 sec 4) 60°C for 45 sec 5) Plate read 6) Go to 3) and repeat 44 times 7) End

Conventional Viral Analyses

Virus Filter Elution and Sample Concentration

After filtration at the field site using the virus sampling apparatus, cartridge housings containing 1MDS cartridge filters were packed in a cooler of ice and shipped or driven to the USEPA virology lab in Cincinnati, Ohio. They were refrigerated at 4°C until elution. Filters were always eluted within 72 hours of sample collection but often within 24 hours, if possible, which is within acceptable quality assurance parameters (Fout et al. 1996). Viruses were eluted from the cartridge filters using a two-day protocol developed at the USEPA in Cincinnati, Ohio (Dahling 2002) that yields improved virus recoveries. Using this elution method, viruses on each filter were eluted into a total of 160 mL of sodium phosphate buffer.

The elution protocol is summarized here; details are provided in Appendix 4-2. The housings and enclosed filters were allowed to come to room temperature. First, 1600 mL of 1.5% beef extract at room temperature (pH 9.5) was pushed through the filter housing containing the cartridge filter using a sterile stainless steel pressure vessel and collected in a large sterile glass beaker. The filter housing was then opened and 800 mL of fresh beef extract was poured directly over the filter. The housing unit was closed tightly and set aside to soak in the dark at room temperature overnight. Viruses in the first elution were concentrated by adding 1.6 grams of celite (diatomaceous earth) to the 1600 mL of eluted beef extract, the pH was lowered to 4.0 with 1 M HCL added drop wise, and the mixture was stirred at room temperature for 15 minutes. Next, the celite was collected on sterile glass fiber prefilters by vacuum filtration. The adsorbed viruses were eluted from the celite by allowing 80 mL of 0.15 M sodium phosphate, pH 9.0 to

9.5, to filter through the celite by gravity filtration. This final eluate was lowered to pH 7.0 with 1 M HCl, filter sterilized, and then frozen at -80°C until cell culture analysis or further concentration prior to molecular analysis. The next day, a fresh 800-mL aliquot of beef extract, along with the 800 mL that had been soaking the filter overnight, was pushed through the filter housing and the 1600 mL were collected in another large sterile glass beaker. Then the entire celite step was repeated for the overnight beef extract elution. Again, the final eluate was frozen at -80°C. The two elutions for each filter were kept separate in the freezer. They were analyzed separately by cell culture but portions of each were combined during the concentration and purification steps necessary before conventional and real-time RT-PCR analysis.

Limited reaction volumes in RT-PCR assays require highly concentrated environmental samples, leading to an increase in inhibitory substances (Ijzerman et al. 1997, Lewis et al. 2000, Shieh et al. 1995). A virus sample concentration and inhibitor removal method was developed by the USEPA (Fout et al. 2003). This inhibitor removal procedure concentrates the viruses if present, removes some of the inhibitory substances from the sample, and improves virus recovery. The detailed sample concentration and inhibitor removal protocol is given in Appendix 4-3.

Briefly, the two celite eluates were removed from the freezer and thawed quickly in a water bath. Twenty milliliters of each eluate were combined. Eighty microliters of 5% bovine serum albumin (BSA) was mixed with the combined sample. Then 30 mL of the sample was pipetted into a sterile SW28 ultracentrifuge tube that had soaked overnight in PBS containing 0.2% BSA (PBSAA). The sample was carefully underlain with 5 mL of a 30% sucrose solution and then another 2 mL of sample was added on top.

This process was repeated for an ultracentrifuge tube with sterile water instead of sample to create a negative process control. Another tube was prepared with sterile water plus the addition of enterovirus to create a positive process control. The viruses in each sample, if present, were pelleted through the sucrose cushion during a 4.5 hour centrifugation in the ultracentrifuge at 131,000 x *g* and 10°C. At the end of the centrifugation, the supernatant was immediately aspirated and the pellet was resuspended in two 100-µL alloquots of PBSAA. Next, 200 µL of a solvent mixture (containing dithiazone, hydroxyquinoline, methanol, butanol, and trichloroethane) prepared fresh for each use, was added to the resuspended pellet. The tube was vortexed for 30 seconds, allowed to sit for 15 seconds, vortexed again for 30 seconds, and allowed to sit for 30 seconds. Then it was centrifuged in a microcentrifuge for 5 minutes at 4°C. The top aqueous layer was removed and pipetted into a Microcon-100 filter unit that had soaked overnight in PBSAA and spun in a microcentrifuge for 30 minutes at 4°C. The filter unit was washed with 80 µL of PBSAA and centrifuged again for 20 minutes at 4°C. Another 10 µL of PBSAA was added to the filter unit, the Microcon-100 filter housing was put into a clean microcentrifuge tube, capped, and vortexed for 15 seconds. The housing was then inverted in the tube and centrifuged for 3 minutes. The filter units were discarded. The volume of the concentrated sample was measured and brought up to 100 µL with PBSAA. The sample was then split into two tubes, one tube of 50 µL of sample for conventional RT-PCR analysis at the USEPA and one tube of 50 µL of sample for real-time RT-PCR analysis at UTCEB. The tubes were frozen at -80°C until analyzed.

Enteroviruses by Cell Culture Analysis

Ground water samples were analyzed at the USEPA in Cincinnati, Ohio for total infectious, culturable enteric viruses using a modified version of the cell culture assay (Total Culturable Virus Quantal Assay) described in the USEPA Information Collection Rule or ICR (USEPA 1995). This test is used to detect primarily infectious enterovirus (Fout et al. 1996) but also detects reovirus, and is based upon direct microscopic viewing of Buffalo Green monkey kidney (BGMK) cell cultures for virus-induced cytopathic effects (CPE). Cytopathic effects are hard to see by an untrained eye but include changes in cell morphology, clumping of cells, and cell sloughing. A brief protocol of the cell culture assay is included in Appendix 4-2.

The first and second (overnight) celite elutions from the virus filter were quickly thawed in a water bath. Twenty milliliters of each celite elution (40 mL total for each virus filter) was inoculated into perforated tissue culture roller bottles containing a monolayer of BGMK cells. After 80 minutes, the samples were poured into another perforated tissue culture bottle. After another 80 minutes, the excess sample was poured off from the second bottle and discarded. This procedure yielded a total of 4 roller bottles inoculated per ground water sample. Next, 200 mL of cell culture media was added to each bottle. The roller bottles were stored on a roller apparatus in a 37°C incubation room, and observed for CPE for two weeks. If CPE was observed at a level 3 (CPE is rated from 1-4, with 4 being the most severe CPE) in any of the roller bottles, the cell culture was immediately frozen at -80°C and viruses were considered to be present in the original water sample. If no CPE was observed at the end of two weeks, the cell culture was frozen at -80°C and the cell culture lysate was later passaged onto test tubes

of BGMK cells using the following procedure. The cell culture from the roller bottles underwent three cycles of freeze/thaw to release any viruses trapped in the cells, creating cell culture lysate. Then 0.5 mL of lysate was inoculated onto each tube, up to 20 tubes per roller bottle. The tubes were incubated at 37°C and observed for CPE for two weeks. If no CPE was observed at the end of two weeks, this passage procedure was repeated up to two more times. With wild strain viruses, it is often necessary to perform three or more passages before CPE is observed. If no CPE was observed in the tubes after the passages, culturable viruses were considered to be absent in the original sample. If CPE was observed, the original water sample was considered to be positive for viruses.

When a ground water sample tested positive for viruses, the most probable number (MPN) test was performed to quantify the viruses in the sample. The MPN test was performed by inoculating multiple tubes of BGMK cells with 0.5 mL per tube of 10-fold dilutions of the original celite eluate and observing them for CPE. As long as there was, for all dilutions of the sample combined, a combination of positive and negative tubes, the MPN could be calculated using the equation,

$$\text{MPN/mL} = \frac{P}{\sqrt{NQ}}$$

where P = total number of positive cultures, N = total mL of sample inoculated, and Q = total mL in all negative cultures (personal communication, USEPA virologists in Cincinnati, Ohio). The results were reported as most probable number of infectious units (PFU) per 100 liters of original water sample.

Enteroviruses by RT-PCR

Ground water samples were analyzed at the USEPA in Cincinnati, Ohio for the presence or absence of enterovirus RNA using RT-PCR analysis followed by dot blot hybridization and agarose gel electrophoresis for confirmation of hybridization results. This method is similar to the one described by Fout and others (Fout et al. 2003) but is an analysis for a single target (enterovirus) instead a multiplex reaction to analyze for multiple virus types at the same time. Since the enterovirus genome is single stranded RNA, this method has a separate reverse transcription step to convert the RNA to cDNA prior to PCR. The primers and probe for this assay are given in Table 4-3 and are specific to a highly conserved region of the enterovirus genome (De Leon et al. 1990). A detailed protocol of the RT-PCR analysis is located in Appendix 4-4 and is summarized here only briefly. All ground water samples collected during the study were analyzed by RT-PCR at the same time on the same PCR plate. They were tested a total of three times, two times undiluted and one time at a 1:10 dilution to check for PCR inhibition. The RT reactions began by mixing 5 µL of each ground water sample (after concentration and inhibitor removal) with 23.25 µL of an RT mix containing 10X PCR Buffer, 25 mM $MgCl_2$, 10mM dNTPs, DEPC treated water, and the RT/downstream primer MRD 13 in separate wells of a 96 well plate. The plate was then heated for 5 minutes at 99°C to release the viral RNA. Next, 1.75 µL of a mix containing the reverse transcriptase MuLV RT and RNasin was added to each well. The plate was placed in a thermocycler and heated to 43°C for one hour and then to 95°C for 5 minutes. Next, PCR was performed by adding 70 µL of a PCR mix containing 10X PCR Buffer, 25 mM $MgCl_2$, nuclease-free water, AmpliTaq Gold polymerase, and the PCR/upstream primer MRD 14

Table 4-3. Primers and probe used for conventional RT-PCR of enterovirus.

Primer/Probe Name	Type	Sequence (5'-3')
MRD 13	Downstream/RT primer	ACCGGATGGCCAATCCAA
MRD 14	Upstream/PCR primer	CCTCCGGCCCCTGAATG
MRD 32	Hybridization Probe	ACTACTTTGGGTGTCCGTGTTTC

to each well. The plate was returned to the thermocycler for a program of 95°C for 10 minutes, 45 cycles of 95°C for 1 minute followed by 50°C for 90 seconds followed by 72°C for 120 seconds, and 72°C for 10 minutes. Next both dot blot hybridization and agarose gel electrophoresis were performed using 5 μL of RT-PCR product from each well with each assay. Electrophoresis was run on a 2% high resolution blend agarose gel stained with ethidium bromide. Dot blot hybridization was performed on Magnagraph nylon membranes. Only samples that were positive by hybridization were considered positive. In other words, samples were reported as positive for enterovirus if one or more of the 3 reactions showed positive signals by dot blot hybridization but not if they showed bands on the agarose gel but did not show a positive signal by dot blot hybridization.

To rule out false positives and false negatives, PCR quality-control samples were run. They included RT-PCR positive controls (sterile water seeded with virus), RT-PCR negative controls (sterile water or buffer), hybridization positive controls (sterile water seeded with virus), and negative hybridization controls (sterile water to check for cross-contamination during hybridization). In addition, the USEPA virology lab in Cincinnati, Ohio has stringent QA/QC procedures which include using different individuals and different rooms for various steps of RT-PCR and gel electrophoresis and hybridization (Fout et al. 2003, USEPA 2004).

Protocol for Real-time RT-PCR Analysis of Ground Water Samples

Ground water samples collected from East Tennessee wells and springs between March 2004 and August 2004 were analyzed for enterovirus using the real-time RT-PCR assay developed by UTCEB (described in Chapter 3). The primers and probes and PCR

mix and conditions for this analysis were given previously in Table 3-1 and Table 3-3, respectively. The real-time RT-PCR assays were run on a DNA Engine Opticon® Continuous Fluorescence Detection System (MJ Research Incorporated; Waltham, MA) using a QuantiTect Probe RT-PCR Kit (Qiagen Incorporated; Valencia, CA). The analyses were set up as follows. Following cartridge filter elution, sample concentration, and inhibitor removal procedures, ground water samples were frozen at -80°C until real-time RT-PCR could be performed at UTCEB. Just before analysis, the samples were removed from the freezer, thawed at room temperature, and put immediately on ice. Next, fresh dilutions of the DNA plasmid and the attenuated poliovirus were made and placed immediately on ice. After determining how many RT-PCR reactions would be needed for the run, a master mix tube was prepared for the planned number of RT-PCR reactions plus 5% extra. Per reaction, the master mix tube contained (added to the tube in the following order) 12.5 µL QuantiTect Probe RT-PCR Master Mix, 7.625 µL nuclease-free water, 0.75 µL (0.68 µM) forward primer (EvUTR24fv3), 0.75 µL (0.68 µM) reverse primer (EvUTR145rv3), 0.625 µL (0.25 µM) probe (EvUTRprobe112rv3), and 0.25 µL QuantiTect RT Mix, for a total of 22.5 µL. Next, the attenuated poliovirus standard and the concentrated ground water samples (only the amount to be analyzed) were heated to 99°C for 5 minutes in a PCR thermocycler to break open virus capsids and release the viral RNA. Then they were immediately quenched on ice. During this time, the master mix was pipetted into the appropriate wells on the PCR plate along with the serial dilutions of the DNA plasmid (2.5 µL per well). The attenuated poliovirus and concentrated ground water samples (diluted 1:5 to reduce the effects of PCR inhibition) were added to the appropriate wells (2.5 µL per well). Lids were placed over the wells,

the plate was put into the real-time PCR machine, and the program was entered in to the computer. The concentration of viruses in each sample was calculated by comparing its C_T values with C_T values generated from the DNA plasmid and/or attenuated poliovirus standard curves.

Quality Assurance and Quality Control for Real-time RT-PCR

Quality assurance and quality control samples are essential with molecular assays such as real-time RT-PCR to rule out false positives, false negatives, and to ensure that basic microbiological laboratory QA/QC practices are being upheld. There were several types of quality control samples for the real-time RT-PCR analyses. PCR negative controls consisted of adding 2.5 μL of sterile, nuclease-free water to PCR wells instead of the target template. These samples ensured that there was no contamination in the RT-PCR reagents and that no contamination occurred during setup of the plate. These were run in triplicate with each real-time RT-PCR run. The next quality assurance measure was to run the serial dilutions of the DNA plasmid as well as the ground water samples in triplicate. Two RT-PCR inhibition controls (or RT-PCR spikes) were run in association with each sample tested. They consisted of two wells that contained the ground water sample (2.5 μL in each well) plus the addition of 2.5 μL DNA plasmid in one well and 2.5 μL attenuated poliovirus in the other well. They were used to quantify the amount of enzyme inhibition associated with each ground water sample. If a quality control sample did not produce a favorable result (such as a PCR negative control coming out positive), the results of ground water samples run that day were discarded and the ground water samples were run again on a different day. Lastly, gel electrophoresis was occasionally performed on the real-time RT-PCR products to confirm results.

CHAPTER V
RESULTS AND DISCUSSION

The purpose of this chapter is to present and discuss the results of the field study. Ground water samples were collected from four wells and four springs between March and August of 2004. Ground water samples were analyzed for physical and chemical parameters, indicator bacteria, and enteric viruses by cell culture, RT-PCR, and the real-time RT-PCR assay for enteroviruses developed by UTCEB. Consistent with the field-based hypotheses for this study, the results for each analysis will be discussed primarily with respect to the "high risk" and "low risk" designations assigned to each sampling site.

Physical and Chemical Parameters

Physical and chemical parameters were measured in association with each ground water sample to characterize the differences in the ground water matrices at each site (which may affect virus recoveries), the geology of bedrock, and the possible effects of anthropogenic processes on the ground water source. Field water-quality parameters were also monitored to detect any unusual changes in water-quality conditions during sampling. In addition, turbidity and pH limits (turbidity < 75 NTU and pH < 8.0), given in the Information Collection Rule (ICR), were met to ensure maximum recovery efficiency of viruses on the 1MDS cartridge filters. Results for ground water temperature, pH, specific conductance, turbidity, alkalinity, and hardness measured in the field are presented in Table 5-1. Results for chemical constituents measured in the lab (chloride, fluoride, nitrate, phosphate, and sulfate) are presented in Table 5-2.

Table 5-1. Field water-quality measurements for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Water Temperature (°C)	pH (standard units)	Specific Conductance (µS/cm)	Turbidity (NTU)	Total Hardness (mg/L as $CaCO_3$)	Calcium Hardness (mg/L)	Calcium/ Magnesium Ratio[#]	Alkalinity (mg/L as $CaCO_3$)
S-1	High	3/22/2004	14.3	7.1	284	3.2	156	92	1.4	147
		8/8/2004	14.4	7.1	305	0.96	170	107	1.7	155
S-2	High	3/29/2004	12.7	7.0	335	0.87	180	135	3.0	155
		8/14/2004	13.6	7.5	354	1.3	189	145	3.3	180
S-4	High	6/7/2004	15.7	6.8	459	26	220	NT	N/A	240
		8/15/2004	14.9	6.8	507	3.0	231	167	2.6	255
W-2	High	4/12/2004	14.4	7.4	284	8.3	150	85	1.3	135
		8/16/2004	16.5	7.5	298	4.1	170	95	1.3	160
W-1	Low	3/1/2004	13.2	7.7	231	0.15	114	80	2.4	99
		8/10/2004	14.3	7.8	229	0.16	113	85	3.0	97
W-3	Low	8/3/2004	13.8	5.3	20	0.29	4	BDL	N/A	9
W-4	Low	8/4/2004	15.9	7.4	230	0.03	119	68	1.3	111
S-3	Low	5/19/2004	14.3	7.6	145	0.07*	69	39	1.3	63
		8/9/2004	14.6	7.6	147	0.35	67	30	0.8	54

*Turbidity was not sampled by the field crew. Turbidity value was obtained from the water treatment plant and was for the finished water after chlorine, phosphate, and fluoride were added (no filtration or settling during treatment).

[#]The calcium/magnesium ratio was calculated by dividing the calcium hardness by the difference between the total hardness and the calcium hardness.

Abbreviations: BDL, below minimum detection level; NT, not tested; N/A, not applicable, °C, degrees Celsius; NTU, nephelometric turbidity units; µS/cm, microsiemens per centimeter; mg/L as $CaCO_3$, milligrams per liter as calcium carbonate.

Table 5-2. Chemical constituents measured in the laboratory for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Chloride (mg/L)	Fluoride (mg/L)	Nitrate (mg/L)	Reactive Phosphate (mg/L)	Sulfate (mg/L)
S-1	High	3/22/2004	2	0.1	1	0.1	2
		8/8/2004	3	0.2	1	0.1	2
S-2	High	3/29/2004	5	0.3	1	0.1	< 2
		8/14/2004	4	0.2	2	< 0.1	2
S-4	High	6/7/2004	5	0.2	1	0.1	19
		8/15/2004	6	0.3	2	0.1	15
W-2	High	4/12/2004	5	0.1	1	0.1	< 2
		8/16/2004	3	0.2	1	< 0.1	< 2
W-1	Low	3/1/2004	5	0.2	1	< 0.1	2
		8/10/2004	6	0.3	1	< 0.1	2
W-3	Low	8/3/2004	0.2	0.1	< 0.1	0.2	4
W-4	Low	8/4/2004	3	0.1	0.6	0.1	2
S-3	Low	5/19/2004	3	0.1	0.6	0.3	8
		8/9/2004	4	0.1	0.6	0.3	6

Abbreviations: <, less than; mg/L, milligrams per liter.

Temperature

Ground water temperatures for all of the wells and springs sampled in this study ranged from 12.7 °C to 16.5 °C with an average temperature of 14.5 ± 1.0 °C. Average temperatures at the sampling sites were slightly higher in the late summer (14.8 ± 1.0 °C) than they were in the spring or early summer (14.1 ± 1.0 °C). The average temperatures from the "high risk" sites and "low risk" sites were 14.6 ± 1.2 °C and 14.4 ± 0.9 °C, respectively. The geometric means of the temperatures from the "high risk" sites and "low risk" sites were 14.5 °C and 14.3 °C, respectively.

pH

Ground water pH for all of the wells and springs sampled in this study ranged from 5.3 to 7.8 with an average pH of 7.2 ± 0.6, close to pH values found in other studies of the region (e.g. median pH of 7.1 by Johnson 2002). Site W-3 had the lowest pH, probably because of its location in a mountain-top fractured sandstone aquifer, which was expected to have less pH buffering capacity than the carbonate aquifers in which most of the other wells or springs were located. Site S-4 also had a slightly lower pH (6.8) than most of the other sites, possibly due to the addition of acidic rainwater to the spring pool, where pH values were measured. The pH at any given site was never ≥ 8.0 and therefore pH reduction with hydrochloric acid during ground water filtration was not necessary. The average pH values from the "high risk" sites and "low risk" sites were 7.2 ± 0.3 and 7.2 ± 1.0, respectively. The geometric means of the pH values from the "high risk" sites and "low risk" sites were 7.1 and 7.2, respectively.

Specific Conductance

Specific conductance of the ground water from all of the wells and springs sampled in this study ranged from 20 µS/cm to 507 µS/cm. The average specific conductance value for all of the wells and springs was 273 ± 125 µS/cm and was primarily attributed to the ions released from dissolution of the carbonate bedrock at most of the sites. Site W-3 had the lowest specific conductance due to its location in a fractured sandstone aquifer which was expected to be relatively resistant to dissolution. Site S-3 had the next lowest specific conductance (average specific conductance of 146 µS/cm) which was likely due to its location in the Rome Formation (dolomite, limestone, and dolomitic sandstones, siltstones, shales) which is more resistant to dissolution than the Cambrian-Ordovician carbonate aquifers in which most of the other wells and springs were located. The highest specific conductance occurred at site S-4 and was probably a result of both dissolution of the carbonate bedrock and also the abandoned zinc mines the ground water flowed through, introducing other ions such as zinc, sulfate, and iron. The average specific conductance values from the "high risk" sites and "low risk" sites were 353 ± 85 and 167 ± 83, respectively. The geometric means of the specific conductance values from the "high risk" sites and "low risk" sites were 345 µS/cm and 132 µS/cm, respectively.

Turbidity

It is important to note that the turbidity values measured by the field crew were often different than the turbidity values reported by the water treatment plants for each site (Table 5-3) because the portable field turbidimeter used by the field crew was not as sensitive (especially at low turbidity values) as the in-line turbidimeters used by the water

Table 5-3. Raw-water turbidity values as measured by the field crew compared to raw-water turbidity values reported by the treatment plants for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Turbidity Measured by the Field Crew (NTU)	Turbidity Reported by the Water Treatment Plant (NTU)	Final Turbidity Value Used for Data Analysis (NTU)
S-1	High	3/22/2004	1.7	3.2	3.2
		8/8/2004	1.1	0.96	0.96
S-2	High	3/29/2004	NT	0.87	0.87
		8/14/2004	1.1	1.3	1.3
S-4	High	6/7/2004	26	NT	26
		8/15/2004	3.0	NT	3.0
W-2	High	4/12/2004	8.3	NT	8.3
		8/16/2004	4.1	NT	4.1
W-1	Low	3/1/2004	0.15	0.15	0.15
		8/10/2004	0.15	0.16	0.16
W-3	Low	8/3/2004	0.29	NT	0.29
W-4	Low	8/4/2004	0.10	0.03	0.03
S-3	Low	5/19/2004	NT	NT	0.07*
		8/9/2004	0.35	NT	0.35

*Turbidity was not sampled by the field crew. Turbidity value was obtained from the water treatment plant and was for the finished water after chlorine, phosphate, and fluoride were added (no filtration or settling during treatment).
Abbreviations: NT, not tested; NTU, nephelometric turbidity units.

treatment plants. When raw-water turbidity values were available from the water treatment plant, those values were used for data analysis because they were expected to be more accurate. However, when there were no raw-water turbidity values available from the water treatment plant at the time of sampling, the turbidity values measured by the field crew were used for data analysis because they best represented conditions at the time of sampling.

Ground water turbidity for all of the wells and springs sampled in this study ranged from 0.03 NTU to 26 NTU with an average turbidity of 3.5 ± 6.9 NTU. The lowest turbidity value was measured at site W-4, which had been designated as "true ground water" by the state. The highest turbidity value was measured at site S-4, a large GWUDI spring draining a well-developed sinkhole plain. Small differences in turbidity between sites had a large effect on how much water could be passed through the 1MDS filters before they began to clog. Turbidity values measured during sampling were not high enough to require the use of prefilters during ground water filtration because sampling was not conducted during storm events, during which many springs and wells under the influence of surface water typically respond with large spikes (sudden increases and decreases) in turbidity.

Average turbidity values at the sampling sites were higher in the spring or early summer (6.4 ± 10.1 NTU) than they were in the late summer (1.3 ± 1.5 NTU). The same observation was made when the geometric mean of the turbidity values from the spring or early summer (1.4 NTU) were compared to the geometric mean of turbidity values from the late summer (0.5 NTU). Although no appreciable rainfall had occurred at any site in the few days prior to sampling (data not shown), these results suggest more elevated

hydrological conditions in the spring and early summer than in the late summer. The average turbidity values from the "high risk" sites and "low risk" sites were 6.0 ± 8.4 NTU and 0.2 ± 0.1 NTU, respectively. The geometric means of the turbidity values from the "high risk" sites and "low risk" sites were 3.1 NTU and 0.1 NTU, respectively.

Hardness

Total hardness values for all of the wells and springs sampled in this study ranged from 4 mg/L as $CaCO_3$ to 231 mg/L as $CaCO_3$. The average total hardness for all of the wells and springs was 139 ± 63 mg/L as $CaCO_3$ and was primarily attributed to the ions released from dissolution of the carbonate bedrock present at most of the sites. The average total hardness values from the "high risk" sites and "low risk" sites were 183 ± 29 mg/L as $CaCO_3$ and 81 ± 44 mg/L as $CaCO_3$, respectively. The geometric means of the total hardness values from the "high risk" sites and "low risk" sites were 181 mg/L as $CaCO_3$ and 55 mg/L as $CaCO_3$, respectively.

Site W-3 was classified as "soft" ground water because it had a hardness of < 75 mg/L as $CaCO_3$ (Durfor and Becker 1964). The total hardness of 4 mg/L as $CaCO_3$ at this site was probably attributed by iron and/or manganese from the sandstone bedrock, not calcium or magnesium. Site S-3 was also classified as "soft" ground water with a mean total hardness of 68 mg/L as $CaCO_3$, which was probably due to the more resistant dolomitic sandstone and shale bedrock underlying this spring versus the more soluble carbonate bedrock underlying most of the other sites. With total hardness values between 75 and 150 mg/L as $CaCO_3$, ground water from wells W-1 and W-4 was considered "moderately hard". With total hardness values between 150 and 300 mg/L as $CaCO_3$, ground water from sites S-1, S-2, W-2, and S-4 was considered "hard". No sites were

classified as having "very hard" ground water. Total hardness at site S-4 was probably higher than at the other sites because it was a large spring and there were likely other contributing solids besides calcium and magnesium due to the abandoned zinc mines through which the ground water flowed.

Calcium hardness was also measured at each site and ranged from below the detection limit (< 1.0 mg/L) to 167 mg/L with an average hardness of 94 ± 40 mg/L. The average calcium hardness values from the "high risk" sites and "low risk" sites were 118 ± 31 mg/L and 60 ± 25 mg/L, respectively. The geometric means of the calcium hardness values from the "high risk" sites and "low risk" sites were 115 mg/L and 29 mg/L, respectively.

Using the assumption that the difference between total hardness and calcium hardness represented the magnesium hardness, the ratio of calcium hardness to the calculated magnesium hardness was used to determine which wells and springs were located in more dolomitic bedrock than others. In a pure dolomite aquifer, the ratio of calcium to magnesium is 1.0. The wells and springs that came closest to that ratio were S-1, W-2, S-3, and W-4. Sites W-1, S-2, and S-4 were less dolomitic and site W-3 had no detectable calcium hardness, verifying its location in a non-carbonate sandstone aquifer. The site with the most dolomitic ground water was S-3, located in the dolomitic Rome Formation. The average calcium to magnesium ratios for the "high risk" sites and "low risk" sites were 2.1 ± 0.9 and 1.8 ± 0.9. The geometric means of the calcium to magnesium ratios for the "high risk" sites and "low risk" sites were 1.9 and 1.6, respectively.

Alkalinity

Alkalinity, a measure of the ability of ground water to resist changes in pH (acid buffering capacity), for all of the wells and springs sampled in this study, ranged from 9 mg/L as $CaCO_3$ to 255 mg/L as $CaCO_3$ with an average alkalinity of 133 ± 68 mg/L as $CaCO_3$. Since the pH values of all of the wells and springs were < 8.3, the alkalinity was probably present mostly as bicarbonate ion. The low alkalinity at site W-3 was due to its location in a non-carbonate aquifer and was reflected in the low pH of the ground water at this site. Site S-3 had the next lowest levels of alkalinity due primarily to its location in the more resistant dolomitic Rome Formation. The highest alkalinity at site S-4 was related to the high amounts of dissolved solids from the carbonate bedrock and the abandoned zinc mines, but may have also reflected local anthropogenic affects on the ground water source. The average alkalinity values from the "high risk" sites and "low risk" sites were 178 ± 45 mg/L as $CaCO_3$ and 72 ± 38 mg/L as $CaCO_3$, respectively. The geometric means of the alkalinity values from the "high risk" sites and "low risk" sites were 174 mg/L as $CaCO_3$ and 57 mg/L as $CaCO_3$, respectively.

Laboratory-Measured Chemical Constituents

Chloride concentrations for all of the wells and springs sampled in this study were low and ranged from 0.2 mg/L to 6 mg/L with an average concentration of 4 ± 2 mg/L. Site W-3 had the lowest levels of chloride because its recharge zone was in a national forest and the ground water was not influenced by industry or other human activities that may have introduced chloride. Also, chloride was probably not a dominant constituent in the sandstone bedrock. The average chloride concentrations from the "high risk" sites and "low risk" sites were 4 ± 1 mg/L and 4 ± 2 mg/L, respectively. The geometric means

of the chloride concentrations of the "high risk" sites and "low risk" sites were 3.9 mg/L and 2.4 mg/L, respectively.

Fluoride concentrations for all of the wells and springs sampled in this study were low and ranged from 0.1 mg/L to 0.3 mg/L with an average concentration of 0.2 ± 0.1 mg/L. The average fluoride concentrations from the "high risk" sites and "low risk" sites were both 0.2 ± 0.1 mg/L. The geometric means of the fluoride concentrations of the "high risk" sites and "low risk" sites were 0.2 mg/L and 0.1 mg/L, respectively.

Nitrate concentrations for all of the wells and springs sampled in this study were low and ranged from below the detection limit (< 0.1 mg/L) to 2 mg/L with an average concentration of 1.0 ± 0.5 mg/L. Nitrate was not found at detectable levels at site W-3 because its recharge zone was in a national forest and the ground water was not influenced by agriculture or other human activities that may have introduced nitrate. The average nitrate concentrations from the "high risk" sites and "low risk" sites were 1.3 ± 0.5 mg/L and 0.6 ± 0.3 mg/L, respectively. The geometric means of the nitrate concentrations of the "high risk" sites and "low risk" sites were 1.2 mg/L and 0.5 mg/L, respectively.

Phosphate concentrations for all of the wells and springs sampled in this study were very low and ranged from below the detection limit (< 0.1 mg/L) to 0.3 mg/L with an average concentration of 0.1 ± 0.1 mg/L. Phosphate concentrations were at or below the detection limit of 0.1 mg/L at sites W-1, S-1, S-2, W-2, S-4, and W-4. The average phosphate concentrations from the "high risk" sites and "low risk" sites were 0.1 ± 0.0 mg/L and 0.2 ± 0.1 mg/L, respectively. The geometric means of the phosphate

concentrations of the "high risk" sites and "low risk" sites were 0.1 mg/L and 0.1 mg/L, respectively.

Sulfate concentrations for all of the wells and springs sampled in this study were low and ranged from below the detection limit (< 2 mg/L) to 19 mg/L with an average concentration of 5 ± 6 mg/L. Sulfate concentrations were at or below the detection limit of 2 mg/L at sites W-1, S-1, S-2, W-2, and W-4. The average sulfate concentrations from the "high risk" sites and "low risk" sites were 5 ± 7 mg/L and 4 ± 3 mg/L, respectively. The geometric means of the sulfate concentrations of the "high risk" sites and "low risk" sites were each 3 mg/L.

Summary of Results for Physical and Chemical Parameters

When the field water-quality parameters and the chemical constituents for each well and spring are analyzed together, a few conclusions can be drawn about the general ground water quality at the sampling sites. The overall chemical-quality of the wells and springs was good. None of the wells or springs had levels of the analyzed chemical constituents above the EPA-recommended National Secondary Drinking Water Regulations for chloride (250 mg/L), fluoride (2 mg/L), sulfate (250 mg/L), and phosphate (5 mg/L), or above the maximum contaminate level (MCL) for nitrate (10 mg/L) in drinking water. The levels of nitrate and phosphate were less than or equal to the national natural ground water concentrations for these nutrients (Johnson 2002). Chloride, fluoride and sulfate concentrations were low and were similar to concentrations found in other studies of ground water quality in East Tennessee (Johnson 2002). There were no elevated concentrations of chloride, phosphate, or nitrate to suggest major anthropogenic impacts on the chemical-quality of the ground water at any site. Bedrock

composition was probably the most influential factor in the higher levels of sulfate found at sites S-4 and S-3, rather than anthropogenic affects, since levels of the other major ions and nutrients were not elevated.

The results for field-water quality parameters also indicated generally good physical and chemical water-quality at the wells and springs. These parameters can be used to distinguish differences in bedrock geology and in the influence of surface water among the sampled wells and springs. The pH, specific conductance, alkalinity, and hardness of the wells and springs (excluding site W-3) were all within typical ranges for ground water of East Tennessee derived from carbonate aquifers (Johnson 2002). The pH, specific conductance, alkalinity, and hardness measured at site W-3 (located in non-carbonate sandstone) were much lower, as was expected, than for the wells and springs in the carbonate units. The bedrock chemistry at site S-4 was clearly different from the other wells and springs in the carbonate units. This was most likely due to its larger recharge area, higher discharge, and the nearby zinc mines which led to higher concentrations of sulfate (and probably other ions not measured in this study).

Table 5-4 presents the averages and standard deviations of the field water-quality parameters and chemical constituents measured for each sampling site with regard to their risk designations for fecal contamination. The "high risk" wells and springs had statistically significant higher average specific conductance, hardness (both total and calcium), alkalinity, and nitrate values than the "low risk" wells and springs. These results partially support field-based hypothesis #2 which stated that "high risk" wells and springs were expected to have higher levels of indicators other than *E. coli*, such as the

Table 5-4. Field water-quality parameters and chemical constituents measured for "high risk" versus "low risk" sites.

Field Water-Quality Parameter or Chemical Constituent	Averages and standard deviations for the "high risk" sites	Averages and standard deviations for the "low risk" sites	Significance (at $p < 0.05$) of difference between "high risk" and "low risk" sites
Water Temperature (°C)	14.6 ± 1.2	14.4 ± 0.9	NS
pH (standard units)	7.2 ± 0.3	7.2 ± 1.0	NS
Specific Conductance (µS/cm)	353 ± 85	167 ± 83	S
Turbidity (NTU)	6.0 ± 8.4	0.2 ± 0.1	NS
Total Hardness (mg/L as $CaCO_3$)	183 ± 29	81 ± 44	S
Calcium Hardness (mg/L)	118 ± 31	60 ± 25	S
Calcium/Magnesium Ratio	2.1 ± 0.9	1.8 ± 0.9	NS
Alkalinity (mg/L as $CaCO_3$)	178 ± 45	72 ± 38	S
Chloride (mg/L)	4 ± 1	4 ± 2	NS
Fluoride (mg/L)	0.2 ± 0.1	0.2 ± 0.1	NS
Nitrate (mg/L)	1.3 ± 0.5	0.6 ± 0.3	S
Reactive Phosphate (mg/L)	0.1 ± 0.0	0.2 ± 0.1	NS
Sulfate (mg/L)	5 ± 7	4 ± 3	NS

Abbreviations: NS, no significant difference between "high risk" sites and "low risk" sites; S, significant difference between "high risk" sites and "low risk" sites; °C, degrees Celsius; µS/cm, microsiemens per centimeter; NTU, nephelometric turbidity units; mg/L as $CaCO_3$, milligrams per liter as calcium carbonate; mg/L, milligrams per liter.

measured field water-quality parameters and chemical constituents. However, many of the field water-quality parameters and chemical constituents (such as turbidity and chloride) were not found to be statistically higher at "high risk" sites than at "low risk" sites. These results also suggest that, as expected based on the criteria used for risk designation, "high risk" wells and springs were more influenced by bedrock dissolution processes, and possible anthropogenic processes than "low risk" wells and springs.

Indicator Bacteria

Ground water samples were analyzed for total coliforms, *E. coli*, and *Bacteroides* to characterize the extent of fecal contamination and to investigate the relationship between the occurrence and concentrations of indicator bacteria and enteric viruses in the sampled wells and springs.

Total coliforms and *E. coli* are the most commonly used indicator organisms for microbial contamination of ground water supplies and are measured routinely by public water systems under the national Total Coliform Rule. Total coliform bacteria are found in feces, but are also naturally present in soils and surface water environments and thus may or may not indicate fecal contamination. Total coliform bacteria, however, are good indicators that contamination from the surface or shallow subsurface has entered the ground water system (Lindsey et al. 2002). Although total coliforms are not considered good fecal indicators, they are routinely measured in virus occurrence studies (and in this study) because currently they are the primary standard (USEPA 2001b) for determining microbial contamination in public water systems using ground water supplies.

E. coli is a much better indicator of fecal contamination than total coliform bacteria. *E. coli* is a subset of total coliform bacteria which indicates the presence of

fecal contamination from warm-blooded animals and therefore the possible presence of enteric pathogens such as viruses. *E. coli*, however, is a facultative anaerobe that may be able to survive and reproduce in the environment (Byappanahalli et al. 2003) and thus may not indicate a recent source of fecal contamination.

Bacteroides is a host-specific fecal anaerobe and its presence in ground water indicates a very recent source of fecal contamination (Layton et al. *in revision*). Concentrations of *Bacteroides* can be used to quantify the amount of fecal contamination in ground water. Since total coliforms and *E. coli* found in ground water supplies may not necessarily be of human origin, the presence and concentration of human *Bacteroides* can be used to distinguish human from non-human sources of fecal contamination (Layton et al. *in revision*).

E. coli and Total Coliforms

A total of 14 ground water samples collected from four wells and four springs were analyzed for *E. coli* and total coliforms using the ColiBlue24™ membrane filtration method. The ColiBlue24™ method was approved by the USEPA only as a presence/absence method for *E. coli* and total coliforms in source water and drinking water. However, for this study, *E. coli* and total coliform colonies were enumerated using methods described in the USGS Field Manual (Meyers and Wilde 1997). The presence/absence results and enumerated concentrations for *E. coli* and total coliforms are presented in Table 5-5. Concentrations of *E. coli* and total coliforms are given in colony forming units per 100 milliliters (CFU/100 mL) of ground water.

Table 5-5. *E. coli* and total coliform data for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	*E. coli* P/A	Total Coliforms P/A	*E. coli* (CFU/100 mL)	Total Coliforms (CFU/100 mL)
S-1	High	3/22/2004	P	P	8 K	70
		8/8/2004	P	P	3 K	>80 E
S-2	High	3/29/2004	P	P	88	180
		8/14/2004	A	P	<10 E	1700 K
S-4	High	6/7/2004	P	P	360	>800 E
		8/15/2004	P	P	20 K	3000 K
W-2	High	4/12/2004	P	P	190 K	870 K
		8/16/2004	P	P	50 K	780
W-1	Low	3/1/2004	A	A	<1 E	<1 E
		8/10/2004	A	P	<1 E	1 K
W-3	Low	8/3/2004	A	A	<1 E	<1 E
W-4	Low	8/4/2004	A	A	<1 E	<1 E
S-3	Low	5/19/2004	P	P	5 K	160 K
		8/9/2004	A	P	<1 E	34

Abbreviations: P/A, presence/absence; P, presence; A, absence; K, an estimated count based on a non-ideal colony count on the membrane filter; < E, maximum estimated number; > E, minimum estimated number; CFU/100 mL, colony forming units per 100 milliliters of water. The ideal count range for *E. coli* and total coliforms is 20-80 colony forming units on a filter.

E. coli concentrations for all of the wells and springs ranged from below the detection limit (< 1 CFU/100 mL) to 360 CFU/100 mL with an average concentration of 91 ± 126 CFU/100 mL and a geometric mean of 8 CFU/100 mL. *E. coli* was detected at 4/4 (100%) of the "high risk" sites and at 1/4 (25%) of the "low risk" sites. When considered on a per sample basis, *E. coli* was detected in 7/8 (88%) of the samples from "high risk" sites and in 1/6 (17%) of the samples from "low risk" sites. The average *E. coli* concentrations at the "high risk" sites and "low risk" sites were 103 ± 131 CFU/100 mL and 5 CFU/100 mL (only one sample point), respectively. The geometric means of the *E. coli* concentrations at the "high risk" sites and "low risk" sites were 39 CFU/100 mL and 5 CFU/100 mL (only one sample point), respectively. Figure 5-1 presents the distributions of *E. coli* concentrations for the "high risk" sites compared to the "low risk" sites.

At three "high risk" sites on three sampling days (S-1 on 8/8/04, S-2 on 8/14/04, and S-4 on 8/15/04), the reported concentrations of *E. coli* were likely biased low because the m- ColiBlue24™ broth was overexposed to sunlight and photobleaching of the colorimetric indicators occurred. The broth is normally blue due to the presence of 5-Bromo-4-Chloro-3-Indolyl-BetaD-glucuronide (BCIG) which reacts with the ß-glucuronidase enzyme of *E. coli* to give the *E. coli* colonies a blue color. On these sampling days, the blue color of the broth had faded significantly. Total coliform concentrations were probably unaffected because the formation and color of their colonies is not related to the blue color of the broth. Although the *E. coli* concentrations from these dates were probably underestimated, the concentrations were still used in

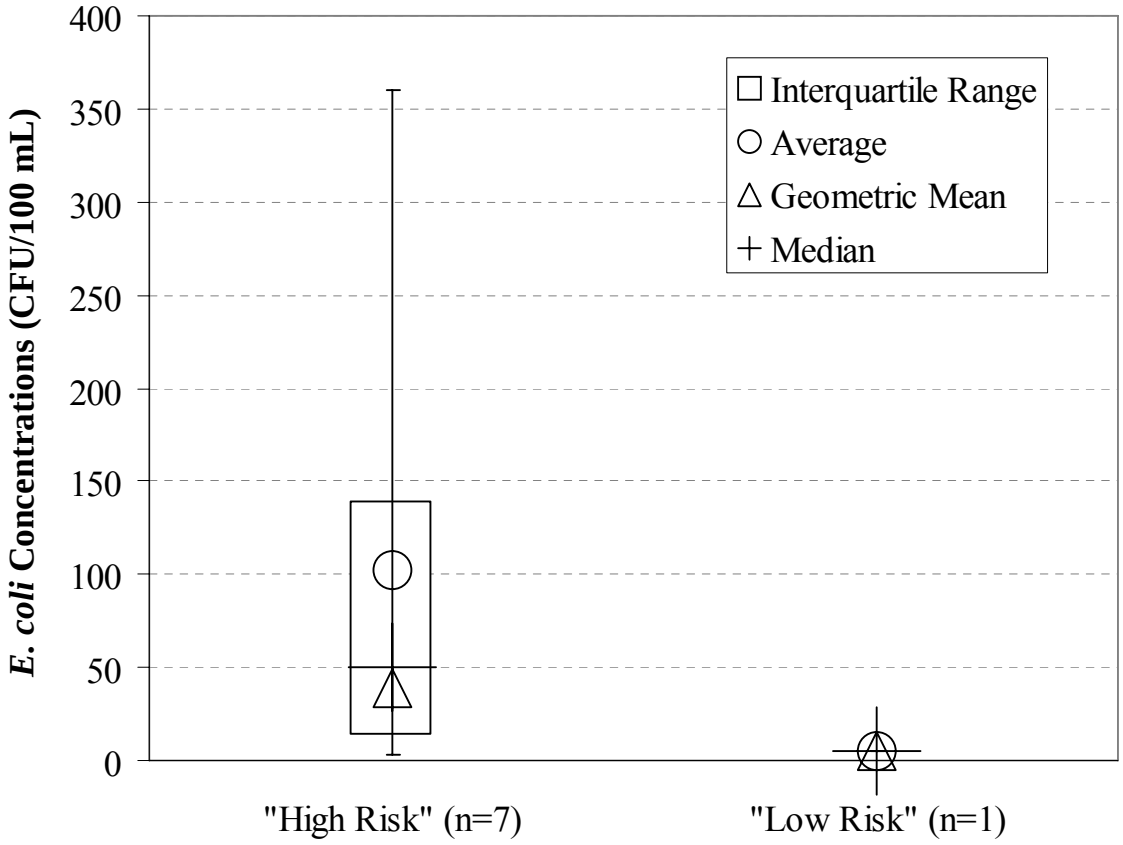

Figure 5-1. *E. coli* concentrations at "high risk" versus "low risk" sites. Box plot whiskers represent the maximum and minimum concentrations.

calculations for descriptive and comparative data analyses if they were above the detection limit.

Total coliform concentrations for all of the wells and springs ranged from below the detection limit (< 1 CFU/100 mL) to > 3,000 CFU/100 mL with an average concentration of 698 ± 928 CFU/100 mL and a geometric mean of 195 CFU/100 mL. Total coliforms were detected at 4/4 (100%) of the "high risk" sites and at 2/4 (50%) of the "low risk" sites. When considered on a per sample basis, total coliforms were detected in 8/8 (100%) of the samples from "high risk" sites and in 3/6 (50%) of the samples from "low risk" sites. The average total coliform concentrations at the "high risk" sites and "low risk" sites were 935 ± 996 CFU/100 mL and 65 ± 84 CFU/100 mL. The geometric means of the total coliform concentrations at the "high risk" sites and "low risk" sites were 479 CFU/100 mL and 18 CFU/100 mL, respectively. Figure 5-2 presents the distributions of total coliform concentrations for the "high risk" sites compared to the "low risk" sites.

Quality-assurance samples to rule out false positives from sample contamination (filter blanks) and to ensure proper rinsing of the filtration funnel during membrane filtration (procedure blanks) were collected in association with each ground water sample and were analyzed for both *E. coli* and total coliforms. All of the quality-assurance samples were negative for *E. coli* and total coliforms.

Bacteroides

Ground water samples collected from the wells and springs were analyzed for *Bacteroides* using real-time PCR assays developed at UTCEB (Layton et al. *in revision*).

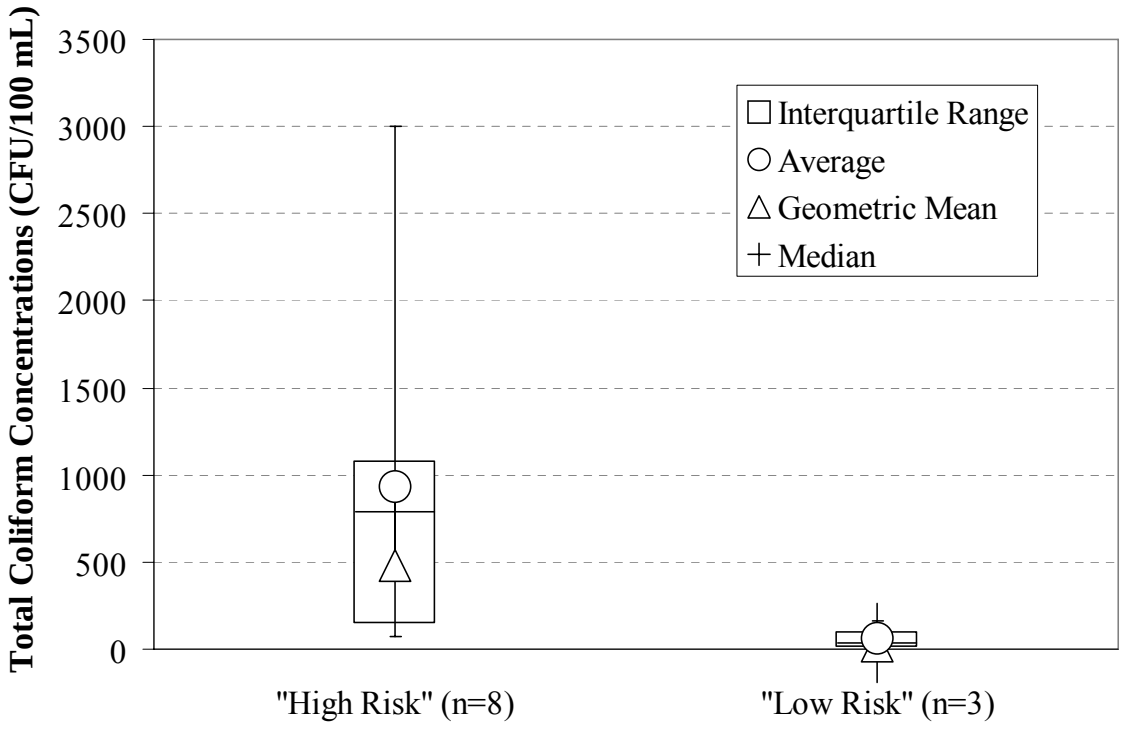

Figure 5-2. Total coliform concentrations at "high risk" versus "low risk" sites. Box plot whiskers represent the maximum and minimum concentrations. A Student's t-test determined that the difference in total coliform concentrations at "high risk" sites versus "low risk" sites was statistically significant at $p < 0.05$.

Fourteen ground water samples were analyzed for total *Bacteroides* and human *Bacteroides* using the AllBac and HuBac assays, respectively. Twelve ground water samples were analyzed for bovine *Bacteroides* using the BoBac assay. The results of all three assays are presented in Table 5-6.

Total *Bacteroides* concentrations for all of the wells and springs sampled in this study ranged from below the detection limit (< 0.0005 mg feces/L of water) to 0.58 ± 0.35 mg feces/L with an average concentration of 0.2017 ± 0.1929 mg feces/L and a geometric mean of 0.0693 mg feces/L. Total *Bacteroides* was detected at 4/4 (100%) of the "high risk" sites and at 2/4 (50%) of the "low risk" sites. When considered on a per sample basis, total *Bacteroides* was detected in 8/8 (100%) of the samples from "high risk" sites and in 3/6 (50%) of the samples from "low risk" sites. The average total *Bacteroides* concentrations at the "high risk" sites and "low risk" sites were 0.234 ± 0.1949 mg feces/L and 0.1155 ± 0.1944 mg feces/L. The geometric means of the total *Bacteroides* concentrations at the "high risk" sites and "low risk" sites were 0.1220 mg feces/L and 0.0153 mg feces/L, respectively. Figure 5-3 presents the distributions of the total *Bacteroides* concentrations for the "high risk" sites compared to the "low risk" sites.

Human *Bacteroides* concentrations for all of the wells and springs sampled in this study ranged from below the detection limit (< 0.0005 mg feces/L) to 0.024 ± 0.024 mg feces/L with an average concentration of 0.0145 ± 0.0111 mg feces/L and a geometric mean of 0.0113 mg feces/L. Human *Bacteroides* was detected at 4/4 (100%) of the "high risk" sites and at 2/4 (50%) of the "low risk" sites. When considered on a per sample basis, human *Bacteroides* was detected in 6/8 (75%) of the samples from "high risk" sites and in 2/6 (33%) of the samples from "low risk" sites. The average human *Bacteroides*

Table 5-6. *Bacteroides* data (averages and standard deviations for triplicate real-time PCR analyses) for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Total *Bacteroides* (mg feces/L)	Human *Bacteroides* (mg feces/L)	Bovine *Bacteroides* (mg feces/L)
S-1	High	3/22/2004	0.12 ± 0.069	0.0039 ± 0.0050	BDL
		8/8/2004	0.0020 ± 0.0024	BDL	BDL
S-2	High	3/29/2004	0.30 ± 0.21	0.013 ± 0.0072	BDL
		8/14/2004	0.17 ± 0.078	0.013 ± 0.012	BDL
S-4	High	6/7/2004	0.45 ± 0.14	BDL	NT
		8/15/2004	0.11 ± 0.032	0.0064 ± 0.0052	BDL
W-2	High	4/12/2004	0.58 ± 0.35	0.037 ± 0.019	BDL
		8/16/2004	0.14 ± 0.025	0.013 ± 0.0093	BDL
W-1	Low	3/1/2004	0.0034 ± 0.0026	0.0053 ± 0.0054	BDL
		8/10/2004	BDL	BDL	BDL
W-3	Low	8/3/2004	BDL	BDL	BDL
W-4	Low	8/4/2004	BDL	BDL	BDL
S-3	Low	5/19/2004	0.34 ± 0.17	0.024 ± 0.024	NT
		8/9/2004	0.0031 ± 0.0011	BDL	BDL

Abbreviations: BDL, below minimum detection level (0.0005 mg feces/L of water); NT, not tested.

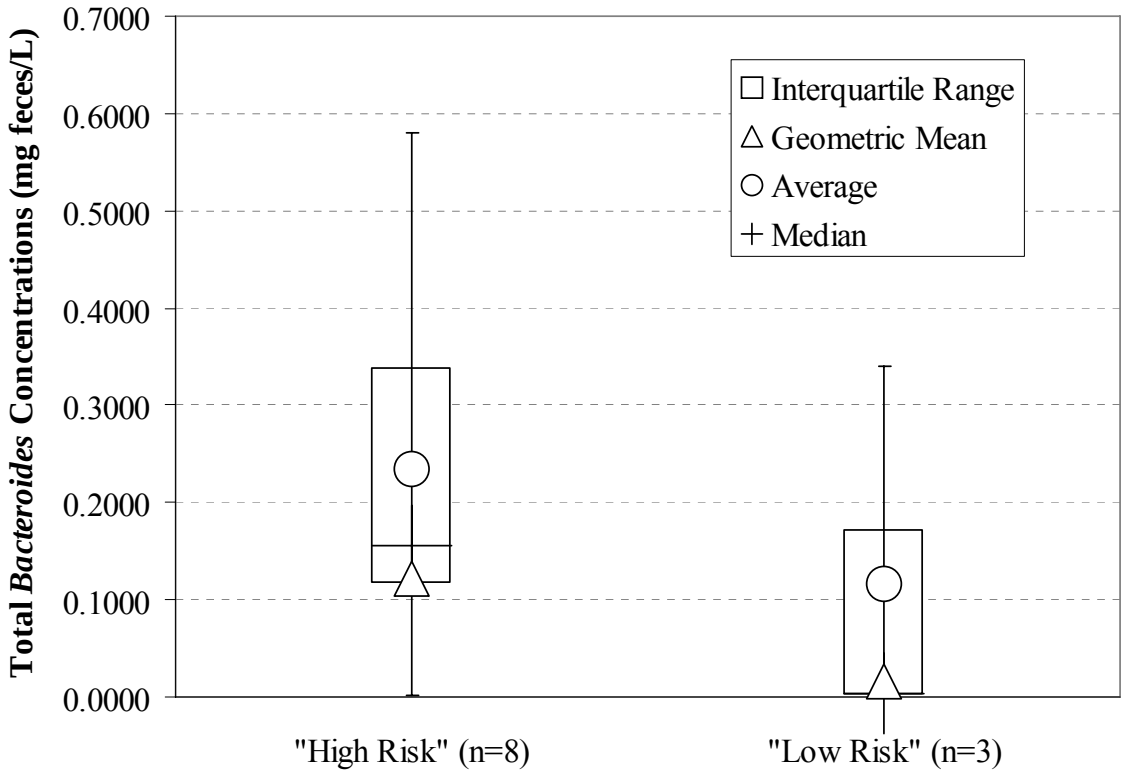

Figure 5-3. Total *Bacteroides* concentrations at "high risk" versus "low risk" sites. Box plot whiskers represent the maximum and minimum concentrations. A Student's t-test determined that the difference in total *Bacteroides* concentrations at "high risk" sites versus "low risk" sites was not statistically significant at $p < 0.05$.

concentrations at the "high risk" sites and "low risk" sites were 0.0144 ± 0.0118 mg feces/L and 0.0147 ± 0.0132 mg feces/L. The geometric means of the human *Bacteroides* concentrations at the "high risk" sites and "low risk" sites were each 0.0113 mg feces/L. Figure 5-4 presents the distributions of the human *Bacteroides* concentrations for the "high risk" sites compared to the "low risk" sites.

Bovine *Bacteroides* was not found at concentrations above the detection limit in any ground water samples collected during the study, suggesting that cows were not a major contributor of fecal contamination in these ground water systems at the time of sampling. Human *Bacteroides* comprised only about 12% of the total *Bacteroides* when the geometric means for total and human *Bacteroides* in all of the samples were compared. This suggests that other fecal sources, such as wildlife, birds, or livestock other than cows, may have been major contributors of fecal loading to these aquifers at the time of sampling. However, the overall very low levels of *Bacteroides* found throughout the study makes fecal source identification in the ground water samples uncertain.

Summary of Results for Indicator Bacteria

The combined results of the indicator bacteria analyses (*E. coli*, total coliforms, and *Bacteroides*) indicate low concentrations of human fecal contamination in the majority of the wells and springs sampled during this study. The results support field-based hypotheses #1 and #2 of this study: (1) "High risk" sites had more frequent detections and higher concentrations of *E. coli* than "low risk" sites (although it was not possible to determine the statistical significance of this difference because there was only one data point for the "low risk" sites) and (2) "High risk" sites had more frequent

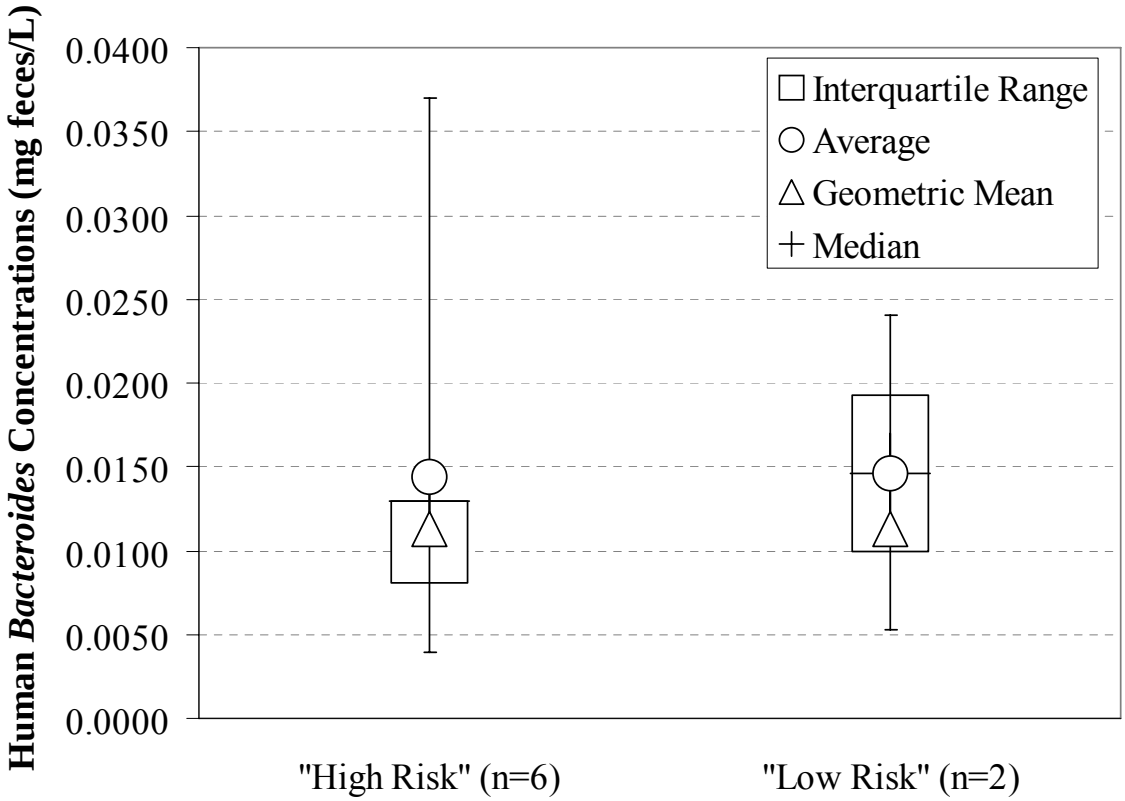

Figure 5-4. Human *Bacteroides* concentrations at "high risk" versus "low risk" sites. Box plot whiskers represent the maximum and minimum concentrations. A Student's t-test determined that the difference in human *Bacteroides* concentrations at "high risk" sites versus "low risk" sites was not statistically significant at $p < 0.05$.

detections and higher concentrations of *Bacteroides* (although concentration differences were not statistically significant) and total coliforms (concentration differences were statistically significant at $p < 0.05$) than "low risk" sites. At least one of the three indicator organisms was detected at 4/4 (100%) of the "high risk" sites and at 2/4 (50%) of the "low risk" sites. When considered on a per sample basis, at least one of the three indicator organisms was detected in 8/8 (100%) of the samples from "high risk" sites and in 3/6 (50%) of the samples from "low risk" sites. This data suggests that "low risk" for fecal contamination does not imply "no" risk for fecal contamination.

One well (W-2) and all 4 springs had total coliform and/or *E. coli* concentrations that exceeded the drinking water standards for public water systems (4 CFU/100 mL for total coliforms and 1 CFU/100 mL for *E. coli*). When the geometric mean concentrations of samples collected from springs were compared to the geometric mean concentrations of samples collected from wells, springs had higher concentrations of all three indicator bacteria (total coliforms, *E. coli*, and total *Bacteroides*) than did wells. However, a Student's t-test determined that none of these differences were statistically significant. There were however, more indicator-positive samples from springs than there were from wells. These results were similar to previous studies of total coliform and *E. coli* concentrations in karst aquifers in East Tennessee which found that although both wells and springs were susceptible to fecal contamination, springs were typically more vulnerable than wells (Johnson 2002 and Hampson 2000). These results were also expected since most of the sampled springs (3/4) were designated as "high risk" while most of the wells (3/4) were designated as "low risk" for fecal contamination. In

addition, these results are likely partly due to sampling from the spring pool (at 3 of the 4 springs) rather than from sub aqueous discharge entering the pool.

It is important to note that interpretation of human fecal contamination at these sampling sites is based on one or two samples from each site which only represent conditions of the ground water quality at the time of sampling (a snapshot). Concentrations of indicator bacteria in ground water can vary throughout the year due to hydrological variations (e.g. rainfall events) and temporal changes in fecal contamination sources such as sudden septic system failures, unintentional wastewater plant overflows, and new application of sludge to the land surface.

Enteric Viruses

Ground water samples collected from four wells and four springs between March and August of 2004 were analyzed for culturable enteroviruses and reoviruses, enteroviruses and reoviruses by separate RT-PCR assays, and enteroviruses by real-time RT-PCR. The original research plan called for analysis of the ground water samples with one cell culture method known as the Total Culturable Virus Quantal Assay (a modified version of the method used for the USEPA ICR). The method uses BGMK cells to detect and enumerate culturable (infectious) enteroviruses and reoviruses. However, based on the results of initial cell culture analyses, virologists at the USEPA recommended an additional cell culture analysis of the ground water samples with a different cell line specific for reoviruses (Madin-Darby bovine kidney or MDBK cells). The USEPA also analyzed the ground water samples for reoviruses by RT-PCR (in addition to the RT-PCR for enteroviruses). Finally, the ground water samples were analyzed for enteroviruses using the real-time RT-PCR assay developed at UTCEB (described in Chapter 3). The

use of a combination of culture-based and molecular-based virus detection methods increased the likelihood of detecting enteric viruses in the ground water samples and also provided information on the infectious state of the detected viruses.

Enteric Viruses by Cell Culture

Ground water samples collected from the wells and springs were analyzed for total culturable viruses using a modified version of the USEPA ICR cell culture method. Total culturable viruses include both infectious enteroviruses and reoviruses. The method uses BGMK cells to detect viruses through microscopic analysis of the cells for cytopathic effects (CPE). The virologists analyzing the ground water samples for this study noticed that upon passage of the roller bottles' cell lysate into tubes of BGMK cells, some of the samples often took more than a week to start showing CPE. It was unclear if this phenomenon was due to slow-growing viruses in the ground water samples (which are often reoviruses) or if it was the result of the cells being over a week old and beginning to die off (which can look similar to CPE).

In order to rule out false positives due to mistaking cell die-off for CPE, each ground water sample was also inoculated onto Madin-Darby bovine kidney (MDBK) cells, which are specific for reoviruses. Samples which showed CPE on the BGMK cells in only a few days but did not show CPE on the MDBK cells were presumed positive for enteroviruses. Samples which showed CPE on the MDBK cells in only a few days but showed either late or no CPE on the BGMK cells were considered positive for reoviruses. The presence of enterovirus in those samples was still possible though unlikely because the samples did not form plaques on BGMK cell monolayers during MPN analyses without the addition of pancreatin to the media. Pancreatin is an enzyme essential for

reovirus to plaque on BGMK cells, but it is not needed for enteroviruses to plaque on BGMK cells. Samples which did not show CPE using either cell line were presumed negative for both enteroviruses and reoviruses.

When a sample was positive on either or both cell lines, a portion of the original concentrated sample was inoculated into tubes of BGMK cells for MPN analysis in order to enumerate the number of total culturable viruses (enteroviruses and/or reoviruses) present in the sample. The presence/absence cell culture results for both cell lines, the volume of ground water filtered in the field for each sample (total sample volume), the total culturable virus MPN results (most probable number of infectious units or PFU per 100 liters of the total sample volume), and the presumed virus type present in each ground water sample are presented in Table 5-7. The effective sample volume analyzed by each cell culture method was 25% of the total sample volume (i.e. 40 milliliters of the 160 milliliters of concentrated ground water sample from each virus filter was analyzed by each cell culture method). Therefore, the results are presented as presence or absence of viruses per 25% of the total sample volume. For example, if 2,000 liters of ground water were filtered in the field, 500 liters of the sample were assayed with each of the cell lines.

Total Culturable Viruses

Total culturable viruses were detected at 4/4 (100%) of the "high risk" sites and at 3/4 (75%) of the "low risk" sites. When considered on a per sample basis, total culturable viruses were detected in 6/8 (75%) of the "high risk" samples and in 3/6 (50%) of the "low risk" samples. Total culturable virus concentrations for the samples from positive wells and springs ranged from 2 MPN/100 L to 156 MPN/100 L with an average

Table 5-7. Results for culturable viruses for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Total Sample Volume Filtered in the Field (Liters)	Volume of Sample Analyzed by each Cell Culture Assay (Liters)	Culturable Viruses by BGMK Cell Culture (P/A per volume of sample analyzed)	Culturable Viruses by MDBK Cell Culture (P/A per volume of sample analyzed)	Presumed Virus Type(s) In Ground Water Sample	Most Probable Number of PFU per 100 Liters of Total Sample Volume
S-1	High	3/22/2004	2364	591	P	A	Enterovirus	6
		8/8/2004	1922	481	A	A	N/A	N/A
S-2	High	3/29/2004	1949	487	P	P	Reovirus*	8
		8/14/2004	1893	473	A	A	N/A	N/A
S-4	High	6/7/2004	396	99	P	P	Reovirus*	13
		8/15/2004	1201	300	A	P	Reovirus	156
W-2	High	4/12/2004	947	237	P	P	Reovirus*	69
		8/16/2004	946	237	A	P	Reovirus	76
W-1	Low	3/1/2004	1894	474	P	A	Enterovirus	7
		8/10/2004	1758	440	A	A	N/A	N/A
W-3	Low	8/3/2004	1980	495	A	P	Reovirus	98
W-4	Low	8/4/2004	1621	405	A	A	N/A	N/A
S-3	Low	5/19/2004	1968	492	P	A	Enterovirus	2
		8/9/2004	1132	283	A	A	N/A	N/A

*The presence of enterovirus in these samples is possible but unlikely.
Abbreviations: P/A, presence/absence; P, presence; A, absence; N/A, not applicable; BGMK, Buffalo Green monkey kidney; MDBK, Madin-Darby bovine kidney; PFU, plaque forming unit.

concentration of 48 ± 55 MPN/100 L and a geometric mean of 21 MPN/100 L. The average total culturable virus concentrations for the "high risk" and "low risk" sites were 55 ± 59 MPN/100 L and 36 ± 54 MPN/100 L, respectively. The geometric means of the total culturable virus concentrations at the "high risk" sites and "low risk" sites were 28 MPN/100 L and 11 MPN/100 L, respectively. Figure 5-5 presents the total culturable virus concentrations for the "high risk" sites compared to the "low risk" sites. These total culturable virus results partially support field-based hypothesis #3 for this study: "High risk" sites had higher occurrences and concentrations of enteric viruses than "low risk" sites. Although "high risk" sites did have more frequent detections than "low risk" sites, the difference in total culturable virus concentrations at "high risk" sites versus "low risk" sites was not statistically significant at $p < 0.05$.

It is difficult to compare the total culturable virus concentrations found in this study to those found in other studies because MPN values are rarely measured or reported for most ground water virus occurrence studies. However, a report focusing on the public health risk of *Giardia* and viruses in drinking water (Regli et al. 1991), determined that a maximum acceptable annual risk of infection from drinking water contaminated with viruses was 1 in 10,000. In order to achieve this level of risk, virus concentrations in finished drinking water must be $\leq 2.2 \times 10^{-5}$ MPN/100 L (based on infectivity studies). Concentrations of total culturable viruses measured in the ground water samples from this study were five to seven orders of magnitude higher than this level. Fortunately, all of the public water systems surveyed in this study disinfect their raw ground water sources, but no monitoring has been carried out to determine whether they are achieving the five to seven logs of reduction/inactivation during treatment necessary to reduce the annual

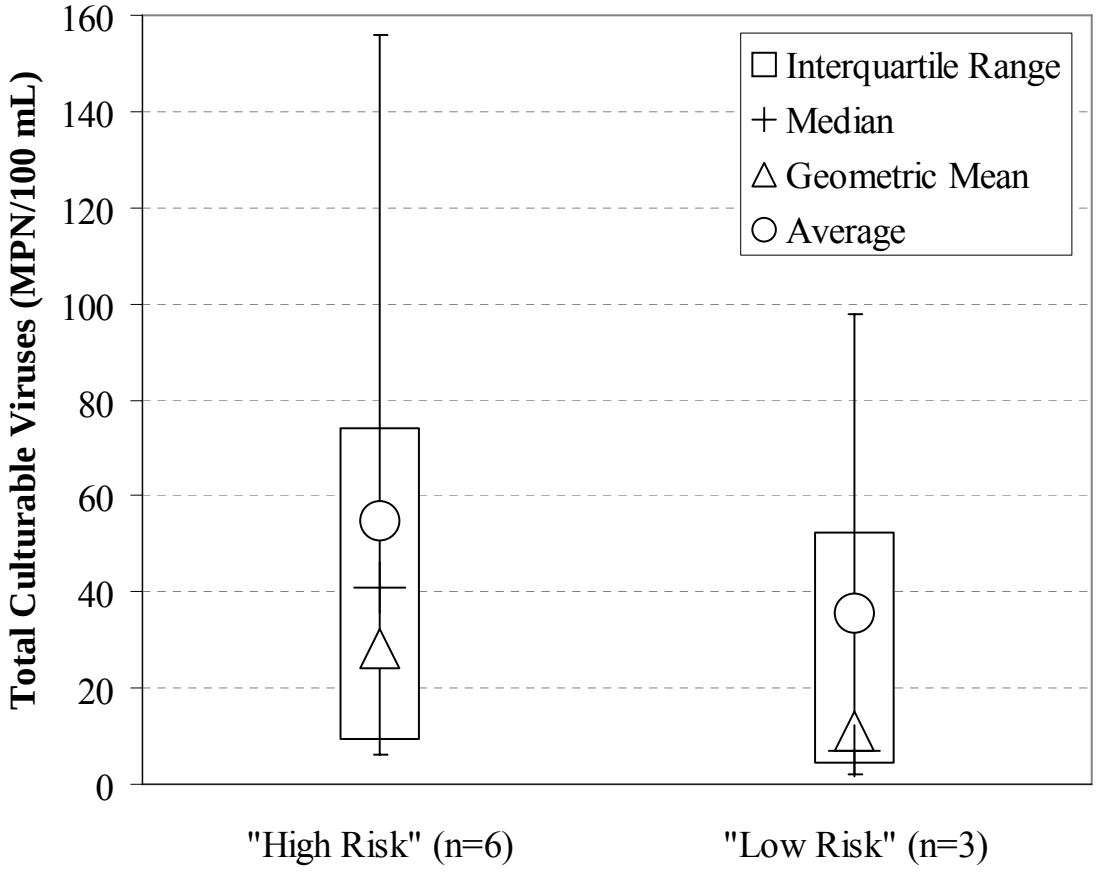

Figure 5-5. Total culturable virus concentrations at "high risk" versus "low risk" sites. Box plot whiskers represent the maximum and minimum concentrations. A Student's t-test determined that the difference in total culturable virus concentrations at "high risk" sites versus "low risk" sites was not statistically significant at $p < 0.05$.

risk of infection to less than 1 in 10,000. Most ground water systems, even those designated as GWUDI, typically assume a four to five-log reduction/inactivation of viruses to be adequate. Therefore, it is not clear whether the levels of total culturable viruses found in the raw ground water sources sampled in this study pose a public health risk for the communities served by the public water systems participating in this study.

Enteroviruses versus Reoviruses

The ability to distinguish between enteroviruses and reoviruses in the ground water samples collected during this study was advantageous because while both enteroviruses and reoviruses are human pathogens, their presence in the ground water samples may be derived from different fecal contamination sources. Reoviruses, like the protozoan, *Cryptosporidium parvum*, are zoonotic pathogens, meaning that although they cause disease in humans, they infect and are transmitted by both humans and animals. Therefore, the presence of reoviruses in ground water indicates possible human and/or animal fecal contamination. Reoviruses have been proposed as useful indicators of fecal contamination (from humans and/or animals), and of viral contamination in particular, in raw water sources (Spinner and Giovanni 2001). The combined enterovirus and reovirus results ("total culturable viruses" discussed in the previous section) have important public health risk implications because both viruses are human pathogens. In addition, most of the previous virus occurrence studies have analyzed ground water samples for total culturable viruses. However, by also analyzing the enterovirus and reovirus results separately, it may be possible to gain additional information about the sources of fecal contamination in the ground water samples collected for this study.

Figure 5-6 presents the enterovirus concentrations versus the reovirus concentrations and Figure 5-7 presents the enterovirus versus reovirus concentrations at the "high risk" versus "low risk" sites. Enteroviruses were detected at 1/4 (25%) of the "high risk" sites and at 2/4 (50%) of the "low risk" sites. When considered on a per sample basis, enteroviruses were detected in 1/8 (13%) of the samples from "high risk" sites and in 2/6 (33%) of the samples from "low risk" sites. Enterovirus concentrations for the samples from positive wells and springs ranged from 2 MPN/100 L to 7 MPN/100 L with an average concentration of 5 ± 3 MPN/100 mL and a geometric mean of 4 MPN/100 L. The average enterovirus concentrations at the "high risk" sites and "low risk" sites were 6 MPN/100 mL (only one sample point) and 5 ± 4 MPN/100 mL, respectively. The geometric means of the enterovirus concentrations at the "high risk" sites and "low risk" sites were 6 MPN/100 L and 4 MPN/100 L, respectively.

Reoviruses were detected at 3/4 (75%) of the "high risk" sites and at 1/4 (25%) of the "low risk" sites. When considered on a per sample basis, reoviruses were detected in 5/8 (63%) of the samples from "high risk" sites and in 1/6 (17%) of the samples from "low risk" sites. Reovirus concentrations for the samples from positive wells and springs ranged from 8 MPN/100 L to 156 MPN/100 L with an average concentration of 70 ± 55 MPN/100 mL and a geometric mean of 45 MPN/100 L. The average reovirus concentrations at the "high risk" sites and "low risk" sites were 64 ± 60 MPN/100 mL and 98 MPN/100 mL (only one sample point), respectively. The geometric means of the reovirus concentrations at the "high risk" sites and "low risk" sites were 39 MPN/100 L and 98 MPN/100 L (only one sample point), respectively.

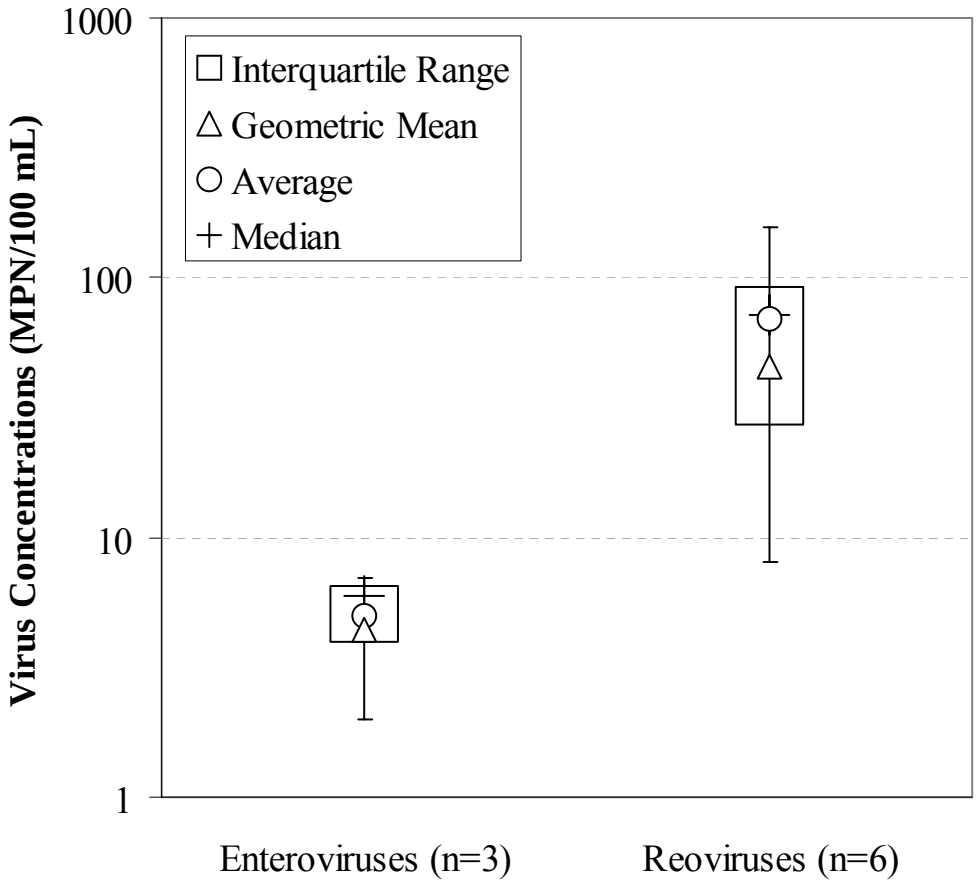

Figure 5-6. Enterovirus versus reovirus concentrations in wells and springs sampled in this study. Box plot whiskers represent the maximum and minimum concentrations. A Student's t-test determined that the difference in enterovirus concentrations versus reovirus concentrations was statistically significant at $p < 0.05$.

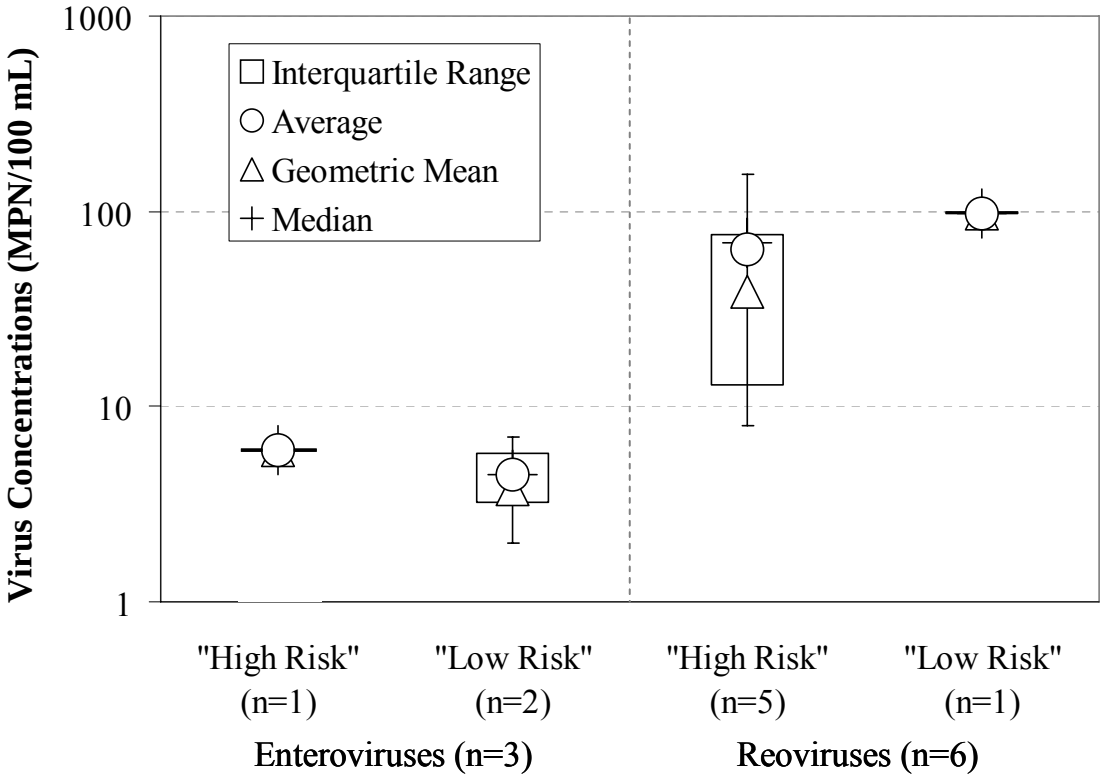

Figure 5-7. Enterovirus versus reovirus concentrations at "high risk" versus "low risk" sites. Box plot whiskers represent the maximum and minimum concentrations.

Reoviruses occurred at concentrations about 10 times greater than enteroviruses in the ground water samples collected in this study and were also detected more frequently than enteroviruses. These higher concentrations (determined to be statistically significant at $p < 0.05$) could be attributed to the fact that reoviruses occur in higher concentrations than enteroviruses in both sewage treatment influent and effluent (Sedmak et al. 2005) and are more resistant to chlorination than enteroviruses (Spinner and Giovanni 2001). These results are also consistent with findings in previous studies that reoviruses are common and occur more frequently and in higher concentrations than enteroviruses in raw water sources (Spinner and Giovanni 2001). Reoviruses may also have been at higher concentrations than enteroviruses in the ground water samples from this study because they were derived from different fecal contamination sources. One study, however, concluded that while reovirus isolates collected in a watershed indicated that animals (cows, pigs, and field mice) contributed to the reovirus contamination, human fecal contamination was the largest contributing source of reoviruses (Matsurra et al. 1988).

While the differences were small, enteroviruses occurred at higher concentrations at "high risk" sites than "low risk" sites and reoviruses occurred at higher concentrations at "low risk" sites than "high risk" sites. The meaning of these results was unclear and statistical tests to determine the significance of these differences were not performed because there was only one enterovirus-positive sample for the "high risk" sites and only one reovirus-positive sample for the "low risk" sites.

Virus Recoveries

In order to determine whether false negative results occurred due to cell culture inhibitors in the concentrated ground water samples or poor virus recovery during the filtration or elution procedures, cell culture (plaque assay with BGMK cells) was performed on the matrix replicate spike samples (QA/QC samples collected in association with each regular ground water sample where ground water was passed through a virus filter in the field, spiked with 10^6 PFU of poliovirus in the lab, and then processed and analyzed as a regular sample). The measured virus recoveries for the matrix replicate spike samples collected in this study were very low and ranged from 3% to 5%. Some additional virus spike and recovery experiments were conducted by virologists at the USEPA in an attempt to determine the cause of the low virus recoveries (data not shown). The results of the experiments showed that most (over 90%) of the spiked polioviruses actually passed through the filter instead of binding to it. Therefore, the virus recoveries probably represented a lowest estimate of virus binding efficiency to the filters and could not be used to determine the efficiency of virus removal from the filters during elution.

In one previous study (Dahling 2002), samples were seeded with $\leq$ 400 PFU of poliovirus and recoveries were as high as 95%. Therefore, it is possible that the low virus recoveries in this study were due to spiking the ground water samples with unrealistically large amounts of poliovirus (10,000 times the amount of poliovirus used in Dahling 2002). However, in another study (Denis-Mize 2004), Ohio River water samples were spiked with 3×10^6 PFU of poliovirus and recoveries, though highly variable, ranged from 16% to 65%. In this study, poliovirus was added to the filters after the equivalent

volume of water collected for the regular sample (sometimes up to 2,000 liters) had been passed through the filter in the field. Therefore, it is also possible that the low virus recoveries in this study were due to the late timing of the spike, thus significantly limiting the surface area available on the filter for binding of viruses. However, previous unpublished virus recovery experiments conducted at the USEPA using Ohio River water (personal communication, Shay Fout, USEPA) found no relationship between recovery efficiency and whether spiked viruses were added before, during, or after the ground water had been passed through the filter. The spike and recovery data in this study could not be used to determine if the different water matrices at each site affected the binding of the viruses to the cartridge filters. However, the results of Denis-Mize 2004 showed that water quality/water components had large impacts on recovery. In conclusion, the reason for the poor virus recoveries associated with the matrix replicate spike samples in this study remains unknown.

The matrix replicate spikes were used to confirm whether any of the concentrated ground water samples caused complete cell culture or PCR inhibition. All of the matrix spike samples tested positive for total culturable viruses by BGMK cell culture, indicating that it was unlikely that any of the concentrated ground water samples completely inhibited the cell culture.

Enteroviruses and Reoviruses by RT-PCR

Ground water samples collected from four wells and four springs between March and August of 2004 were analyzed by the USEPA for enteroviruses and reoviruses using two separate RT-PCR assays. The results of these analyses are presented in Table 5-8. The effective sample volume analyzed by each RT-PCR assay was 3% of the total sample

Table 5-8. Results for enteroviruses and reoviruses by RT-PCR for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Total Sample Volume Filtered in the Field (Liters)	Amount of Total Sample Volume Analyzed by each RT-PCR Assay (Liters)	Enteroviruses by RT-PCR (P/A)	Reoviruses by RT-PCR (P/A)
S-1	High	3/22/2004	2364	71	A	A
		8/8/2004	1922	58	A	A
S-2	High	3/29/2004	1949	58	A	A
		8/14/2004	1893	57	A	A
S-4	High	6/7/2004	396	12	A	A
		8/15/2004	1201	36	A	A
W-2	High	4/12/2004	947	28	A	A
		8/16/2004	946	28	A	A
W-1	Low	3/1/2004	1894	57	A	A
		8/10/2004	1758	53	A	A
W-3	Low	8/3/2004	1980	59	A	A
W-4	Low	8/4/2004	1621	49	A	A
S-3	Low	5/19/2004	1968	59	A	A
		8/9/2004	1132	34	A	A

Abbreviations: P/A, presence/absence; A, absence.

volume passed through the virus filter in the field. Therefore, the RT-PCR results are presented as presence or absence of viruses per 3% of the total sample volume. For example, if 2,000 liters of ground water were filtered in the field, 60 liters of the sample were analyzed in each RT-PCR assay.

Neither enteroviruses nor reoviruses were detected in any of the ground water samples by RT-PCR. Since the presence of enteric viruses in the ground water samples was confirmed by cell culture, reasons for these negative RT-PCR results may have included one or a combination of the following possibilities: (1) The primers and probe used in the RT-PCR and hybridization analyses were not specific for the viruses in the ground water samples, (2) RT-PCR inhibitors in the concentrated ground water samples led to false negative results, (3) The concentrations of the viruses in the ground water samples were below the detection levels for the RT-PCR assays (theoretically one virus per RT-PCR reaction).

With regards to the first possibility, the primers and probe used in the RT-PCR and hybridization assays for enterovirus were expected to be pan-specific for all known enterovirus strains and therefore it was assumed that there would not be any specificity problems with regard to enteroviruses in the ground water samples. However, additional sequencing of the enteroviruses in the ground water samples is needed to determine whether there were any mismatches between the enterovirus strains present and the primers and probe used in the RT-PCR assay (even one mismatch can greatly reduce binding efficiency of the primers and probe). Sequencing would also be useful to ensure that false positives due to lab contamination did not occur (i.e. if all of the enterovirus-

positive samples were positive for poliovirus, then lab contamination would be suspected).

Most of the ground water samples positive for culturable viruses contained reoviruses. Reoviruses are less-studied than enteroviruses and new strains are frequently added to GenBank® (Duan et al. 2003). The primers and probe used in the RT-PCR and hybridization assays for reoviruses may not have been pan-specific to all of the possible strains of reoviruses present in the ground water samples. Sequencing of the reoviruses present in the samples is needed to determine whether the ground water samples contained novel reovirus strains not able to be picked up by the primers and probe.

In order to determine whether false negative results were caused by RT-PCR inhibition in the concentrated ground water samples (possibility #2), RT-PCR was performed on the matrix spike samples (QA/QC samples where ground water was passed through a virus filter in the field, spiked with poliovirus in the lab, and then processed and analyzed as a regular sample). All of the matrix spike samples were positive for enteroviruses by RT-PCR and hybridization assay, indicating that PCR was not completely inhibited by any of the ground water samples. However, the amount of inhibition in the matrix spike samples was not quantified with RT-PCR. Also, the matrix spike samples were spiked with concentrations of viruses much higher than those expected in actual ground water samples so that the viruses in the matrix spikes could be detected even if high amounts of inhibition occurred. The concentrations of viruses in the ground water samples were low enough (as measured by MPN analysis of the cell cultures) that small amounts of RT-PCR inhibition may have lead to false negative results. In an attempt to dilute the effects of any possible inhibitors, 1:10 dilutions of the

concentrated ground water samples were made and analyzed by RT-PCR. These diluted samples also tested negative for enteroviruses and reoviruses. Additional QA/QC samples, where one PCR well containing the concentrated ground water sample was spiked with a small amount of virus immediately prior to RT-PCR, were not performed but would have been helpful in determining if RT-PCR inhibition was resulting in false negative results.

The theoretical detection limit for RT-PCR analysis is one virus per RT-PCR reaction. Assuming viruses in the concentrated ground water samples were well-dispersed, and there was 100% recovery in the sample concentration and inhibitor removal procedures prior to RT-PCR, concentrations of culturable viruses in the ground water samples (as determined by MPN analysis) should have been above the theoretical detection limit for RT-PCR.

A combination of inhibition, low virus concentrations, and nonspecific primers and probe may have led to the negative RT-PCR results for the analyzed ground water samples. One other possibility for why the ground water samples may have tested negative for enteroviruses and reoviruses by RT-PCR is that some of the chemicals used in the sample concentration/inhibitor removal procedure performed prior to RT-PCR (Appendix 4-3) had expired. It is possible that the expired chemicals lead to decreased virus recovery efficiencies during the sample concentration/inhibitor removal procedure and increased inhibition during RT-PCR.

Enteroviruses by Real-time RT-PCR

Ground water samples collected from four wells and four springs between March and August of 2004 were analyzed for the presence and concentration of enteroviruses

using a real-time RT-PCR assay developed at UTCEB. The results of these analyses are presented in Table 5-9. The effective sample volume analyzed by the real-time RT-PCR assay was 0.3% of the total sample volume passed through the virus filter in the field. This effective sample volume takes into account the 1:5 dilution of each concentrated ground water sample before real-time RT-PCR analysis. Therefore, the real-time RT-PCR results are presented as presence or absence per 0.3% of the total sample volume. For example, if the total sample volume was 2,000 liters of ground water, 6 liters of the sample were analyzed by the real-time RT-PCR assay.

Enteroviruses were not detected in any of the ground water samples by real-time RT-PCR. These results are consistent with the negative conventional RT-PCR results for enteroviruses. Since the presence of enteroviruses in at least three of the ground water samples was confirmed by cell culture, reasons for these negative real-time RT-PCR results may have included one or a combination of the following possibilities: (1) The primers and probe used in the real-time RT-PCR assay were not specific for the enteroviruses in the ground water samples, (2) RT-PCR inhibitors in the concentrated ground water samples led to false negative results, (3) The concentrations of the enteroviruses in the ground water samples were below the detection levels for the real-time RT-PCR assay (0.5 PFU per reaction based on the attenuated poliovirus standard and 10 viral RNA copies per reaction based on the DNA plasmid).

With regard to possibility #1, the primer and probe sequences used for the enterovirus real-time RT-PCR assay were analyzed in GenBank® (Benson et al. 2003) and confirmed to be pan-specific for most human enterovirus strains in the database at the time of assay design. However, additional sequencing of the enteroviruses in the ground

Table 5-9. Results for enteroviruses by real-time RT-PCR for wells and springs sampled in this study.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Total Sample Volume Filtered in the Field (Liters)	Amount of Total Sample Volume Analyzed by the Real-time RT-PCR Assay (Liters)	Enteroviruses by Real-time RT-PCR (P/A)
S-1	High	3/22/2004	2364	7	A
		8/8/2004	1922	6	A
S-2	High	3/29/2004	1949	6	A
		8/14/2004	1893	6	A
S-4	High	6/7/2004	396	1	A
		8/15/2004	1201	4	A
W-2	High	4/12/2004	947	3	A
		8/16/2004	946	3	A
W-1	Low	3/1/2004	1894	6	A
		8/10/2004	1758	5	A
W-3	Low	8/3/2004	1980	6	A
W-4	Low	8/4/2004	1621	5	A
S-3	Low	5/19/2004	1968	6	A
		8/9/2004	1132	3	A

Abbreviations: P/A, presence/absence; A, absence.

water samples is needed to determine whether there were any mismatches between the enterovirus strains present and the primers and probe used in the real-time RT-PCR assay since even one mismatch can greatly reduce binding efficiency of the primers and probe. Sequencing is also needed to ensure that false positives due to lab contamination did not occur (i.e. if all of the enterovirus-positive samples were positive for poliovirus, then lab contamination would be suspected).

As with the regular samples, all of the matrix replicate spike samples (QA/QC samples where ground water was passed through a virus filter in the field, spiked with poliovirus in the lab, and then processed and analyzed as a regular sample) were diluted 1:5 before real-time RT-PCR in an attempt to reduce PCR inhibition in the samples. Even with the dilution, two of the matrix replicate spike samples tested negative for enteroviruses by real-time RT-PCR, indicating that there were possible virus recovery problems during sample concentration or PCR inhibition problems with those samples (possibility #2). Since all of the matrix replicate spikes tested positive for enteroviruses by conventional RT-PCR, these results also suggests that the real-time RT-PCR assay for enteroviruses may be slightly less sensitive than the conventional RT-PCR assay for enteroviruses. However, this may simply be due to the fact that the real-time RT-PCR assay analyzed only a tenth of the sample volume analyzed in the conventional RT-PCR assay (0.3% versus 3%).

Additional QA/QC samples, where one real-time RT-PCR well containing the concentrated ground water sample was spiked with a known amount of the attenuated poliovirus and another well containing the concentrated ground water sample was spiked with a known amount of the P29 DNA plasmid, were analyzed to quantify the amount of

RT-PCR inhibition associated with each regular ground water sample. These results are presented in Table 5-10 as a percent recovery for each standard (i.e. 100% recovery = 0% inhibition and 0% recovery = 100% inhibition). The average percent recovery of the attenuated poliovirus for all of the ground water samples was 66 ± 18% and the average percent recovery of the DNA plasmid for all of the ground water samples was 106 ± 29%. These results suggest some loss of recovery in the virus preheating step or the RT step of the real-time RT-PCR assay because recovery for the DNA-based plasmid (which does not require preheating or reverse transcription) was higher (statistically significant at $p < 0.05$) than the recovery for the RNA-based attenuated poliovirus. The attenuated poliovirus, because it is an actual enterovirus, probably provides a more accurate measure of the inhibition present in each ground water sample.

With regards to possibility #3, the real-time RT-PCR assay for enteroviruses was determined to have a detection limit of 0.5 PFU per reaction based on the attenuated poliovirus standard and 10 viral RNA copies per reaction based on the DNA plasmid. Assuming enteroviruses in the concentrated ground water samples were well-dispersed, there was 100% recovery in the sample concentration procedures prior to real-time RT-PCR, and there was no PCR inhibition, the concentrations of enteroviruses in the ground water samples (as determined by MPN analysis) should have been very low but still above the detection limits for the real-time RT-PCR assay. However, as discussed above, there was some inhibition present in the ground water samples as measured by the real-time RT-PCR assay and therefore the combination of inhibition and low virus concentrations (and possibly nonspecific primers and probe) may have led to the negative real-time RT-PCR results for the analyzed ground water samples. The ground water

Table 5-10. Percent recoveries of spiked attenuated poliovirus and P29 DNA plasmid in ground water samples analyzed for enteroviruses by real-time RT-PCR.

Site ID	Risk for Fecal Contamination Designation	Sample Date	Total Sample Volume Filtered in the Field (Liters)	% Recovery of Attenuated Poliovirus	% Recovery of P29 DNA Plasmid
S-1	High	3/22/2004	2364	57	118
		8/8/2004	1922	98	97
S-2	High	3/29/2004	1949	92	118
		8/14/2004	1893	63	69
S-4	High	6/7/2004	396	46	78
		8/15/2004	1201	52	82
W-2	High	4/12/2004	947	31	98
		8/16/2004	946	64	86
W-1	Low	3/1/2004	1894	58	180
		8/10/2004	1758	76	97
W-3	Low	8/3/2004	1980	62	137
W-4	Low	8/4/2004	1621	81	128
S-3	Low	5/19/2004	1968	74	98
		8/9/2004	1132	76	90

samples may have also tested negative for enteroviruses by real-time RT-PCR because some of the chemicals used in the sample concentration/inhibitor removal procedure (Appendix 4-3) had expired. The expired chemicals may have contributed to decreased virus recovery efficiencies during the sample concentration/inhibitor removal procedure and increased inhibition during the real-time RT-PCR.

The results of the real-time RT-PCR analysis of the ground water samples support part of the methodological hypothesis for this study. A real-time RT-PCR method was developed to be quantitative (as proven in Chapter 3), but when applied to actual ground water samples, it was difficult to determine whether the method provided equivalent sensitivity to conventional RT-PCR since all of the ground water samples were negative for the targeted viruses by both conventional and real-time RT-PCR analyses.

Quality Assurance and Quality Control Samples

Several types of QA/QC samples other than the matrix replicate spikes were collected throughout this study to ensure the quality of the enteric virus results for ground water samples analyzed by cell culture, conventional RT-PCR, and real-time RT-PCR. Three field blanks were collected on three separate days during the study to ensure proper sterilization of the sampling equipment and to rule out cross contamination between samples from filter elution through analysis. All three of the field blanks were negative for enteric viruses by cell culture, RT-PCR, and real-time RT-PCR.

At each site on each sampling day, ground water was passed through two virus filters yielding two ground water samples (pictured in Figure 4-10) to determine reproducibility between samples with each of the enteric virus analyses. The results for both samples from each site were identical during the entire study. For example, both

samples (filters) from site W-2 on 8/16/04 were positive for reovirus by cell culture and negative for enteric viruses by RT-PCR and real-time RT-PCR. The results indicate low sample-to-sample variation using each of the enteric viral analyses.

Negative and positive process controls were used during the concentration and inhibitor removal process prior to the molecular analyses to ensure no cross contamination between ground water samples and adequate virus recoveries, respectively. The negative process controls were negative for enteric viruses by RT-PCR and real-time RT-PCR and the positive process controls were positive for enteric viruses by RT-PCR and real-time RT-PCR.

PCR negative controls performed to rule out false positive results in the RT-PCR and real-time RT-PCR analyses were negative, and PCR positive controls used to ensure successful reaction completion in the RT-PCR and real-time RT-PCR analyses were positive.

Lastly, in addition to the QA/QC samples collected throughout this study, it is important to note that the cell culture and RT-PCR analyses were conducted by highly experienced USEPA virologists in facilities with strict QA/QC standards. Ground water samples were collected and analyzed using the same USEPA-approved methods used in some other important ground water virus occurrence studies (Fout et al. 2003 and Francy et al. 2004).

Summary of Enteric Virus Results

The results of the cell culture analyses for enteric viruses in the fourteen ground water samples suggest enteric viral contamination (reoviruses and/or enteroviruses) in at least 7/8 (88%) of the raw ground water sources sampled during this study. The enteric

viruses were detected using cell culture methods and were therefore considered infectious. Enteric viruses were present in the raw ground water sources at concentrations high enough to warrant possible public health concerns. Supporting field-based hypothesis #3 for this study, sites designated as "high risk" for fecal contamination had more occurrences of enteric viruses than sites designated as "low risk" for fecal contamination. However, although the average total culturable virus concentration from "high risk" sites was slightly higher than the average total culturable virus concentration from "low risk" sites, the difference was not statistically significant at $p < 0.05$. Therefore, designation of "low risk" using the criteria described in Chapter 2 should certainly not be used to rule out the possibility of viral contamination of a raw ground water source (i.e. "low risk" ≠ "no" risk). The sampling sites were positive for different types of viruses (enteroviruses and reoviruses) and both virus types were not detected together in any ground water sample, which is a common finding in virus occurrence studies (Francy et al. 2004). Reoviruses were detected more often and at higher concentrations (statistically significant at $p < 0.05$) than enteroviruses.

The results of this study highlight some of the limitations of enteric virus detection methods. Enteric viruses in the cell culture-positive samples were not detected by the molecular methods because the RT-PCR and real-time RT-PCR methods analyzed a much smaller effective sample volume than the cell culture methods, were much more prone to inhibition than the cell culture methods, and may have been affected by mismatches between the virus strains and the primers and probes used in the assays. Therefore, if only molecular methods had been chosen to analyze the ground water

samples from this study, the results may have incorrectly indicated no enteric viral contamination in the samples (due to false negatives).

The results of this study emphasize the importance of repeat sampling of wells and springs for enteric viruses. Of the six sites sampled for enteric viruses twice during the study, four of them were positive for enteric viruses by cell culture on only one of the sampling dates. Only two sites were positive for enteric viruses by cell culture on both sampling dates. These results suggest that sampling ground water systems one or even two times may not be sufficient. If the sites had been sampled 3 times, more of the sites may have been found to be positive for enteric viruses. This need for rigorous temporal monitoring programs of enteric viruses in ground water systems is one reason why virus occurrence studies (and studies of other waterborne pathogens) are done so rarely.

No virus detection method can analyze for all of the more than 100 types of enteric viruses that may be present in ground water. In this study, ground water samples were analyzed for enteroviruses and reoviruses only. Even if a ground water sample was not positive for either of these viruses using the chosen virus detection methods, it may have been positive for viruses not analyzed for such as HAV or rotavirus. In addition, many studies (Metcalf et al. 1995) have found that the types of viruses in ground water systems change temporally due to variations in concentrations of viruses in the watershed according to outbreaks of enteric illness (i.e. viruses are only shed into the environment when people are infected). In a study of small public water supplies in Michigan (Francy et al. 2004), for example, wells tested positive for enterovirus and HAV, but never in the same sample or on the same sampling date.

It is important to note that the raw ground water sources of all of the public water systems participating in this study were disinfected prior to the water entering the distribution system, many of them receiving full surface water treatment. However, the Ground Water Rule (USEPA 2000) acknowledges that disinfection of viruses is not always successful (and treatment plants occasionally fail). Therefore, understanding contaminant occurrence in source waters is essential as part of a multi-barrier approach to protecting public health. The ground water systems surveyed in this study tested positive for infectious enteroviruses and reoviruses, human enteric viral pathogens which pose human health risks and indicate fecal contamination of the raw water source. These ground water systems therefore have the potential to be contaminated with other enteric pathogens capable of transport and survival through the subsurface from the contamination source to the well or spring. The presence of reoviruses also indicates the possibility of animal fecal contamination and thus the potential for the presence of enteric pathogens found in animal waste such as *Cryptosporidium parvum* and *E. coli* O:157 H7.

Although the enteric virus results for this study suggest fecal contamination at sites S-1, S-2, S-3, S-4, W-1, W-2, and W-3, fecal contamination or the possible presence of viral pathogens at site W-4, which did not have detectable levels of enteroviruses or reoviruses, cannot be ruled out. All that can be concluded for site W-4 is that it was not positive for enteroviruses or reoviruses by cell culture, RT-PCR, or real-time RT-PCR on the day it was sampled. It may have been positive for pathogens not analyzed for or it may have been positive for the targeted viral pathogens on other days of the year, depending on factors such as the hydrological conditions and the presence of infected individuals in the community.

Comparison of Occurrence of Enteric Viruses and Indicator Bacteria

The enteric virus and indicator bacteria results for this study are presented in Table 5-11 for direct comparison. Statistical analysis of the absolute values from the indicators and enteric viruses was performed to determine whether there was a relationship between the concentrations of indicators and enteric viruses in the sampled wells and springs. The first statistical test was a simple calculation of the Spearman's rank correlation coefficients between the concentrations of each of the indicator bacteria organisms and the enteric virus MPN values for each ground water sample. For this analysis, enteroviruses and reoviruses were tested together as total culturable viruses and reoviruses were also tested separately. Since enteroviruses only occurred in three samples, it was determined that it was not valid to perform this statistical test separately with the enterovirus MPN values. Furthermore, this test was only performed for ground water samples in which both of the measured variables (indicators and viruses) co-occurred. If the correlation was significant at $p < 0.05$, the highest correlation coefficients corresponded to data sets with the strongest correlation.

The results of the Spearman's rank correlation are presented in Table 5-12. A statistically significant positive correlation was found between total culturable virus MPN values and total coliform concentrations (correlation coefficient of 0.86 and a p-value of 0.01). However, reovirus MPN values were not correlated to the total coliform concentrations. Total culturable virus and reovirus MPN values were not correlated (at $p < 0.05$) to *E. coli* concentrations or *Bacteroides* concentrations. However, as described earlier in this chapter, some of the *E. coli* concentrations were considered suspect and may have correlated with the total culturable virus or reovirus MPN values if they had

Table 5-11. Combined detection results for enteric viruses and indicator bacteria in wells and springs sampled in this study.

Indicator Bacteria or Enteric Virus	"HIGH RISK" SITES		"LOW RISK" SITES	
	Percent of Wells or Springs Positive	Percent of Samples Positive	Percent of Wells or Springs Positive	Percent of Samples Positive
Total coliforms	100	100	50	50
E. coli	100	88	25	17
Total *Bacteroides*	100	100	50	50
Human *Bacteroides*	100	75	50	50
Bovine *Bacteroides*	0	0	0	0
Enteroviruses or Reoviruses by Cell Culture	100	75	75	50
Enteroviruses by RT-PCR	0	0	0	0
Enteroviruses by Real-time RT-PCR	0	0	0	0

Table 5-12. Results of Spearman's rank correlation analyses between concentrations of total culturable viruses and reoviruses and indicator bacteria in wells and springs sampled in this study.

Indicator Bacteria	Spearman Rank Correlation Coefficient with Total Culturable Virus MPN Values	Number of Pairs Tested	Significance at $p < 0.05$	Spearman Rank Correlation Coefficient with Reovirus MPN Values	Number of Pairs Tested	Significance at $p < 0.05$
E. coli	0.39	7	NS	0.7	5	NS
Total Coliforms	0.86	7	S ($p=0.013$)	0.7	5	NS
Total *Bacteroides*	0	8	NS	0.2	5	NS
Human *Bacteroides*	0.14	7	NS	0.4	4	NS

Abbreviations: NS, correlation is not statistically significant; S, correlation is statistically significant.

been valid. The results of this analysis did not provide any information on whether the reoviruses in the ground water samples were due to human or animal fecal contamination. In addition to the indicator bacteria, the coefficient of correlation analysis was also performed with the turbidity values measured at each site (data not shown). No statistically significant relationship was found between MPN values for total culturable viruses or reoviruses and the turbidity values.

A simple co-occurrence analysis for the presence of enteric viruses and indicator bacteria was done in previous studies on the occurrence of viruses in ground water (Lindsey et al. 2002 and Francy et al. 2004). This co-occurrence analysis is useful because it (1) allows for comparison with results from previous studies, (2) can be used with a small data set that may not lend itself to more rigorous statistical analyses, and (3) uses presence or absence of viruses and indicators rather than absolute values/concentrations (which may alleviate some of the problems of the suspect *E. coli* values mentioned above).

Table 5-13 presents the co-occurrence results for the indicator bacteria and total culturable viruses detected in this study on a per-site and per-sample basis. The co-occurrence results were presented on a per-site and per-sample basis mainly to compare the results of this study to results from previous virus occurrence studies. The co-occurrence results indicated that all of the indicator bacteria had high co-occurrence with total culturable viruses on a per-site and per-sample basis. Other studies found lower co-occurrence rates for viruses and any indicator (44% by Francy et al. 2004 and 80% by Lindsey et al. 2002). The indicator with the highest co-occurrence with total culturable viruses on a per-sample basis was *Bacteroides*. On a per-site basis, total coliforms and

Table 5-13. Co-occurrence of total culturable viruses and indicator bacteria in wells and springs sampled in this study.

Indicator Bacteria	BY SITE		BY SAMPLE	
	Overall Percent of Occurrence of Indicator	Co-occurrence* with Total Culturable Viruses	Overall Percent of Occurrence of Indicator	Co-occurrence* with Total Culturable Viruses
Total coliforms	75	86	79	78
E. coli	63	71	57	78
Bacteroides	75	86	79	89
At least one indicator	75	86	86	89

*Co-occurrence = (Number of times the indicator co-occurred in a ground water sample with total culturable viruses) ÷ (Number of samples positive for total culturable virus) X 100%

Bacteroides had equally high co-occurrence with total culturable viruses. The lower co-occurrence values for *E. coli* on a per-sample and per-spring basis may be due to the suspect *E. coli* values discussed earlier in this chapter. At least one indicator was present at 86% of the sites where total culturable viruses were detected and in 89% of thesamples in which total culturable viruses were detected. In general, for this study, the indicator bacteria were useful in predicting the occurrence of total culturable viruses.

Table 5-14 presents the co-occurrence results for the indicator bacteria and total culturable viruses detected in this study with respect to ground water samples collected from "high risk" versus "low risk" sites. Table 5-15 presents the co-occurrence results for the indicator bacteria and enteroviruses detected in this study with respect to ground water samples collected from "high risk" versus "low risk" sites. Table 5-16 presents the co-occurrence results for the indicator bacteria and reoviruses detected in this study with respect to ground water samples collected from "high risk" versus "low risk" sites. The important findings from these co-occurrence analyses were: (1) At "high risk" sites, there was 100% co-occurrence of all three indicators and total culturable viruses, enteroviruses, and reoviruses, (2) At "low risk" sites, *Bacteroides* had the highest co-occurrence with total culturable viruses and enteroviruses, and (3) At "low risk" sites, no indicators co-occurred with reoviruses (although this may a result of having only one reovirus detection for the "low risk" sites). In general, *Bacteroides*, a potential alternative measure of fecal contamination, was a better predictor of virus occurrence than both of the commonly-used indicators of fecal contamination in ground water systems (total coliforms and *E. coli*).

Table 5-14. Co-occurrence of total culturable viruses and indicator bacteria in ground water samples from "high risk" versus "low risk" sites.

Indicator Bacteria	"HIGH RISK" SITES		"LOW RISK" SITES	
	Overall Percent of Occurrence of Indicator	Co-occurrence* with Total Culturable Viruses	Overall Percent of Occurrence of Indicator	Co-occurrence* with Total Culturable Viruses
Total coliforms	100	100	50	33
E. coli	88	100	17	33
Bacteroides	100	100	50	67
At least one indicator	100	100	67	67

*Co-occurrence = (Number of times the indicator co-occurred in a ground water sample with total culturable viruses) ÷ (Number of samples positive for total culturable virus) X 100%

Table 5-15. Co-occurrence of enteroviruses and indicator bacteria in ground water samples from "high risk" versus "low risk" sites.

Indicator Bacteria	"HIGH RISK" SITES		"LOW RISK" SITES	
	Overall Percent of Occurrence of Indicator	Co-occurrence* with Enteroviruses	Overall Percent of Occurrence of Indicator	Co-occurrence* with Enteroviruses
Total coliforms	100	100	50	50
E. coli	88	100	17	50
Bacteroides	100	100	50	100
At least one indicator	100	100	67	100

*Co-occurrence = (Number of times the indicator co-occurred in a ground water sample with enteroviruses) ÷ (Number of samples positive for enterovirus) X 100%

Table 5-16. Co-occurrence of reoviruses and indicator bacteria in ground water samples from "high risk" versus "low risk" sites.

Indicator Bacteria	"HIGH RISK" SITES		"LOW RISK" SITES	
	Overall Percent of Occurrence of Indicator	Co-occurrence* with Reoviruses	Overall Percent of Occurrence of Indicator	Co-occurrence* with Reoviruses
Total coliforms	100	100	50	0
E. coli	88	100	17	0
Bacteroides	100	100	50	0
At least one indicator	100	100	67	0

*Co-occurrence = (Number of times the indicator co-occurred in a ground water sample with reoviruses) ÷ (Number of samples positive for reovirus) X 100%

Results of This Study Compared to Other Enteric Virus Occurrence Studies

The results of this study were compared to the two most similar virus occurrence studies described in Chapter 1 of this thesis. Table 5-17 compares the enteric virus results from this study (which focused mostly on karst aquifers) to the results for the wells in karst aquifers only from the large USEPA study (also known as the Lieberman Study) and the USGS Pennsylvania study (Lindsey et al. 2002). Although the studies had some differences in experimental design, they did use the same ground water filtration methods and the same viral analysis methods. In fact, the specific virus identification work for both of these studies was performed at the USEPA virology lab in Cincinnati, Ohio (where most of the viral analyses for this study were also performed).

The current study had a much higher frequency of sampling sites/samples positive for enteric viruses by cell culture than did the USGS and USEPA studies. However, the USGS study sampled more wells in karst aquifers than did the current study and the USEPA study had a much higher sampling frequency than did the current study and the USGS study. Although the viruses detected in the current study were not typed to identify the specific strains present in the ground water samples, the MPN values from the current study reasonably compare with the MPN values from both the USGS and the USEPA studies. Specific virus identification in the enterovirus-positive ground water samples from the current study is needed to confirm that the enteroviruses present in the ground water samples were wild-type enteroviruses and not poliovirus (the lab control virus). Sample contamination could explain the higher virus detection rates for this study compared to the other studies. However, due to the extensive QA/QC performed in the current study, sample contamination is unlikely. It is more likely that the higher virus

Table 5-17. Major findings from this study compared to major findings from karst sites tested in previous studies.

Reference for Study	Number of Karst Sites Tested in Study	Number of Times Each Site was Sampled	Percent of Sites Positive for Enteric Viruses by Cell Culture	Percent of Samples Positive for Enteric Viruses by Cell Culture	Virus Identification Results (Echoviruses and Coxsackieviruses are specific types of Enteroviruses)	MPN of infectious units/100 L
Lindsey et al. 2002 (USGS Study)	25	1	8	8	Echovirus 20, Echovirus 13, and Poliovirus 3	18
					Reovirus	56
Lieberman et al. 2002, Dahling 2002, and Fout et al. 2003 (known as the USEPA Study or the Lieberman Study)	7	12	43	15	Reovirus	7
					Coxsackievirus B4, Echovirus 11	12
					Coxsackievirus B4, Echovirus 11	27
					Coxsackievirus B1	1
					Coxsackievirus B4	4
					Coxsackievirus B4, B5, Echovirus 15	2
					Coxsackievirus B1, B3, B4, Echovirus 11, 15, 27	11
					Coxsackievirus B4, Echovirus 11, Reovirus	45
					Coxsackievirus B1, Echovirus 21,24	3
					Echovirus 11	44
					Coxsackievirus B4, Echovirus 15	48
					Echovirus 15	53
					Coxsackievirus B4	25
Current Study (Detection of Enteric Viruses in East Tennessee Public Ground Water Systems)	7*	1-2	86	62	[#]Enterovirus	6
					Reovirus	8
					Reovirus	13
					Reovirus	156
					Reovirus	16
					Reovirus	76
					Enterovirus	7
					Reovirus	98
					Enterovirus	2

*For this comparison, results from site W-3 were excluded because it is located in a fractured sandstone aquifer, not a karst/carbonate aquifer.
[#]Virus-positive ground water samples from this study were not typed for specific enteroviruses, therefore only general virus types are presented.

detection rates for the current study were a factor of sampling primarily ground water sources designated as GWUDI, large springs (and not just wells as in the other studies), having a low sampling frequency, and purposely selecting some sites with a very high risk of fecal contamination.

CHAPTER VI
SUMMARY AND CONCLUSIONS

A two-part study was conducted by UTK, the USGS, and the USEPA-NERL to (1) develop, validate, and test a real-time RT-PCR assay for enteroviruses in ground water samples and to (2) perform the first survey of enteric viral occurrence in the karst aquifers of East Tennessee. This study consisted of sampling four wells and four springs that were raw water sources for public water systems serving communities in East Tennessee. The wells and springs were sampled one to two times from March to August of 2004 for enteroviruses and reoviruses by cell culture methods, enteroviruses and reoviruses by RT-PCR, enteroviruses by the real-time RT-PCR assay developed at UTCEB, indicator bacteria (total coliforms, *E. coli*, and *Bacteroides*), water-quality field parameters, and basic chemistry.

The real-time RT-PCR assay for enteroviruses was developed and confirmed to be quantitative but provided slightly less sensitivity than conventional RT-PCR assays for enteroviruses. However, the utility of the real-time RT-PCR assay for detecting and measuring the concentration of enteroviruses in ground water samples compared to the performance of conventional RT-PCR assays was difficult to assess because all of the ground water samples collected during this study were negative for enteroviruses using both the real-time and conventional RT-PCR methods. However, the real-time RT-PCR and conventional RT-PCR assays were comparable in that they yielded the same results for ground water samples throughout the study (i.e. all of the ground water samples were negative with both methods). This outcome emphases the importance of using multiple detection methods (both culture-based and molecular-based) in order to maximize the

likelihood of detecting enteric viruses present in ground water samples. More research is needed to fully assess the performance and capabilities of the real-time RT-PCR assay for enteroviruses in ground water samples. Future research could include investigation of an integrated cell culture-real-time-RT-PCR (ICC-real-time RT-PCR) method which could provide a quantitative detection assay for infectious enteric viruses that is less time-consuming than cell-culture and more sensitive than real-time RT-PCR used alone. Also, additional real-time RT-PCR assays could be developed for enteric viruses other than enteroviruses, such as reoviruses, HAV and rotaviruses. Another important future research area is the development of new ground water sample processing techniques to increase the effective sample volume analyzed by real-time and conventional RT-PCR methods and thereby reduce the negative impacts of inhibitors.

The main findings of the field survey were: (1) 88% of the sites were positive for enteric viruses by cell culture on at least one of the sampling days and 75% of the ground water samples were positive for enteric viruses by cell culture, (2) Although sites previously designated as "high risk" for fecal contamination had a higher prevalence of enteric viruses and indicator bacteria than sites previously designated as "low risk" for fecal contamination, this was only statistically significant for total coliforms (3) 75% of the sites were positive for at least one of the indicator bacteria, and (4) None of the sites were positive for enteric viruses using the RT-PCR or real-time RT-PCR methods. Although there was a higher prevalence of enteric viruses at the sites designated as "high risk", enteric viruses were found at three of the four "low risk" sites. Also, one of the "low risk" sites was negative for two of the three indicator bacteria and another "low risk" site was negative for all of the indicator bacteria on the same days they tested

positive for enteric viruses. These results indicate that absence of indicators cannot ensure the absence of pathogens and that the methods used to designate "low risk" sites (although they included a host of factors) may not be adequate for ensuring absence of pathogens (i.e. "low risk" does not equal "no" risk).

In this study, co-occurrence rates for any indicator bacteria and enteric viruses both on a per-site and per-sample basis were high (86% and 89% respectively) as compared with previous studies (Francy et al. 2004 and Lindsey et al. 2002). The previous studies found that of the individual indicator organisms, total coliforms were the best predictors of, and had the highest co-occurrence with, detections of enteric viruses. This study found that *Bacteroides* was the best predictor of, and had the highest co-occurrence with, detections of enteric viruses. However, the current study confirmed, as did the previous studies, that the highest co-occurrence with detections of enteric viruses was found when all of the indicator bacteria were analyzed together. Therefore, the best predictor of viral occurrence in karst ground water systems may be a suite of indicators, rather than just one.

There are many possibilities for future research in the karst aquifers of East Tennessee which could help further the understanding of enteric viral contamination (as well as human fecal contamination in general) in these vulnerable ground water systems. One of the top priorities could be the establishment of a virus monitoring program to better characterize temporal and spatial changes in virus occurrence and concentrations in the wells and springs of East Tennessee. With more data through regular viral monitoring, the relationships between indicator organisms, physical and chemical parameters of the aquifers, land use and inventories of contamination sources, and details

of geologic settings versus occurrence and concentration of enteric viruses could be evaluated in a rigorous statistical manner. Other possible research areas include microbial source tracking to determine the sources of fecal contamination in the aquifers, dye tracing studies to determine both if point sources of fecal contamination to the source waters can be identified and to better characterize the transport of fecal contamination to wells and springs, and storm sampling to investigate the relationship between enteric virus occurrence and variations in hydrologic conditions. Research directions to better address the public health implications of the viral contamination in the ground water systems of East Tennessee could include sampling for multiple viruses such as HAV, Norwalk, and rotaviruses to better characterize the types and sources of pathogens in the ground water (and also to correlate the detected virus types with any infectious illness data available at local health departments) and testing of the finished waters of public water systems (whose raw source waters have tested positive for viruses in this study) as well as distribution systems to determine if viruses in the source waters are surviving the treatment process.

The occurrence rate of enteric viruses in the wells and springs sampled in this study is much higher than rates of enteric viral occurrence found in previous studies. This study is the first study of virus occurrence in ground water to focus primarily on karst aquifers, most of which were considered GWUDI by the state of Tennessee. The findings of this study confirm that raw ground water sources derived from the karst aquifers of East Tennessee (and perhaps karst aquifers, in general) are vulnerable to contamination with enteric viral pathogens. These results emphasize the importance of source water protection and adequate treatment of ground water used for drinking water

supplies in East Tennessee. Local governments, drinking water utilities, and the public of East Tennessee can all be active participants in a multi-barrier approach to reduce the public health risk associated with consumption of pathogens in their ground water supplies.

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APPENDICES

Appendix 2-1. *E. coli* results from preliminary sampling of East Tennessee wells and springs.

Name of Well or Spring	Number of Times Sampled	Maximum *E. coli* Concentration (CFU/100 mL)	Minimum *E. coli* Concentration (CFU/100 mL)	Geometric mean of *E. coli* Concentrations (CFU/100 mL)
Site S-1	6	1553	6	65
Site S-2	6	>2419	144	
Site S-3	4	9	<1	2
Site W-1	1	<1	<1	
Site W-2	4	>2419	190	>190
Site W-4	1	<1	<1	
Athens U.D., Well #7	1	32	32	
Athens U.D., Ingleside Spring	1	29	29	
Athens U.D., Well #4	1	15	15	
Ocoee U.D., Carpenter Well	4	91	13	34
Ocoee U.D., Wildwood Spring	1	5	5	
Watts Bar U.D., Well #1	2	6	<1	
Hixon U.D., Cave Springs	1	2	2	
Hixon U.D., Walker's Corner Well #2	1	<1	<1	
Maynardville U.D., Davis Spring	5	378	2	51
Maynardville U.D., Lay Spring	2	62	2	
Maynardville U.D., Davis Well	2	<1	<1	
Maynardville U.D., Lay Well	2	<1	<1	
Hallsdale-Powell U.D., Fowler Spring	4	579	1	37
Hallsdale-Powell U.D., Granny Bright Spring	2	111	72	
Dandridge U.D., Water Plant Well	5	517	<1	11
Dandridge U.D., Water Plant Spring	2	4	1	
Mountain City U.D., Rambo Spring	1	261	261	
Mountain City U.D., Lowe Spring	2	5	2	
Erwin U. D., O'brien Spring	1	16	16	
Erwin U. D., Birchfield Well	1	8	8	
Erwin U. D., Railroad Well	1	<1	<1	
Siam U.D. Well	1	<1	<1	
Hampton U.D. Spring	1	1	1	
Capshaw Resurgence, Cookeville	3	>2419	157	
Love Spring (TWRA), Erwin	1	78	78	
LaMerde Spring, Monterey	1	46	46	
Birchfield Spring, Erwin	1	44	44	
Fountain City Park Spring, Fountain City	2	41	15	
Mead's Quarry Spring, South Knoxville	1	31	31	
Carpenter Spring, Ocoee	4	91	1	8
Gill Spring, Powell	2	30	2	
Hunter Spring, Halls	1	14	14	
Ijams Spring, South Knoxville	1	5	5	

Appendix 2-2.
Letter sent to each public water system participating in this study.

THE UNIVERSITY OF TENNESSEE
Department of Geological Sciences
306 Geological Sciences Building
Knoxville, Tennessee 37996-1410

Hydrogeology Research Program
Phone: (865) 974-0821
Fax: (865) 974-2368
http://web.utk.edu/~hydro/

To whom it may concern,

The University of Tennessee, in cooperation with the United States Geological Survey (USGS), is conducting sampling for enteric viruses at a limited number of public water systems. This effort is sponsored by the Tennessee Department of Environment and Conservation and is a first attempt to gather data on viral occurrence in East Tennessee ground water. We appreciate the help your staff extended to us over the past months as we gathered preliminary data to select candidate sites for viral sampling. The results of our preliminary sampling are enclosed with this letter.

We are now beginning to collect samples for viral analysis and hope we can continue to work with the (insert name of public water system) staff. We will need to attach our collection system to a tap from the wellhead and filter a few gallons per minute of ground water for several hours to obtain a composite sample. It is anticipated that we will commence sampling in your area soon, and should visit (insert name of public water system) in (insert month) 2004. We will wish to sample at least twice subsequently, once in the spring and once in the summer or fall.

Specific well or spring locations, the identity of participating water systems, and other detailed data that might compromise the security of a water supply will not be disclosed in reports or publications. University of Tennessee and USGS staff that may be present during sampling include:

Trisha Baldwin, UTK Department of Geological Sciences Master's Candidate
Sid Jones, UTK Department of Geological Sciences Research Associate
Greg Johnson, USGS Hydrologist
Dan Williams, UTK Center for Environmental Biotechnology Technician

We will be in touch soon by phone to set up an acceptable date for sampling. Once again, we thank your staff for their willingness to take time to work with us.

Sincerely,

Dr. Sid Jones, Research Associate Dr. Larry McKay, Program Director

Appendix 2-3.
Driller's report for site W-2.

TENNESSEE WATER WELL DRILLERS REPORT

DEPARTMENT OF ENVIRONMENT & CONSERVATION

THIS REPORT TO BE SUBMITTED BY DRILLER WITHIN 30 DAYS AFTER COMPLETION OF DRILLING WATER WELL WITH REQUIRED FEE TO: DIRECTOR, DIVISION OF WATER SUPPLY, 401 CHURCH ST. L & C TOWER 6TH FLOOR, NASHVILLE, TENNESSEE 37243-1549

PLEASE PRINT

OFFICE USE ONLY
Well No.: 1789
Date Rec'd: 5/31/01
Check#: 9873
Amount Rec'd: 250.00 175.00
Receipt# 14976 Cd# 710-116457

(1) **WELL CONTRACTOR**
Firm Name [redacted] Lic. No. 571
Rig. Operator
Driller Tag# WD0048968

(2) **WELL LOCATION**
County [redacted]
Driller Map No. 2 (NUMBER) E (LETTER) (W) (X) (Y) (Z)
or Latitude: DEG MIN SEC Longitude DEG MIN SEC
Well Address: [redacted] (ROAD OR STREET)
City: [redacted] Zip
5 1/2 Miles (N) (E) (S) (W) of Liberty (LANDMARK)

(3) **TYPE OF WORK**
Date drill rig left site: 5/10/01
New Well ☑ Deepen ☐ Rework ☐
Backfill And Abandon ☐

(4) **WELL COMPLETION DATA**
Date Completed 5/10/01 Static Level 7 Ft.
Total Depth 111 Ft. Estimated Yield 1000# GPM
Depth to bedrock 31 Ft.

(5) **WATER-BEARING ZONES**

DEPTH IN FT.	GPM	WATER QUALITY
56-60	500#	Good
60-83	500#	Good

(6) **WELL TEST**
Tested By: Pumping ☐ Blowing ☑ Bailing ☐
Static Level Ft. Pumping Level Ft. After Hr.
Min. At GPM
Development Time Prior to Test Hr. Min.

(7) **FORMATION LOG**

DEPTH IN FT. FROM	TO	DESCRIPTION (DENOTE ROCK COLOR & TYPE & CAVES)
0	31	Overburden
31	35	LS
35	38	Soft zone Ft LS
38	42	LS
42	46	Soft zone Ft LS
46	47	LS
47	50	Ft LS weathered
50	56	Ft LS
56	60	Gravel
60	83	Ft LS + gravel seams
83	111	LS (Solid)

(If additional space is needed, use back of form or use comments section)

(8) **COMMENTS**
Used 20' 14" Casing from 0-20', used 10" Casing to 72', Casing Perf 65-72
Used 8" Slotted Casing w/ drive shoe on top and bottom from 72-83
8" hole from 83-111

(9) **WELL OWNER**
Name
FIRST LAST
or Company [redacted]
Address
City [redacted] State TN Zip
Phone # ( )

(10) **PROPOSED USE OF WELL**
Residential ☐ Commercial ☐ Industrial ☐ Monitor ☐ Test ☐
Farm ☐ Irrigation ☐ Heat Pump ☐ Municipal ☑ Other ☐
(Specify)

(11) **PRIMARY CASING**
Diameter 10 Inches Top Set 36 Above In Ground
From Land Surface to 72 Ft. below ground
Type: Plastic ☐ Steel ☑ Galvanized ☐
Concrete ☐ Other ☐ None ☐
Wall Thickness .250 or SDR#

(12) **WELL FINISH**
Open Hole ☐ Screen ☐ Slotted or Perf. Pipe ☑ 8" dia
From 71 Ft. To 83 Ft.
If Screen, Plastic ☐ Metal ☐ Slot Size In.
Gravel Pack From: Ft. to Ft.

(13) **BACK FILL MATERIAL**
Bentonite ☑ Cement ☐ From 8 Ft. to 10 Ft.

From	To	From	To
Cuttings 30	60	Sand	
Bentonite 0	30	Cement	
Other (Specify) Hole 71	Gravel		

(14) **LINER CASING** Yes ☑ No ☐
Type: Plastic ☐ Steel ☑ Diameter 8 In.
From: 71 Ft. to 83 Ft.
Packers Installed? Yes ☐ No ☑ Drive shoes
Location: Ft. and Ft.

(15) **ANTICIPATED WATER QUALITY**
Clear ☑ Cloudy ☐ Dingy ☐ Muddy ☐
Good ☑ Fair ☐ Bad ☐ Iron ☐ Sulfur ☐
Gas ☐ Oil ☐ Salt ☐
Other ☐ (specify)

(16) **GENERAL INFORMATION**
Well Disinfected: Yes ☑ No ☐
Well Capped: Yes ☑ No ☐
Well located greater than fifty feet from septic tank & field line: Yes ☑ No ☐
From information provided by:
☐ Property owner (provide written statement by owner)
☐ Driller determination
☐ Health Department permit
Drilling process water obtained from:
Well ☐ Springbox ☐ Public Supply ☑ Surface Supply ☐
Pump Installed by Driller: Yes ☑ No ☐

I certify that the above information is true and accurate to the best of my knowledge. Signed [redacted] Licensed Driller

Distribution: White - Central Office Canary - Driller Pink - Homeowner

Appendix 2-4.
Driller's report and log for site W-1.

TENNESSEE WATER WELL DRILLERS REPORT
Department of Health and Environment

This Report To Be Submitted Within 30 Days After Well Completion
To: Director, Tennessee Division of Water Management
T.E.R.R.A. Bldg., 150 Ninth Ave., North, Nashville, TN 37203

OFFICE USE ONLY 3753
Well No.
Date Rec'd
Lat: Long:
Update No. 255 Source

(1) WELL CONTRACTOR
Firm Name [redacted] Lic. No. [redacted]
Rig Operator [redacted]
Well No. or Name Test Well no 4

(2) WELL LOCATION
County [redacted] Town [redacted]
Driller Map No. 2 DY Lot or Parcel No.
Well Is On [redacted] Rd. Or St.
Approx. 2 1/2 Miles N S E W From [redacted]

(3) DRILLING METHOD 1
Air Rotary ☑ Mud Rotary ☐ Cable ☐
Auger ☐ Other ☐ (Specify)

(4) TYPE OF WORK 1
New Well ☑ Deepen ☐ Rework ☐ Set Pump ☐
Plug And Abandon ☐

(5) WELL COMPLETION DATA
Date Completed 8/13/93
Total Depth 305 Ft.
Total Yield 1200 GPM
Static Level 23 Ft.
Depth To Bedrock 141 Ft.

(6) WATER-BEARING ZONES

DEPTH IN FT.	GPM	WATER QUALITY
216-230	400+	Good
245-250	400+	Good
253-261	400+	Good

(7) FORMATION LOG 1

DEPTH IN FT. FROM	TO	DESCRIPTION
		Attached

(If additional space is needed, use back of form)

(17) COMMENTS
Installed 10" casing To 120' with drive shoe. Installed 8" To 223' with drive shoe

(8) WELL OWNER
Name [redacted] Phone No.
Address [redacted]

(9) PROPOSED USE OF WELL 5
Residential ☐ Commercial ☐ Industrial ☐ Monitor ☐ Test ☑
Farm ☐ Irrigation ☐ Heat Pump ☐ Municipal ☐
Other ☐
(Specify)

(10) CASING 2
Type: Plastic ☐ Steel ☑ Galvanized ☐
Concrete ☐ Other ☐ (Specify)
Diameter 8 Inches Wall Thickness .250 Inches
Bottom Set At 223 Ft. Top Set 2 Feet Above Ground

(11) WELL FINISH 1
Open Hole ☑ Screen ☐ Slotted or Perf. Pipe ☐
From 223 Ft. To 305 Ft. Diameter 8 Inches
Type Screen: Plastic ☐ Metal ☐ Gravel Pack From: To Ft.

(12) BACK FILL MATERIAL

	From	To		From	To
Sand		Ft.	Puddled Clay		Ft.
Cuttings	0	223 Ft.	Cement Grout		Ft.
Other ☐ (Specify) Bentonite Clay					Ft.

(13) WELL TEST
Tested By: Pumping ☐ Blowing ☑ Bailing ☐
Static Level Ft. Pumping Level Ft. After Hrs. Min.
Pumping GPM Date
Development Time Prior To Test Hrs. Min.

(14) PUMP
Pump setter Lic. No.
Pump Intake At Ft. Date Set
Type: Sub ☐ Turbine ☐ Jet ☐ Cylinder ☐ Other ☐
H.P. Rated Yield GPM Wiring Size
Well and Pump Disinfected: Yes ☐ No ☐
Well Cap: Sanitary Seal ☐ Pitless Adapter ☐ Other ☐

(15) WATER QUALITY 3 1 1 8
Appearance: Clear ☐ Cloudy ☐ Dingy ☑ Muddy ☐
Odor: None ☑ Musty ☐ Sulfur ☐ Other ☐
Taste: Good ☑ Fair ☐ Bad ☐ Iron ☐
Analysis: Chemical ☐ Bacterial ☐ Radiological ☐
Analysis By

I certify that the above information is true and accurate to the best of my knowledge. Signed [redacted]

[redacted] WELL DRILLING

[redacted]

Formation Log Test well no. 4

From	to	Description
0	12	Dirt & Clay
12	15	Shaley Clay & Shale
15	70	Gravel, Sand, Cobble Stones
70	130	Gravel (Water Zone 100 Plus gpm)
130	141	Shale & Gravel Seams
141	145	Rock
145	150	Soft Zone - Shale & Gravel
150	152	Rock
152	156	Shale
156	159	Rock
159	160	Soft Zone
160	162	Shale
162	163	Rock
163	214	Softer Zone, Shale & gravel
214	217	Rock
217	224	Soft Shale
224	226	Rock (Hard)
226	229	Shale
229	230	Water 500 plus gpm
230	232	Shale
232	233	Hard Rock
233	235	Hard Shale
235	245	Hard L.S.
245	247	Softer
247	249	Hard L.S.
249	251	Soft Zone (Shale)
251	252	Harder
252	258	Softer Shale (Water)
258	260	Harder
260	261	Softer Shale (Water)
261	262	Harder
262	271	Softer Zone (Water
271	293	Real Soft (Water)
293	298	Hard L.S.
298	302	Softer
302	305	Harder Rock

Appendix 2-5.
Driller's report and log for site W-4.

TENNESSEE WATER WELL DRILLERS REPORT
Department of Health and Environment

This Report To Be Submitted Within 30 Days After Well Completion
To: Director, Tennessee Division of Water Management
T.E.R.R.A. Bldg., 150 Ninth Ave., North, Nashville, TN 37203

OFFICE USE ONLY 3402
Well No.
Date Rec'd
Lat: Long:
Update No. 254 Source

(1) WELL CONTRACTOR
Firm Name [redacted] Lic. No. [redacted]
Rig Operator [redacted]
Well No. or Name [redacted] Production Well

(2) WELL LOCATION
County [redacted] Town [redacted]
Driller Map No. 40X Lot or Parcel No.
Well Is On [redacted] Rd. Or St.
Approx. 3 Miles N (S) E W From [redacted]

(3) DRILLING METHOD
Air Rotary ☑ Mud Rotary ☐ Cable ☐
Auger ☐ Other ☐ (Specify)

(4) TYPE OF WORK
New Well ☑ Deepen ☐ Rework ☐ Set Pump ☐
Plug And Abandon ☐

(5) WELL COMPLETION DATA
Date Completed 7/29/93
Total Depth 415 Ft.
Total Yield 2000+ GPM
Static Level 88 Ft.
Depth To Bedrock 155 Ft.

(6) WATER-BEARING ZONES

DEPTH IN FT.	GPM	WATER QUALITY
225	1000+	Good
357	1000+	Good

(7) FORMATION LOG

DEPTH IN FT. FROM	TO	DESCRIPTION
		Attached

(If additional space is needed, use back of form)

(17) COMMENTS
Open hole from 257-415 8" diameter, Will Test Pump later

(8) WELL OWNER
Name [redacted] Phone No.
Address [redacted]

(9) PROPOSED USE OF WELL
Residential ☐ Commercial ☐ Industrial ☐ Monitor ☐ Test ☐
Farm ☐ Irrigation ☐ Heat Pump ☐ Municipal ☑
Other ☐
(Specify)

(10) CASING
Type: Plastic ☐ Steel ☑ Galvanized ☐
Concrete ☐ Other ☐ (Specify)
Diameter 16 Inches Wall Thickness .375 Inches
Bottom Set At 225 Ft. Top Set 2 Feet Above Ground

(11) WELL FINISH
Open Hole ☐ Screen ☑ Slotted or Perf. Pipe ☐
From 221 Ft. To 257 Ft. Diameter 14 Inches
Type Screen: Plastic ☐ Metal ☑ Stainless Gravel Pack From: To Ft.

(12) BACK FILL MATERIAL

	From	To		From	To
Sand		Ft.	Puddled Clay		Ft.
Cuttings		Ft.	Cement Grout	0	160 Ft.
Other ☐ (Specify)					Ft.

(13) WELL TEST
Tested By: Pumping ☐ Blowing ☑ Bailing ☐
Static Level Ft. Pumping Level Ft. After Hrs. Min.
Pumping GPM Date
Development Time Prior To Test Hrs. Min.

(14) PUMP
Pump setter Lic. No.
Pump Intake At Ft. Date Set
Type: Sub ☐ Turbine ☐ Jet ☐ Cylinder ☐ Other ☐
H.P. Rated Yield GPM Wiring Size
Well and Pump Disinfected: Yes ☐ No ☐
Well Cap: Sanitary Seal ☐ Pitless Adapter ☐ Other ☐

(15) WATER QUALITY
Appearance: Clear ☐ Cloudy ☑ Dingy ☐ Muddy ☐
Odor: None ☑ Musty ☐ Sulfur ☐ Other ☐
Taste: Good ☑ Fair ☐ Bad ☐ Iron ☐
Analysis: Chemical ☐ Bacterial ☐ Radiological ☐
Analysis By

I certify that the above information is true and accurate to the best of my knowledge. Signed [redacted]

[redacted] WELL DRILLING

[redacted]

Formation Log

Depth in Feet		Description
From	To	
0	112	Clay & Chert
112	130	Gravel (Brown)
130	140	Clay & Gravel
140	155	Gravel
155	157	Limestone
157	161	Gravel
161	183	Limestone
183	184	Faulty Zone
184	185	Limestone
185	186	Faulty Zone
186	187	Opening
187	190	Broken Rock & Gravel
190	191	Limestone
191	194	Gravel
194	195	Limestone
195	257	River Gravel w/ Clay Seams
257	290	Dark Limestone
290	387	Limestone
387	388	Fracture Zone
388	415	Limestone (Solid)

Water Bearing Zones

115	140	30 gpm
145	160	500 + gpm
183		200 + gpm
185		200 + gpm
190	191	200 + gpm
210		200 + gpm
225	235	1000 + gpm
248	257	1000 + gpm

Appendix 3-1. Design protocol used by UTCEB for real-time PCR primers and probe.

Probe:

- Needs to be designed first, before primers.
- GC content should be 30-80%, or mirror the CG content of the template.
- Avoid runs of identical nucleotides over 3 bases (especially guanine).
- Do not put Gs on the 5' end.
- Select strand that gives the probe more Cs than Gs.
- T_m should be between 68-70°C
  http://www.basic.nwu.edu/biotools/oligocalc.html, use salt adjusted method
- Should be as close as possible to the primer (5-10 bases).
- Blast your probe in GenBank® (http://www.ncbi.nlm.nih.gov/BLAST/), use Blastn or search for short nearly exact matches.
- Add fluorescent dye in 5' end and quencher in 3' end (BHQ the best).

Primers:

- Should be 18-24 nucleotides in length.
- T_m should be between 58-60°C
  http://www.basic.nwu.edu/biotools/oligocalc.html, use salt adjusted method
- GC content should be 40-60%, or mirror the CG content of the template.
- Avoid complementary sequences at the 3' end to reduce primer-dimers.
- Avoid GC-rich 3' ends (only 2 in the last 5 nucleotides).
- Avoid mismatches, especially at the 3' end.
- Avoid sequences with the potential to form internal secondary structure.
- Avoid polyXs.
- Avoid a 3'-end T (greater tolerance of mismatch).
- Gs or Cs in the 5' end and central regions confer hybridization stability.
- Both primers should anneal at the same temperature.
- Always blast primers in GenBank® (http://www.ncbi.nlm.nih.gov/BLAST/), use Blastn or search for short nearly exact matches.

Appendix 3-2. Enterovirus taxonomy report generated by GenBank®.

EvUTRprobe112rv3

```
Enterovirus ......................   100 hits   11 orgs [root; Viruses; ssRNA positive-strand
viruses, no DNA stage; Picornaviridae]
. Human enterovirus B ............    64 hits   10 orgs
. . Human echovirus 6 ............     5 hits    1 orgs
. . Swine vesicular disease virus .     1 hits    1 orgs
. . Human echovirus 18 ...........    35 hits    1 orgs
. . Human echovirus 30 ...........     7 hits    1 orgs
. . Human echovirus 9 ............     6 hits    1 orgs
. . Human coxsackievirus B5 ......     3 hits    1 orgs
. . Human echovirus 11 ...........     3 hits    1 orgs
. . Human coxsackievirus A9 ......     1 hits    1 orgs
. . Human echovirus 13 ...........     2 hits    1 orgs
. . Human echovirus 25 ...........     1 hits    1 orgs
. Human poliovirus 1 .............    36 hits    1 orgs [Poliovirus]
```

EvUTR24fv3

```
Enterovirus ................   100 hits   22 orgs [root; Viruses; ssRNA positive-strand
viruses, no DNA stage; Picornaviridae]
. Human enterovirus B ........    52 hits    9 orgs
. . Human echovirus 2 ........     1 hits    1 orgs
. . Human echovirus 7 ........     1 hits    1 orgs
. . Human echovirus 11 .......     4 hits    1 orgs
. . Human echovirus 13 .......     6 hits    1 orgs
. . Human coxsackievirus B5 ..     3 hits    1 orgs
. . Human echovirus 6 ........     2 hits    1 orgs
. . Human echovirus 30 .......     3 hits    1 orgs
. . Human coxsackievirus B4 ..     1 hits    1 orgs
. Human enterovirus 71 .......     1 hits    1 orgs [Human enterovirus A]
. Poliovirus .................    37 hits    2 orgs
. . Human poliovirus 1 .......    35 hits    1 orgs
. . Human poliovirus 2 .......     2 hits    1 orgs
. Human enterovirus C ........    10 hits   10 orgs
. . Human coxsackievirus A22 .     1 hits    1 orgs
. . Human coxsackievirus A20 .     1 hits    1 orgs
. . Human coxsackievirus A19 .     1 hits    1 orgs
. . Human coxsackievirus A18 .     1 hits    1 orgs
. . Human coxsackievirus A17 .     1 hits    1 orgs
. . Human coxsackievirus A15 .     1 hits    1 orgs
. . Human coxsackievirus A13 .     1 hits    1 orgs
. . Human coxsackievirus A11 .     1 hits    1 orgs
. . Human coxsackievirus A1 ..     1 hits    1 orgs
. . Human coxsackievirus A21 .     1 hits    1 orgs
```

EvUTR145rv3

```
root .................................   100 hits   31 orgs
. Enterovirus ........................    99 hits   30 orgs [Viruses; ssRNA positive-strand
viruses, no DNA stage; Picornaviridae]
. . Poliovirus .......................    33 hits    5 orgs
. . . Human poliovirus 1 .............    25 hits    3 orgs
. . . . Human poliovirus 1 strain Sabin .     1 hits    1 orgs
. . . . Human poliovirus 1 Mahoney ......     1 hits    1 orgs
. . . Human poliovirus 2 .............     5 hits    1 orgs
. . . Human poliovirus 3 .............     3 hits    1 orgs
. . Human coxsackievirus A4 ..........     1 hits    1 orgs [tentative species in the genus
Enterovirus]
. . Human enterovirus B ..............    40 hits    7 orgs
. . . Human coxsackievirus A9 ........     1 hits    1 orgs
. . . Human echovirus 2 ..............     1 hits    1 orgs
. . . Human echovirus 6 ..............     2 hits    1 orgs
. . . Human echovirus 7 ..............     1 hits    1 orgs
. . . Human echovirus 13 .............     4 hits    1 orgs
. . . Human coxsackievirus B5 ........     1 hits    1 orgs
. . Human enterovirus C ..............    14 hits   11 orgs
. . . Human coxsackievirus A20 .......     4 hits    2 orgs
. . . . Human coxsackievirus A20b ....     1 hits    1 orgs
. . . Human coxsackievirus A1 ........     2 hits    1 orgs
. . . Human coxsackievirus A22 .......     1 hits    1 orgs
. . . Human coxsackievirus A19 .......     1 hits    1 orgs
```

```
. . . Human coxsackievirus A18 .........     1 hits    1 orgs
. . . Human coxsackievirus A17 .........     1 hits    1 orgs
. . . Human coxsackievirus A15 .........     1 hits    1 orgs
. . . Human coxsackievirus A13 .........     1 hits    1 orgs
. . . Human coxsackievirus A11 .........     1 hits    1 orgs
. . . Human coxsackievirus A21 .........     1 hits    1 orgs
. . Porcine enterovirus B ..............     4 hits    2 orgs
. . . Porcine enterovirus 10 ...........     1 hits    1 orgs
. . . Porcine enterovirus 9 ............     3 hits    1 orgs
. . Human enterovirus A ................     6 hits    3 orgs
. . . Human coxsackievirus A5 ..........     2 hits    1 orgs
. . . Human coxsackievirus A7 ..........     2 hits    1 orgs
. . . Human enterovirus 71 .............     2 hits    1 orgs
. . Human enterovirus 70 ...............     1 hits    1 orgs [Human enterovirus D]
. synthetic construct ..................     1 hits    1 orgs [other sequences; artificial
sequences]
```

Appendix 4-1. USEPA sample data sheet for collection of ground water samples for enteric viruses using 1MDS filters.

SAMPLE DATA SHEET
SAMPLE NUMBER:
SITE OR UTILITY NAME:
SITE OR UTILITY ADDRESS: **STREET:** **CITY:** **STATE:** **ZIP:**
SAMPLER'S NAME:
WATER TEMPERATURE:
WATER pH:
WATER pH after adjustment (if initially >8.0):
WATER DISINFECTED: **(CHECK) __ YES __ NO**
THIOSULFATE ADDED: **(CHECK) __ YES __ NO**
INIT. METER READING: **CHECK UNITS:** **____gallons ____ft^3** **Date:** **Time:**
FINAL METER READING: **CHECK UNITS:** **____gallons ____ft3** **Date:** **Time:**
TOTAL SAMPLE VOLUME (L): **(Final-Initial meter readings × 3.7854 (for readings in gallons) or × 28.316 (for readings in ft^3))**
SHIPMENT DATE:
ARRIVAL DATE:
CONDITION ON ARRIVAL:
DATE ELUTED: **TIME:**
DATE CONCENTRATED: **TIME:**
FINAL CONCENTRATED SAMPLE VOLUME: **mL**
Comments:

Appendix 4-2. Protocol for 1MDS filter elution and cell culture analysis.

1.5% Adam Beef Extract:
Two portions of 1.5% Adam Beef extract were made by dissolving 24 g of beef extract powder in 1.6 L of Millipore water. The pH was adjusted to 9.5 and the solutions were autoclaved at 121°C for 15 minutes.

0.15 M Sodium Phosphate, dibasic:
A 0.15 M solution of sodium phosphate was made by dissolving 8.214g of sodium phosphate, dibasic, in 200 mL of Millipore water. The pH was adjusted to 9.5 and the solution was autoclaved at 121°C for 15 minutes.

Protocol:
The samples were sent in the form of water that had been filtered through a 1 MDS electropositive cartridge filter. The filter and housing were mailed on ice overnight or driven to the EPA from the sampling site in Tennessee.

1. Connect the air hose to the inlet of a pressure vessel.
2. Connect a hose to the outlet of the pressure vessel and the opposite end to the inlet of the cartridge housing.
3. Connect a hose to the outlet of the cartridge housing and place the opposite end of the hose into a beaker for collection of the eluate.
4. Pour 1.6 L of 1.5% Adam Beef Extract into the pressure vessel.
5. Open the air vent valve and allow the beef extract to slowly be forced out of the pressure vessel and into the cartridge housing.
6. When the beef has completely covered the filter, shut off the vent valve and allow the filter and beef extract to come into contact for one minute.
7. Open the vent valve and force the rest of the beef extract through the filter and into the beaker.
8. Disconnect all tubes from the filter housing. Remove the lid of the housing and pour approximately 800 mL of 1.5% Adam Beef, pH 9.0, onto the filter until it completely covers the filter and fills the housing. Replace the lid tightly; cover the inlet and outlet with foil, and allow it to site overnight.
9. Transfer the beaker containing the beef extract to a stir plate.
10. Add 1.6 g of analytical celite and mix.
11. Drop the pH to 4 by adding 1 M HCl dropwise and allow it to mix for 15 minutes.
12. Filter the sample through a sterile pre-filter to collect the celite.
13. Elute the sample with 80 mL of 0.15 M Sodium Phosphate solution, dibasic, pH 9.5.
14. Allow the sodium phosphate to drip through the celite and into a side-arm flask for collection.
15. When the majority of the sodium phosphate has dripped through, apply a slight vacuum to the flask/filter to pull the remaining drops through the filter/celite and into the flask.
16. Drop the pH to 7 and filter sterilize the sample.
17. Repeat steps 4-7 and 9-16 the following day for the overnight elution.
18. Samples should be frozen at -80°C until concentration/inhibitor removal and tissue culture assay.

19. Assay 20 mL of sample A (first elution) on a perforated tissue culture roller bottle (#1). After 80 minutes, pour this sample into another perforated tissue culture bottle (#2) for 80 minutes. Pour off the sample and add 200 mL of media (M-L15 without serum) to each bottle. Repeat this for sample B (overnight elution). Observe for 2 weeks for CPE. (This gives four bottles: A#1, A#2, B#1, B#2, plus an uninoculated control bottle).

Passage of samples from perforated tissue culture roller bottles to tissue culture tubes:
If no CPE is observed following observation of the perforated tissue culture roller bottles for 2 weeks, it is necessary to passage a portion of each sample into tissue culture tubes. Sometimes with wild strain viruses it is necessary to do 2 or 3 passages before CPE is observed. In this case, BGM cells were planted into tubes. The following procedure was used:

1. Do a series of freeze/thaws of the perforated tissue culture bottles containing the sample. (In this case 3 freeze/thaws in total were done, including the final thaw before inoculation.) Be sure to use the slushy ice to swirl around the bottle in order to scrape the cells from the sides of the bottle.
2. Pour off the media from the tubes.
3. Add 2 mL of EBSS (Earle's Balanced Salts Solution) with 0.5% lactalbumin, 2% calf serum, and antibiotics to each tube.
4. Inoculate each tube with 0.5 mL of the appropriate sample.
5. Incubate at 37°C and observe 2 weeks for CPE.

Appendix 4-3. Protocol for ground water sample concentration and inhibitor removal.

Solutions needed:

Phosphate Buffered Saline (PBS)-Dissolve the following in 900 mL distilled water:

8 g Sodium Chloride (NaCl)
0.2 g Potassium Chloride (KCl)
0.2 g Potassium Phosphate (KH_2PO_4)
0.92 g Sodium Phosphate, dibasic ($Na_2HPO_47H_20$)
Adjust the pH to 7.3. Bring final volume to 1 L with distilled water. Autoclave.

5% Bovine Serum Albumin (BSA)-Dissolve 5 g of BSA in 100 mL of DEPC treated water. Filter sterilize.

PBSAA-Mix 4 mL of 5% BSA with 96 mL of PBS.

30% Sucrose Buffer-Dissolve 300 g sucrose in 700 mL of 20mM Tris, 1 mM EDTA, 5mM EGTA, and 0.1% BSA.

0.01% Dithiozone-Dissolve 0.01 g of dithiozone in 100 mL chloroform.

0.01 M 8-Hydroxyquinoline-Dissolve 0.1425 g hydroxyquinoline in 100 mL of chloroform.

Solvent Mix-Mix the following:

0.1 mL of 0.01% dithiozone
0.9 mL of 0.01 M 8-Hydroxyquinoline
1 mL of butanol
0.25 mL of methanol
0.25 mL of trichloroethane

Day 1: Preliminary Procedures

1. Wipe the inside of the hood in room 345 and still air hood in room 395 with 10% bleach. Turn on the UV light and wait 15 to 30 minutes before proceeding.
2. Open a box of SW28 centrifuge tubes in the hood and place 6 in a rack. Fill each tube with PBSAA. Cover with parafilm and soak overnight at 4°C.
3. Fill 6 microcon-100 filter units, 6 microcon-100 microcentrifuge tubes, and 6 1.5 mL microcentrifuge completely with PBSAA in the still air hood. Place the filter units in microcentrifuge tubes without PBSAA and cap them before removing them from the hood. Let them soak overnight at 4°C.

Day 2:

Sample Concentration by Ultracentrifugation

1. Wipe the inside of the hood in room 345 with 10% bleach. Turn on the UV light and wait for 15-30 minutes before proceeding.

2. Soak SW28 rotor buckets in 10% bleach for 10 minutes. Dechlorinate with sterile 0.005% sodium thiosulfate. Rinse the buckets with sterile distilled water and let dry.
3. Thaw the water sample concentrates and place them on ice.
4. Place 40 mL of sterile PBSAA into each of two sterile 50 mL centrifuge tubes. One will be for the negative process control, one will be for the positive process control. To the positive process control, add 5µL of virus (echovirus 7 stock).
5. Add 80 µL of 5% BSA to each 40 mL water sample concentrate and the sterile distilled water.
6. Discard the PBSAA in the ultracentrifuge tubes and label each tube with the appropriate sample number.
7. Add 30 mL of sample to the appropriate tube.
8. Underlay each sample with 5 mL of 30% sucrose.
9. Add 2 additional milliliters of each sample to the appropriate tubes. Place tubes in SW28 buckets.
10. Balance the buckets using sterile PBSAA.
11. Centrifuge at 27,000 rpm for 2.5 hours at 10°C with the brake on.
12. Immediately after the rotor stops, remove the tubes and aspirate off the supernatant as quickly as possible. Place the tubes upside down on a paper towel to drain.
13. After draining, place the tubes upright in a rack and add 10 µL of sterile PBSAA to each pellet.
14. Using an aerosol-free P200 pipette tip, thoroughly scrape the pellet to remove it from the centrifuge tube. Draw the liquid back and forth to dissolve the pellet.
15. Wash the sides of the curved area of the centrifuge tube with the dissolved material and then transfer it to a labeled sterile 1.5 mL microcentrifuge tube.
16. Wash the centrifuge tube with another 100 µL of PBSAA and combine the second wash with the first.
17. Repeat steps 14-16 for all samples.

<u>Inhibitor Removal</u>

1. Pour off and discard the PBSAA from the microcon units prior to use.
2. Prepare the solvent mix.
3. Add 200 µL of solvent mix to each microcentrifuge tube containing the dissolve water sample pellets.
4. Vortex for 30 seconds at room temperature. Let sit for 15 seconds. Vortex for 30 seconds. Let sit for 30 seconds.
5. Centrifuge in the microcentrifuge at 4°C for 5 minutes.
6. Carefully transfer the aqueous (top) layer from each sample to the PBSAA-treated microcon-100 filter unit labeled with the sample number.
7. Cap and centrifuge the filter units at 6,000 rpm for 30 minutes at 4C.
8. Add 80 µL of PBSAA to each sample and then centrifuge again at 6,000 rpm for 20 minutes at 4°C.
9. Take the units to the hood in room 345 and add 10 µL of PBSAA.
10. Place the housing in a clean microcentrifuge tube, cap and vortex for 15 seconds.
11. Invert the housing into the microcentrifuge tubes treated with PBSAA.

12. Centrifuge at 3,500 rpm for 3 minutes.
13. Discard the filter housings.
14. Measure the volume of the concentrated sample.
15. Bring up to desired volume (if volume is less than desired volume) with PBSAA and record the final volume.
16. Store samples on ice or at 4°C until assayed, but freeze at -80C if they cannot be analyzed on the day of processing.

Appendix 4-4. Protocol for RT-PCR and dot-blot hybridization analysis (enterovirus and reovirus) of ground water samples.

Step 1. Purchase the oligonucleotides shown below (Integrated DNA Technologies, Coralville, IA). *Steps 2-7 are all performed in a clean room which does not contain any virus or PCR products. Within this room work is performed in still air hood which has been wiped with bleach*

Step 2. Resuspend the oligonucleotides to 10pmol/µl in DEPC treated water.

Primer and Probes used for RT-PCR			
Name	**Virus**	**Type**	**Sequence (5'-3')**
MRD 13	Enterovirus	RT	ACC GGA TGG CCA ATC CAA
MRD14		PCR	CCT CCG GCC CCT GAA TG
MRD32		Probe	ACT ACT TTG GGT GTC CGT GTT TC
MRD 188	Reovirus	RCR	ACG TTG TCG CAA TGG AGG TGT
MRD 189		RT	GTG CTG AGA TTG TTT TGT CCC AT
REOP 1		Probe	AAC GGT CAT CAG ATC G
REOP 2		Probe	ACG GTC ATC AGG TCG
REOP 3		Probe	AAT GGT CAT CAG GTC G

Step 3. Prepare the RT mixes as shown below.
Prepare all mixes (steps 2-4) and store on ice until used.

RT Mix Preparation	
RT Mix	Amount per Reaction (µl)
10X PCR Buffer (0 mM Mg)[a]	3
25 mM $MgCl_2$[a]	1.8
10mM dNTPs	2
DEPC-dH_2O	11.5[b]
RT primer [c]	5
Total	23.3

[a]10 X PCR Buffer II and 25 mM $MgCl_2$ can be ordered separately (Applied Biosystems Cat. No. N808-0010) or purchased with AmpliTaq Gold (Applied Biosystems Cat. No. N808-0241).
[b]The amount of water per reaction is calculated for adding 5 µL of sample per reaction.
[c] MRD13 and MRD189 at 10 pmol/µl (200nM final concentration)

Step 4. Prepare the PCR mixes as shown below.

PCR Mix Preparation	
PCR Mix	Amount per Reaction (µl)
10X PCR Buffer (0 mM Mg)	7
25 mM $MgCl_2$	10.2
DEPC-dH_2O	46.8
PCR A[a]	5
AmpliTaq Gold	1
Total	70

[a] MRD14 and MRD 188 at 10 pmol/µl (200nM final concentration)

Step 5. Prepare RT/RNasin Mix as shown below:

RT/RNasin Mix Preparation	
Ingredient	Amount per Reaction (µl)
MuLV RT[a]	1.0
RNasin[b]	0.75
Total	1.75

[a]Applied Biosystems Cat. No. N808-0018.
[b]Promega Cat. No. N2515.

Step 6. Dispense 23.25 µL of RT mix A into plate and cover with Microfilm A (MJ Research)

Step 7. In all subsequent steps use a hood or clean area for sample preparation for working with virus samples. Add 5 µL of samples to 96-well plate. Pipet sample into the bottom of the well and pipet up and down to mix. Cover with Microfilm A.

Step 8. Heat for 5 minutes at 99EC in the Thermal Cycler. Quench immediately on ice.

Step 9. Add 1.75 µL of RNasin/MuLV RT mix to each well. Mix by pipeting.

Step 10. Place the plate into a thermal cycler and run using a program to cycle the samples one time at 43EC for 60 min followed by 95EC for 5 min and hold the samples at 4EC indefinitely.

Step 11. Add 70 µL of PCR to each well. Mix by pipeting. Cover plate with foil seal.
Step 12. Place the tubes back into a thermal cycler and run on a program to cycle the samples 1 time at 95EC for 10 min., then 45 cycles of 95EC for 60 sec followed by 50EC

for 90 sec followed by 72EC for 120 sec., followed by a final extension of 72EC for 10 min., then hold the samples at 4EC indefinitely. *Store leftover PCR products at -20EC.*

AGAROSE GEL ELECTROPHORESIS:

5µl of each PCR product was run on a 2% high resolution blend agarose gel. Stained with ethidium bromide in the agarose. Visualize the DNA using a transilluminator at 302 nm. Image is recorded using the Kodak gel documentation system.

3'-END LABELING OF OLIGONUCLEOTIDE HYBRIDIZATION PROBES:

Step 1. For each oligonucleotide probe to be labeled, label a sterile 0.5 mL microcentrifuge tubes with "DIG" followed by the name of the oligonucleotide, the date of labeling and the initials of the one doing the labeling.

Step 2. Add the following ingredients from a DIG Oligonucleotide 3'-End Labeling Kit (Genius 5; Boehringer Mannheim Cat. No. 1362 372) to each tube:

a. 20 µL (200 pmol) of the appropriate oligonucleotide to be labeled.

b. 8 µL of tailing buffer (kit vial 1).

c. 8 µL of $CoCl_2$ (kit vial 2).

d. 2 µL digoxigenin-ddUTP (kit vial 3).

e. 2 µL of terminal transferase (kit vial 4).

Step 3. Mix and incubate the tubes for 30 minutes at 37EC. Place on ice.

Step 4. Add 1 µL of glycogen (kit vial 8) to 200 µL of 0.2 M EDTA, pH 8.0. Mix and add 2 µL to each reaction tube to stop the end labeling reaction.

Step 5. Add 58 µL of DNA dilution buffer (kit vial 9) to each tube. Mix and store at -20EC.

HYBRIDIZATION OF WATER SAMPLES:
Date: 2/23/05
Experiment: Hybridization of PCR products for UT concentrated water samples
Purpose: Although some samples do look positive on the gels, most are smears and difficult to see. Plus any positive must be proven real by a secondary method i.e. hybridization or sequencing.
Protocol:
1.) For each sample to be tested add the following to a 96 well Dynatech v-bottom tray:
    a.) 46.2ul of dH2O in wells with samples

b.) 13.8ul of 1M NaOH, 0.4M EDTA into wells with PCR products
c.) Add 15.75ul of each PCR samples as shown

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	34	35	36
D	37	38	39	40	41	42	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}
E	1*	2*	3*	4*	5*	6*	7*	8*	9*	10*	11*	12*
F	13*	14*	15*	16*	17*	18*	19*	20*	21*	22*	23*	24*
G	25*	26*	27*	28*	29*	30*	31*	32*	33*	34*	35*	36*
H	37*	38*	39*	40*	41*	42*	10^{-6}	10^{-6}	10^{-7}	10^{-7}	10^{-8}	10^{-8}

Key: See list on page 57 for sample number, * indicates 1:10 dilution of sample, 10- isa dilution of polio virus stock for positive controls

2.) Incubate at least 10 minutes at room temperature

3.) Prepare a 96-well S&S manifold by adding filter paper, with two nylon blots cut to size on top of filter paper. Add wells to manifold and lock in place. Polio blot is cut in upper right corner and lower right corner.

4.) Add 13.8ul of 10M NH4Ac to wells

5.) Mix and Spot 50ul onto corresponding well of manifold

6.) Wash membrane 2 times with 150ul of 6X SSC per well

7.) Stratalink the blots. (Autocross link)

8.) Prepare 50mL of pre-hybridzation fluid for each blot—mix as below:
a.) 12.5mL 20XSSC
b.) 5.0mL of 10% blocking solution
c.) 0.5mL of 10% sarkosyl
d.) 0.1mL of 10% SDS
e.) 31.9mL of dH20
9.) Prehybe membrane for 1hr at 51° C in 50mL of hybe buffer
10.) Hybridize overnight at 51°C in 5mL of probe mix for MRD 32 or internal control.

11.) Turn on floor water bath shaker to 51°C for the next day. Check water level.

Next Day:

1.) Transfer membrane to a tray containing 50mL of 2X SSC, using forceps specific for polio and internal control. (Refreeze the hybridization fluid at -20°C for future use.) Wash the membranes twice in 50mL of 2X SSC for 5 minutes at room temp.

2.) Wash each membrane twice in 50mL of the correct astringency wash solution (pre-warmed to 51°C) at 51°C.

Washing solutions 100mL
MRD 32 (0.05X SSC)
250ul 20X SSC
1mL 10% SDS
H_2O to 100mL

Perform all remaining steps at room temperature.

3.) Wash the membrane in 50mL of Genius buffer 1 with 0.3% tween 20 (150ul tween 20 to 50mL of Genius buffer) for 5 minutes.

4.) Prepare 100mL of 1% blocking solution for each membrane (10mL of blocking solution + 90mL of Genius buffer). Wash membranes in 50mL for 30 minutes.

5.) Add 5µl of α-dig-alkaline phosphatase to the remaining 50mL of 1% blocking solution. Add to membrane and incubate 30 minutes.

6.) Wash twice for 15 minutes with 50mL of Genius buffer 1.

7.) Wash twice for 5 minutes with 25mL of diethanolamine buffer.

8.) Transfer membrane to a zip-lock plastic bag. Add 20µl of CSPD reagent to 2mL of diethanolamine buffer. Mix and add to the bag, agitate for 5 minutes.

9.) Pour off the CSPD reagent, leaving just enough to keep membrane wet. Seal bag and transfer to a film cassette.

10.) Add film in the darkroom and expose for 30 minutes at room temp. (If necessary, re-expose for 60-90 minutes; the filter can be stored at 4°C overnight and then warmed to room temp. for a second exposure.)

11.) Develop film in the darkroom with GDX developer for 5 minutes. Rinse in a tray of tap water. Fix with GDX fixer for 2-3 minutes. Rinse thoroughly with tap water and dry.

The same procedure is followed with the samples testing with the Reo1,2,3 probes however the wash was 10.6ml 20XSSC, 1ml 10% SDS, to 100mL of water

VITA

Trisha Baldwin Johnson was born in Richmond, Virginia on January 25, 1979. She was raised in Quinton, VA and graduated from New Kent High School in 1997. From there, she went to the College of William and Mary in Williamsburg, VA and received a B.S. in biology with a minor in geology in 2001. In December of 2005, she will receive a M.S. in geology from the University of Tennessee, Knoxville.

Trisha and her husband, Brandon, currently reside in Laramie, Wyoming where Trisha is employed as a geologist with Trihydro Corporation, an environmental and engineering consulting firm.