TORQUE TENO VIRUS:

A POTENTIAL INDICATOR OF ENTERIC VIRUSES

By

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Abstract

To protect public health, drinking water systems are monitored for indicator organisms that correlate with fecal contamination and suggest the presence of human pathogens. Total coliforms, fecal coliforms, and *E. coli* are the most commonly used indicator organisms. These bacteria generally colocate with fecal pollution, but some limitations exist. In particular, the ability of indicator bacteria to predict the presence of enteric viruses is questionable because of distinct transport and survival characteristics of bacteria and viruses. Although viral indicators of enteric viruses have been proposed, none have been implemented into the current regulatory framework. In this thesis, the correlation of bacteria and viruses in drinking water sources and treatment systems is reviewed, and the potential of Torque Teno virus (TTV) to qualify as an indicator virus is discussed. TTV is unique among enteric viruses as it infects approximately 80% of healthy individuals worldwide, is transmitted by the fecal-oral route, causes no observable illness, and lacks seasonal fluctuations.

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CHAPTER 1 – INTRODUCTION

Drinking water contamination with fecally deposited bacteria, parasites, and viruses presents a consistent and significant threat to public health. Regulatory bodies have promulgated rules to protect surface water and ground water sources of drinking waters from enteric pathogens. These rules depend on monitoring water bodies and treatment systems for indicator organisms that are expected to colocate precisely with fecal pollution. Total coliforms, fecal coliforms, and *E. coli* are the most commonly used indicator organisms. In theory, routine detection and removal of these bacteria from water supplies ensures that colocated waterborne pathogens will be removed as well.

Typically, viruses exhibit greater resistance than bacterial indicators to environmental stressors and treatment processes. The small size of viruses compared to bacteria may give rise to enhanced transport in surface waters and the subsurface. These characteristics lead to instances of virus presence in the absence of indicator bacteria and thus a public health risk where none is predicted. Alternatively, the imperfect association of coliforms with fecal contamination and the potential of these organisms to replicate in receiving waters may lead officials to anticipate a public health risk where none exists.

To more accurately detect pathogenic virus presence in drinking waters, bacteriophages and representative human enteric viruses have been proposed as alternatives to bacterial indicators based on similar sizes and resistance patterns. However, bacteriophages may continue to replicate in bacterial hosts following fecal excretion or may be physically removed (*e.g.*, by filtration) before egressing from bacterial cells. Therefore, the utility of

bacteriophages as indicators of enteric viruses is questionable. The use of a single pathogenic enteric virus species to indicate all other enteric viruses has been unsuccessful to date because of seasonal fluctuations and epidemic spikes that differ across members of this virus group. Instead of colocating precisely and consistently with fecal pollution, enteric viral pathogens are only present when fecal contamination is derived from infected individuals. These caveats have precluded viral indicators from being implemented as a monitoring strategy to complement bacterial indicators.

The recently described Torque Teno virus (TTV) is unique among enteric viruses. TTV is a small, unenveloped DNA virus that infects approximately 80% of healthy individuals worldwide. It elicits persistent, productive infections in various human tissues but is not associated with illness. TTV is transmitted primarily by the fecal-oral route, and it is neither demographically localized nor does it exhibit seasonal variance. A small number of studies have been conducted to assess the indicator potential of TTV. Although standard, accepted protocols for TTV detection using cell culture and polymerase chain reaction (PCR) are still in the development phase, preliminary results support the utility of TTV as an indicator virus.

In this thesis, source water contamination with viruses and consequent waterborne disease outbreaks are reviewed in light of regulations that focus on monitoring and removal of indicator bacteria. The usefulness of viral indicators, particularly TTV, is discussed, and a monitoring strategy for TTV in source waters and treatment systems is proposed.

CHAPTER 2 – PUBLIC DRINKING WATER SYSTEMS

Most U.S. residents obtain drinking water from the 156,000 public drinking water systems distributed throughout the United States (U.S. Environmental Protection Agency [USEPA] Factoids, 2007). Public water systems supply drinking water to at least 25 people or have at least 15 service connections. They are further classified as community water systems (CWS), nontransient noncommunity water systems (NTNCWS), or transient noncommunity water systems (TNCWS). CWS serve 25 or more year-round residents. Noncommunity water systems include NTNCWS, in which 25 or more people are served for at least 6 months in any given year (e.g., schools, hospitals), and TNCWS, which provide drinking water to people on a very short-term basis (e.g., campgrounds).

Approximately 286 million people in the United States depend on CWS for potable water. Large systems that serve more than 10,000 residents each supply the majority of consumers, with 8% of systems providing water to 82% of the population. Drinking water systems are sourced by surface water—such as lakes, rivers, and reservoirs—or ground water. Whereas ground water is used as the source for most (78%) CWS, a majority (68%) of the U.S. population is served by surface water systems. Surface water bodies may interact significantly with ground water aquifers via runoff, percolation, recharge, or depletion. These interactions involve an exchange of solutes and volume.

Table 2.1. Types of drinking water systems across population size and water source.

CWS = community water system; NTNCWS = nontransient, noncommunity water system; TNCWS = transient noncommunity water system. Adapted from USEPA Factoids, 2007.

		Serving ≤ 500	Serving 501 - 3,300	Serving 3,301 - 10,000	Serving 10,001 - 100,000	Serving > 100,000	Ground Water Systems	Surface Water Systems
CWS	Systems	29,282	13,906	4,822	3,702	398	40,646	11,449
	Population	4.86×10^6	1.98×10^7	2.79×10^7	1.05×10^8	1.29×10^8	9.05×10^7	1.96×10^8
	% Systems	56	27	9	7	1	78	22
	% Pop.	2	7	10	37	45	32	68
NTNCWS	Systems	16,034	2,662	120	22	1	18,151	679
	Population	2.25×10^6	2.71×10^6	6.40×10^5	5.34×10^5	2.03×10^5	5.50×10^6	7.88×10^5
	% Systems	85	14	1	0	0	96	4
	% Pop.	35	43	10	8	3	87	13
TNCWS	Systems	81,873	2,751	102	15	3	82,851	1,878
	Population	7.23×10^6	2.68×10^6	5.46×10^5	4.25×10^5	2.87×10^6	1.11×10^7	2.67×10^6
	% Systems	97	3	0	0	0	98	2
	% Pop.	53	19	4	3	21	81	19

2.1. Drinking Water Contamination

Water pollution can originate from point and nonpoint sources. Point source pollution generally describes pollutant discharge from industrial or sewage treatment plants that is released from a conduit such as a pipe. Point source pollution levels are federally regulated through the National Pollutant Discharge Elimination System (NPDES) permitting program. Nonpoint source pollution is more difficult to track and characterize. In this case, pollutants are collected and carried by runoff from rain or snowmelt into surface and ground waters. Individual states develop and implement programs to control nonpoint source pollution.

Water may become contaminated chemically or microbiologically; in both cases, humans may become ill from ingestion, dermal exposure, or inhalation of droplets. Chemical-induced illness is likely to be chronic and may occur via ingestion of copper in corrosive water; lead leachate from lead-soldered pipe; or nitrate, soap concentrate, or fluoride following back siphonage of water (Craun *et al.*, 2002). Microbiological contamination most often occurs via introduction of feces from individuals infected with pathogenic viruses, bacteria, protozoa, or helminths (Bull *et al.*, 1990). Infection and illness may result when fecally contaminated water is ingested (*i.e.*, the fecal-oral, or enteric, route). Microbiologically derived illnesses typically are acute and self-limiting. The scope of this thesis is limited to the detection of virological pollution in drinking water.

The USEPA Information Collection Rule (ICR, see Section 4.6) reported that source waters were positive for virus contamination at more than 80% of 207 surface water treatment plants that conducted monthly monitoring (Shaw *et al.*, 2003). Quantifying viruses by the Most Probable Number (MPN) method (see Section 3.3), half of the treatment plants measured virus concentrations higher than 0.4 MPN/100 L. Ten percent detected virus above 5 MPN/100 L. A subset of the 207 treatment plants also measured virus in finished waters. Of these, 16% reported at least one virus-positive result.

CHAPTER 3 – INDICATOR ORGANISMS

More than 150 known enteric pathogens may be present in untreated waste (Gerba and Smith, 2005; Reynolds *et al*., 2008), and this may include more than 100 different species of enteric viruses alone (Glass, 1995; Macler, 1995). Infectious enteric viruses have been isolated from various water sources, including rivers, streams, coastal waters, ground water, treated sewage, aerosols, and wells. From a strictly public health standpoint, direct monitoring of waterborne enteric pathogens may be the ideal option to detect contamination and protect water supplies (Yates, 2007). However, the number of enteric microbial species—particularly viral species—that may be present in a fecally contaminated water sample makes it economically impractical and time-prohibitive to test directly for each pathogen. In addition, tissue culture, which informs water utility managers about virus infectivity, is beyond the technical capabilities of some water utility laboratories. Moreover, certain waterborne pathogenic viruses of great public health significance (*e.g.*, norovirus) have not been adaptable to facile tissue culture methods (Nuanualsuwan and Cliver, 2002). Norovirus recently has been cultured using a three-dimensional organoid model of human small intestine epithelium (Straub *et al*., 2007), but this technique is beyond the analytical capabilities of typical water testing laboratories. Instead, water quality professionals monitor for surrogate organisms, called indicators, that are expected to colocate with waterborne pathogens transmitted by the fecal-oral route. The presence of indicator organisms in a water sample suggests fecal contamination and potential pathogenic risk.

3.1. Indicator Organism Criteria

In 1966, Bonde described the requirements for an appropriate indicator organism, including that the indicator should:

(1) be exclusively and predictably associated with pathogenic species whenever pathogens are present to such a degree that the public health is at risk;

(2) exist more abundantly than pathogens in environmental waters and be as resistant to disinfectants and environmental stressors as the most resistant correlated pathogen; and

(3) grow readily and independently of other organisms and be uniformly distributed in samples to facilitate unambiguous, straightforward identification in the laboratory.

Since then, others have amended Bonde's criteria, adding that indicators should exhibit similar transport characteristics to pathogens, correlate only with infectious (rather than inactivated) pathogens, be cost-effective to monitor, allow for rapid presence/absence measurement, and be of low risk to the analyst (*i.e.*, the indicator is not itself pathogenic) (Payment *et al.*, 2003; National Research Council [NRC], 2004; Yates, 2007). Some researchers have supported the selection of indicator organisms from innocuous gut microbes that happen to correlate with illness (Cabelli *et al.*, 1979; Seyfried *et al.*, 1985a; Seyfried *et al.*, 1985b, Zmirou *et al.*, 1987; Cheung *et al.*, 1990; Payment *et al.*, 1991; Payment *et al.*, 1997; Hellard *et al.*, 2001; Colford *et al.*, 2002). Others have proposed choosing potential indicators among any of the microbes that happen to be detected during conditions of elevated pathogen concentration (Gerba *et al.*, 1979; LaBelle *et al.*, 1980; Robertson, 1984; Seyfried *et al.*, 1984; Havelaar, 1993; Leclerc *et al.*, 2000).

Notably, the colocation of an indicator with one pathogenic species does not translate to a correlation between the indicator and all pathogenic species (Yates, 2007), nor does it guarantee that the indicator is exclusively associated with a given pathogen at all times and in all geographic locations.

In some cases, the viability of the pathogen (*i.e.*, its capacity to cause infection) is more important than its presence/absence. For instance, in a treatment system, an appropriate indicator should only be detected when pathogens to which it is correlated are infectious. Ideally, the indicator would be absent if a treatment system were effectively inactivating pathogens, regardless of whether the pathogens were being physically removed from the water. Alternatively, in ground water sources, even the threat of contamination—evidenced by viable and nonviable pathogens—should correlate with indicator presence in order to identify a putative "path of contamination" (Yates, 2007).

Indicator organisms can be chosen for a number of purposes, including detection of (1) fecal contamination; (2) wastewater contamination; (3) correlated pathogenic organisms; (4) treatment system efficiency; and (5) subsurface transport (Yates, 2007). Whereas the same indicator organism may suffice for more than one of these purposes, the intent of a given water monitoring process must be considered before an indicator is chosen. For instance, similar sensitivity and resistance to manmade treatment processes is particularly important when an indicator is used to determine treatment system efficiency, whereas similar size, electrostatic properties, and environmental decay rates are important if an indicator is used for information about subsurface transport of a pathogen.

3.2. Coliform Bacteria

In 1892, Schardinger proposed that fecal contamination—and by extension, potential pathogenic contamination and human health risk—could be inferred by collecting water samples and assaying for the ability of *Escherichia coli* to ferment glucose and lactose (Feng *et al.*, 2002). *E. coli* is almost exclusively of fecal origin, so in theory, this method was expected to be sufficient to detect all fecal contamination without generating false-positives. In practice, this method was complicated because other bacteria exhibit similar phenotypes and fermentation properties to *E. coli*. This includes several nonfecally derived genera within the family *Enterobacteriaceae*, such as *Escherichia* spp., *Citrobacter*, *Klebsiella*, and *Enterobacter*. As a result, these similar bacterial genera were grouped under the term "coliforms" and a single assay was used to enumerate all of them without distinction (*i.e.*, total coliform).

Coliforms are defined as Gram-negative, nonsporulating, rod-shaped, facultative anaerobes that ferment lactose with acid and gas production within 48 hours at 35°C. This bacterial group generally is nonpathogenic to humans, and the presence of coliforms in water indicates a broad range of bacteriological contamination. However, because coliforms include bacterial species that are indigenous to soil, water, vegetation, and the digestive systems of humans and animals, coliforms do not specifically and exclusively detect human fecal contamination (Toranzos and McFeters, 1997; Craun *et al.*, 2002; NRC, 2004).

The subset of coliform species that populates the intestinal tracts of humans and animals and is abundant in feces is termed fecal coliforms. *Escherichia* spp. (mainly *E. coli*) as well as *Klebsiella* spp. and *Citrobacter* spp. compose the fecal coliforms. Sixty to ninety percent of total coliforms in contaminated waters are fecal coliforms, and *Escherichia* spp. compose 90% or more of the fecal coliforms (American Public Health Association [APHA] *et al.*, 1992). This subgroup grows and ferments lactose at 44.5°C rather than 35°C, hence its alternate name, thermotolerant coliforms. The bacterial species that are grouped as fecal coliforms correlate more precisely with fecal contamination than do total coliforms. However, even fecal coliforms do not exclusively indicate fecal pollution (Feng *et al.*, 2002). For instance, some *Klebsiella* species are associated with textile and paper mill wastes.

Total coliforms and fecal coliforms have been isolated from tropical waters far removed from human activity. These isolates likely were deposited by animals or birds and multiplied in receiving waters (Santiago-Mercado and Hazen, 1987). For instance, geese and swans may shed 10^7–10^9 fecal coliforms per day (Hussong *et al.*, 1979). In addition, Leclerc *et al.* (2002) reported that 30% of drinking water samples that tested positive for fecal coliforms since 1962 contained strains of the environmentally ubiquitous *Aeromonas* bacterium that would have triggered a coliform-positive result even in the absence of fecal contamination. Other researchers substantiated this report (Burke *et al.*, 1984; Havelaar *et al.*, 1990; Schubert, 1991). False-positive results likely would cause drinking water utilities to accrue unnecessary costs in excessive filtration and disinfection procedures.

Methods have been developed that rapidly and specifically identify *E. coli*, consequently reintroducing this species as a recommended indicator of recent fecal contamination. Yet the strategy of solely relying on bacterial indicators for all fecally derived microbiological contamination remains inadequate because waterborne viruses and protozoa exhibit different transport and survival characteristics from bacteria (see Section 5.4).

3.3. Laboratory Detection of Total Coliforms, Fecal Coliforms, and *E. coli*

The two approved methods for coliform monitoring under the USEPA Total Coliform Rule (TCR) (see Section 4.4) are the Most Probable Number (MPN) method (a.k.a. Multiple Tube Fermentation [MTF]) and the Membrane Filtration (MF) technique. By the MPN method, serial dilutions of water samples are prepared and coliform positive/negative responses are recorded for each dilution after the appropriate culture conditions are met. Statistical tables then are consulted to estimate the coliform, fecal coliform, or *E. coli* density in the original sample (APHA *et al*., 2005).

The MPN method detects total coliforms based on the coliform group's ability to ferment lauryl tryptose broth or lactose broth and produce acid and gas within 24 ± 2 hours or 48 ± 3 hours at 35 ± 0.5°C (Standard Methods 9221B and 9221C, APHA *et al*., 2005). Serial dilutions of a sample are inoculated into nutrient broth, and gas-positive dilutions are scored. However, several Gram-positive organisms also produce gas under these conditions, and therefore a presumptive positive result must be confirmed. This is accomplished using brilliant green lactose bile (BGLB), which inhibits growth of Gram-

positive, noncoliform bacteria. BGLB is not used for the initial test because it is toxic to low densities of bacteria and to injured, but viable, bacteria. Once confirmed, coliform-positive results can be further substantiated by streaking samples on eosin ethylene blue agar and verifying microscopically that the bacteria are Gram-negative, nonsporulating rods.

The presence of fecal coliforms is determined by inoculating *E. coli* (EC) broth with an aliquot of each presumptive sample, incubating at $44.5 \pm 0.2°C$, and assaying for gas production within 24 ± 2 hours. *E. coli* is identified by inoculating presumptive positive samples into media containing 4-methylumbelliferyl-β-D-glucuronide (MUG) substrate (Standard Methods 9221F, APHA *et al*., 2005). *E. coli* expresses the glucuronidase enzyme, which hydrolyzes MUG substrate, resulting in turbidity and fluorescence under a long wavelength ultraviolet (UV) lamp.

The MF method involves passing a water sample through a 0.45 μm filter, which traps bacteria and other particulate matter, and transferring the filter to a saturated pad of m-Endo or Lawrence Experimental Station (LES)-Endo broth (for total coliforms) or m-FC broth (for fecal coliforms) in a Petri dish (Standard Methods 9222B and 9222C, APHA *et al*., 2005). Petri dishes are incubated at $35 \pm 0.5°C$ (for total coliforms) or $44.5 \pm 0.2°C$ (for fecal coliforms). After 24 ± 2 hours on m-Endo or LES-Endo broth pads, total coliforms appear as pink or dark red colonies with a metallic green surface sheen. Colonies are counted under a dissecting microscope and reported as colony forming units (cfu) per 100 mL sample. Fecal coliforms are identified as blue colonies on

m-FC broth pads and contrast with gray or cream-colored nonfecal coliforms. *E. coli* is confirmed by subculturing colonies onto nutrient agar containing MUG substrate (Standard Method 9222 G, APHA *et al.*, 2005). Samples are incubated for 4 hours at $35 \pm 0.5°C$ during which *E. coli*-positive colonies become delineated with blue fluorescence.

3.4. Other Bacterial Indicator Systems

Fecal streptococci and fecal enterococci occur in the intestinal tracts of humans and many animals and rarely multiply in the environment. These bacteria are more resistant to treatment systems and environmental stressors and persist longer than coliform bacteria (Yates, 2007). Fecal streptococci and fecal enterococci are generally employed as indicators in marine waters where the concentration of other bacterial indicators is low, but the USEPA also has suggested their use as fecal indicators in ground water (USEPA, 2000).

The ratio of fecal coliforms to fecal streptococci previously was thought to be useful in determining whether fecal contamination was human- or animal-derived. Ratios higher than 4 were considered human fecal contamination, whereas ratios below 0.7 were considered animal fecal contamination. This method is now obsolete because of its inability to indicate contamination type for ratios between 0.7 and 4 and the observation that the FC/FS ratio demonstrates significant spatial and temporal variability. The latter occurs because fecal coliforms and fecal streptococci exhibit different survival and

regrowth rates (Gannon and Busse, 1999) and react differently to temperature and sediment particle size (Howell *et al.*, 1996).

Heterotrophic plate count (HPC) bacteria are defined as the aerobic and facultatively anaerobic bacteria that obtain carbon and energy from organic sources (Bartram *et al.*, 2003). The HPC bacteria test is used to enumerate pathogenic and nonpathogenic microorganisms alike. A high HPC result does not necessarily suggest a public health risk, although sudden changes in HPC may suggest contamination of a water source. Interestingly, HPC bacteria grow well at point-of-use filters and may out-compete pathogenic bacteria (Yates, 2007).

CHAPTER 4 – DRINKING WATER REGULATIONS IN THE UNITED STATES

Contaminated drinking water has been recognized as a vector for disease spread since 1855, when the epidemiologist John Snow demonstrated that cholera was transmitted by common use of a polluted well. In the 1880s, Louis Pasteur described the germ theory of disease, in which contagious microscopic organisms—later identified as bacteria, viruses, and protozoa—could be spread through water and other media. These discoveries led to the realization that pathogens or indicators of pathogens could be monitored in water sources to protect the nation's drinking water supplies.

In 1914, the U.S. Public Health Service adopted the strategy of removing coliform bacteria from drinking water. This indirectly protected the public from the threat of correlated pathogens (Feng *et al*., 2002). However, the legislation only applied to water supplies serving interstate transportation and was meant to safeguard travelers (Pontius and Clark, 1999). Subsequent revisions of the U.S. Public Health Service legislation set the framework for contaminant limits in drinking water and monitoring of bacteriological presence. By 1962, the legislation had expanded to include regulation of 28 waterborne contaminants (U.S. Department of Health, Education, and Welfare, 1969). Yet the potential health detriment from pollutants that entered source waters through factory discharges, farm runoff, and sewage leaks continued to concern citizens. The federal government responded by performing a number of analyses of the nation's drinking water systems. In 1969, the U.S. Public Health Service reported that 40% of the nation's water systems were substandard, and many suffered from severe deficiencies in treatment (U.S. Department of Health, Education, and Welfare, 1970).

4.1. The Safe Drinking Water Act

As a response to intensifying concerns about the environment, the USEPA was formed on December 2, 1970, as a centralized federal agency for environmental research, monitoring, and regulation. Control of drinking water was transferred to the USEPA from the Bureau of Water Hygiene of the Department of Health, Education, and Welfare. On December 16, 1974, the Safe Drinking Water Act (SDWA) was passed in response to foul odors and tastes and increased recognition of the health effects of putative carcinogens, lead, and waterborne pathogens in the waters of urban and rural communities (USEPA, 1994). The intent of the SDWA was to treat and maintain the quality of the nation's drinking waters. Contaminant limits were to be set after the states, public utilities, scientists, environmentalists, and consumers provided insight into the necessary balance of needs and capabilities to maintain water quality. In addition, the National Academies was to conduct a study of water contaminants and the contaminant concentrations that could exist in water without posing a health hazard. The SDWA arranged a program to protect ground water aquifers, thus preventing source contamination before water reaches the treatment stage. In addition, 80 cities were selected for water sampling and analysis to detect organic chemicals, such as chlorine byproducts.

On June 25, 1977, the Safe Drinking Water Standards went into effect. Requirements included regular sampling of the 40,000 community drinking water systems and the 200,000 other public water systems. Consumers were to be notified if sampling or standards were not met. Standards addressed microbiological contamination—using

coliform bacteria as indicators—as well as pollution from inorganic chemicals, organic pesticides, turbidity, and radioactivity.

4.2. National Primary Drinking Water Regulations

The SDWA gave rise to the National Interim Primary Drinking Water Regulations (NIPDWR) in 1975, which put forth the maximum contaminant levels (MCLs) for a number of drinking water-associated chemicals. These standards consider both public health and cost-effectiveness and are enforced and met by water providers. Maximum Contaminant Level Goals (MCLGs), which also were introduced in the NIPDWR, do not account for economic feasibility and were considered nonenforceable ideal contaminant levels. In 1985, the NIPDWR transitioned to the National Primary Drinking Water Regulations (NPDWR).

4.3. SDWA Amendments

In 1986, amendments to the SDWA were signed into law. Under these amendments, certain water systems using surface waters were required to treat by filtration, and certain ground water systems were required to use disinfection treatment. MCL-setting was required for 83 contaminants within the first 3 years following the 1986 amendments, and MCLs were required for no fewer than 25 contaminants during each subsequent 3-year period. Additional amendments in 1996 established the framework for future MCLs including that enforceable levels would be established after considering public input. The amendments also mandated public water systems to distribute consumer confidence reports to the public.

4.4. The Total Coliform Rule

The Total Coliform Rule (TCR) was passed in June 1989 to control fecal contamination in drinking waters by monitoring and controlling indicator bacteria (USEPA, 1989a). Total coliforms, fecal coliforms, and *E. coli* were chosen as indicators of fecal pollution, and all public water systems are required to sample for coliforms at representative sites in the distribution system. Individual states may choose which indicator organism is most appropriate for their monitoring practices (Yates, 2007). The frequency of routine monitoring depends on the population serviced by the water system and ranges from 1 sample per month for systems serving 25–1,000 residents to 480 samples per month for systems serving more than 3.96 million consumers.

The USEPA set a MCLG of zero for coliforms. The MCL was based on the presence or absence of total coliforms (*i.e.*, a positive or negative result using a variation of the MPN technique with a single sample) rather than a measure of the coliform concentration, although the USEPA also has approved quantitative measures of coliform density. For small systems that sample fewer than 40 times per month, any more than one coliform-positive sample is considered a violation of the MCL. Systems serving larger populations that consequently sample more frequently are allotted no more than 5% of samples to be positive for total coliforms. Some researchers have noted that the monthly frequency of water monitoring for small systems is too low to foresee a waterborne disease outbreak because most cases of fecal contamination occur as transient spikes rather than protracted high densities of fecal indicators (Craun *et al.*, 2002). During 1991–1998, only 22% of

CWS outbreaks and 9% of NTNCWS and TNCWS outbreaks had violated the USEPA's MCL for coliforms in the 12-month period before the outbreak (See Section 5.5).

If a routine sample is positive for total coliforms, then the culture is to be further tested for fecal coliforms or *E. coli*, and repeat sampling upstream and downstream of the positive site is performed 3–4 times within 24 hours (USEPA, 2001a). Samples then are taken at least five more times during the following month. The MCL violation is considered acute if fecal coliforms or *E. coli* are detected upon repeat sampling or if a fecal coliform/*E. coli*-positive routine sample is followed by a total coliform-positive repeat sample. In both cases, the public and state are to be notified of the MCL violation.

4.5. The Surface Water Treatment Rule

Concurrent with the TCR, the Surface Water Treatment Rule (SWTR) was promulgated in 1989 (USEPA, 1989b). The SWTR requires filtration and disinfection of all surface water systems and ground water systems in which the ground water interacts directly with surface water (*i.e.*, ground water under direct influence of surface water [GWUDI]). The GWUDI classification is determined if a microscopic particulate analysis (MPA) of the ground water indicates the presence of algae, rotifers, *Giardia*, or other microorganisms common to surface water (USEPA, 1992). Notably, this method may be flawed in assessing viral pathogen risk because the MPA indicators are 100–1,000 times larger than viruses and might be selectively filtered (Borchardt *et al.*, 2004).

The SWTR set MCLGs of zero for waterborne viruses, *Giardia lamblia*, and *Legionella*. In place of MCLs, the SWTR specifies treatment techniques (*e.g.*, filtration and disinfection) that translate to log reduction and/or inactivation "credits." The rule required *Giardia* to be reduced by 3 log (or 99.9%) and viruses to be reduced by 4 log (or 99.99%). Any log reduction of *Giardia* and viruses that is not achieved by filtration credit is accomplished by disinfection. If a water system employs alternative practices to remove pathogens, it can receive credits that translate to log reduction rates (USEPA, 1995). Filtered water turbidity must be maintained below 5 nephelometric turbidity units (NTU) in 100% of the samples and 0.5 NTU in 95% of the samples. Water systems can receive a waiver from filtration if:

(1) the source water demonstrates acceptable coliform levels and turbidity is less than 5 NTU;

(2) the disinfection plan is effective at reducing *Giardia* by 99.9% (3-log reduction) and viruses by 99.99% (4-log reduction); and

(3) a 0.2 mg/L disinfectant residual is maintained throughout the distribution system, which includes the pumping, piping, and storage networks.

Moreover, the system needs to have a watershed control program, demonstrate two or fewer coliform MCL violations in any given year, and have no regional history of waterborne disease. Coliforms are monitored at least weekly with increased monitoring for larger systems.

The Science Advisory Board announced in 1990 that drinking water contamination was one of the greatest current environmental risks and cited microbiological pollution as the

greatest health risk among the nation's potable waters (USEPA, 1990). In 1995, the USEPA put forth an "Agenda for Action" to protect drinking water against microbiological contaminants. The USEPA partnered with water suppliers to assess operations, maintenance, and management. However, the SWTR requirements for viruses are met on the basis of treatment alone; that is, there are no specific monitoring practices to verify that virus inactivation and/or reduction actually results from a treatment method. Depending on the source water quality, some utilities may be treating unnecessarily and other may be removing virus insufficiently.

4.6. The Information Collection Rule

In July 1997, the USEPA began an 18-month monitoring period for infectious bacteria, viruses, and protozoa in an effort to study the benefits and risks of disinfecting drinking water. Included in monitoring were water systems that use surface water and serve at least 100,000 people and water systems that use ground water and serve at least 50,000 people. For viruses, these systems were required to monitor their source water by inoculating buffalo green monkey kidney (BGMK) cells and assaying for virus replication. Systems finding more than one infectious enteric virus per liter were required to monitor finished water as well. Resulting information regarding pathogen contamination at specific water systems was made available to the public so that people could determine whether their local water quality utilities were functioning appropriately and comparably to other water quality utilities across the United States. The ICR reported that more than 80% of source waters were positive for virus during monthly sampling at

207 surface water treatment plants (Shaw *et al.*, 2003). Of a subset of plants monitoring for virus in finished waters, 16% detected virus at least once.

4.7. Enhanced Surface Water Treatment Rules

The Interim Enhanced Surface Water Treatment Rule (IESWTR), promulgated in December 1998, recognized that:

(1) disinfection reduced waterborne disease outbreaks but may inadvertently generate disinfection byproducts such as trihalomethanes and haloacetic acids that are deleterious to human and environmental health; and

(2) pathogens such as *Cryptosporidium* had emerged as resistant to chlorine disinfection leading to twelve outbreaks in the late 1980s and early 1990s (USEPA, 2002).

The IESWTR mandated that public drinking water systems using surface water or GWUDI and serving a population of 10,000 or greater must achieve a 2-log reduction of *Cryptosporidium* by filtration. The turbidity requirements were lowered from 0.5 to 0.3 NTU for 95% compliance and from 5 to 1 NTU for maximum turbidity with monitoring of individual filters rather than blended water.

The Stage 1 Disinfection/Disinfectant Byproduct Rule (D/DBP) simultaneously limited chlorine residuals and disinfectant byproducts in drinking water to increase protection from chemical exposure (USEPA, 1998). The Long Term 1 Enhanced Surface Water Treatment Rule (LT1), passed in January 2002 and effected in January 2005, extended the IESTWR to smaller water systems servicing fewer than 10,000 people.

The Long Term 2 Enhanced Surface Water Treatment Rule (LT2), published in January 2005, addressed the need for additional protection measures among public water systems at high risk for microbiological contamination where a 2-log reduction of *Cryptosporidium* may be insufficient. High-risk systems include all unfiltered systems and filtered systems with high levels of *Cryptosporidium* in the source water. Some of these water systems are required to enhance or add treatment processes to achieve an additional 1–2.5 log reduction of *Cryptosporidium*. Large, high-risk systems must monitor *Cryptosporidium* directly, whereas smaller, filtered systems are given the less expensive option to sample *E. coli* as an indicator of *Cryptosporidium*. Systems that employ the maximum level of treatment are exempt from monitoring. However, the USEPA acknowledged that the options for *Cryptosporidium* control are costly. They include covering reservoirs to protect source water, treating reservoir discharge, upgrading filters, and adding UV or ozone disinfection.

Concurrent with increased protection against *Cryptosporidium* contamination, extended exposure to high concentrations of disinfectant byproducts were identified as a health risk. Therefore, enhanced chlorine disinfection is not approved as a method to improve water quality. In addition, the Stage 2 D/DBP, published simultaneously with the LT2, increased the stringency of DBP compliance by requiring that individual monitoring locations in the distribution system remain below a specific DBP concentration average rather than allowing a treatment system to average all locations (USEPA, 2005).

4.8. The Ground Water Rule

To address public health issues in drinking water systems using ground water sources, the Ground Water Rule (GWR) was published in November 2006 and should be fully effected in 2009. The GWR focuses on the subset of ground water sources that are susceptible to fecal contamination and on mixed surface water and ground water systems in which the ground water is not treated. Private ground water wells are not included in the GWR and instead are the responsibility of individual homeowners.

Under the GWR, sanitary surveys and triggered source monitoring are required. Sanitary surveys are conducted by state regulators to determine the physical, managerial, and operational quality of their treatment systems. Triggered source monitoring involves sampling source water only if a system obtains a coliform-positive sample. Before the GWR was enacted, treated drinking water typically was monitored instead of source water (Yates, 2007). Triggered source monitoring applies only to systems that do not already achieve 4-log virus reduction and that cannot attribute the coliform-positive sample to a distribution system failure.

4.9. Current Drinking Water Quality Issues

The American Academy of Microbiology has reported that drinking water is not safe in terms of viral, bacterial, and protozoan pathogens (Ford and Colwell, 1996). More recently, the USEPA reported that 94% of the U.S. population was served by a CWS compliant with drinking water standards of treatment and source water protection (Reynolds *et al.*, 2008). However, an internal audit estimated that the actual value was

81% (USEPA, 2004). Populations that are not served by public water systems generally do not perform monitoring, and information is lacking from this demographic regarding exposure to pathogens (Reynolds *et al.*, 2008). As the concern about waterborne disease illness increases, regulatory frameworks based solely on monitoring bacterial indicators may need to be reassessed.

CHAPTER 5 –COLIFORMS AND VIRAL PATHOGEN RISK

The use of fecal indicators facilitates timely and cost-effective monitoring of water sources, whereas direct measurement of every known waterborne pathogen is not feasible as a monitoring strategy. During the past few decades, the USEPA has promulgated regulations based on coliform monitoring to assess the quality of the nation's drinking waters and to ensure that water systems are compliant with current, accepted treatment practices. In some instances, however, coliform indicators do not colocate with viruses or protozoa because of differences in size and resistance to environmental conditions and water treatment processes. Consequently, coliforms may be absent, suggesting that water is potable, even in the presence of viral or protozoan pathogens. Conversely, the natural occurrence or regrowth of coliforms in water sources (Caplenas and Kanarek, 1984) may imply a public health risk where none exists. Researchers have proposed alternative indicators, specifically for enteric viruses (See Section 6.2), but none have been adopted for widespread usage. Current research supports the inadequacy of bacteria as an indicator system for protozoa and viruses. The proposal of a more relevant viral indicator is the focus of this discussion.

5.1. Virology Primer

Viruses are astoundingly diverse and pervasive. In fact, for every organism on the planet, there exists one or more viruses that have evolved to infect it (Flint *et al*., 2004). Collectively, viruses are obligate intracellular parasites that exist extracellularly as colloidal particles ranging in size from 20–350 nm. The mature virus particle is composed of nucleic acid—RNA, DNA, or a combination of these—surrounded by a

proteinaceous capsid. The capsids of many viral species are icosahedral, although other geometries exist. In some cases, a host-derived lipid envelope surrounds the capsid. Exterior proteins projecting from the viral capsid or envelope often are amphoteric, which allows the virus to interact with cellular receptors and other substances over a range of pH levels.

The viral infectious cycle in a host cell includes binding, entry, replication of the viral genome, assembly of new particles, and egress (Flint *et al*., 2004). Depending on the virus species and host immune status, viral infections may be acute, latent, or persistent. An acute infection involves rapid replication in an index host and transmission to other hosts before immunological clearance or host death occurs. Viruses that induce latent infections may remain with the host for life, either integrating into the host cell genome or remaining unintegrated in the cell as a circular episome. A host infected with a latent virus may not show any symptoms of infection, but the viral genome may be triggered (*e.g.*, by host stress or illness) to replicate and generate progeny virions (*i.e.*, mature virus particles) for transmission to other hosts. Persistent viral infections last for long periods because the virus is capable of evading the immune system. For instance, the virus may circulate at extremely low titers or continually undergo mutation of its capsid proteins during a persistent infection. Alternatively, host immune dysfunction may allow a virus to infect persistently.

5.2. Enteric Viruses

Viruses from the families *Picornaviridae* (*e.g.*, hepatitis A virus and the enteroviruses, poliovirus, coxsackie virus, and echovirus), *Adenoviridae* (*e.g.*, enteric adenovirus), *Caliciviridae* (*e.g.*, the noroviruses: Norwalk and Norwalk-like virus), *Astroviridae* (*e.g.*, astrovirus) and *Reoviridae* (*e.g.*, rotaviruses and reoviruses) are classified as enteric viruses (Flint *et al.*, 2004). The genomes of most enteric viruses are composed of single-stranded RNA (ssRNA), although adenoviral genomes are double-stranded DNA (dsDNA). Enteric viruses are specialized to be transmitted via the fecal-oral route—infecting and replicating in the host gastrointestinal tract following ingestion of contaminated water or food.

Even at low exposure doses, enteric viruses may infect and manifest as gastroenteritis with diarrhea and/or vomiting (Abbaszadegan *et al.*, 1993; Griffin *et al.*, 2003; Fong and Lipp, 2005). In immunocompetent individuals, enteric virus infections are self-limiting because illness symptoms serve to purge virus particles from the host. However, for pregnant women, the elderly and very young, and the immunocompromised and immunosuppressed, these infections may lead to chronic or fatal secondary infections of the skin, respiratory system, and circulatory system as well as conjunctivitis, hepatitis, meningitis, encephalitis, and paralysis (Macler and Merkle, 2000; Fout *et al.*, 2003; Griffin *et al.*, 2003; Fong and Lipp, 2005; Gerba and Smith, 2005; Reynolds *et al.*, 2008; Table 5.1).

Table 5.1. Waterborne enteric viruses of public health concern and their associated illnesses. Adapted from Reynolds *et al*., 2008.

Virus Family	Virus Group/Species	Associated Illness(es)
Adenoviridae	Enteric adenovirus	Diarrhea, upper respiratory disease, eye infections, heart disease
Astroviridae	Astrovirus	Diarrhea
Caliciviridae	Noroviruses (e.g., Norwalk virus)	Diarrhea, flu-like symptoms, vomiting
Picornaviridae	Enteroviruses (e.g., poliovirus, coxsackie virus)	Diarrhea, fever, upper respiratory disease, meningitis, encephalitis, myocarditis, birth defects
	Hepatitis A virus	Hepatitis, liver damage
Reoviridae	Rotavirus	Diarrhea

Different virus species are associated with different infectious doses, measured in the laboratory as a particle-to-plaque forming unit (PFU) ratio. A plaque is an isolated region of cell death in culture; it indicates that a productive virus infection had occurred. The particle-to-PFU value indicates how many virus particles are necessary to initiate a productive infection in cell culture. The ratio translates to the exposure level that would be sufficient to establish an infection in a host organism. For poliovirus, the particle-to-PFU ratio ranges from 30 to 1,000, for adenovirus it ranges from 20 to 100, and for reovirus it is 10 (Flint *et al*., 2004). A high ratio may indicate that the virus is highly mutable and many nonviable progeny are created with each infectious cycle. It may also indicate that virus particles were unsuccessful to initiate a full infectious cycle, which is a very complex set of biochemical reactions. Alternatively, it may indicate that virus particles were damaged during replication or sample purification in the laboratory.

In general, the infectious dose for enteric viruses is very low, and in the case of rotavirus, may even approach one particle (Ward *et al*., 1986; Payment and Morin, 1989). This

indicates that exposure to extremely dilute enteric virus could lead to an infection (Leclerc *et al*., 2002). The infectious dose of most enteric bacteria (*e.g.*, *Salmonella*, *Shigella*, and *E. coli*) is remarkably higher than that of viruses, approaching 10^7–10^8 cells, although certain bacterial species can establish an infection upon host exposure to only a few hundred cells (*e.g.*, *Shigella* spp., *Campylobacter* spp., and *E. coli* O157:H7) (Leclerc *et al*., 2002). Although enteric bacteria exhibit a replication preference for the host intestine, they may multiply in receiving waters if temperature and nutrient conditions are favorable. Viruses, in contrast, are completely inert outside of a susceptible host.

Individuals with viral gastroenteritis may shed 10^5 to 10^{11} virus particles per gram of stool (Bosch, 1998; Leclerc *et al*., 2002). In raw sewage, 10^2 to 10^3 infectious enteric viruses (*i.e.*, PFU) may be detected per liter (Gerba *et al*., 1979; Leclerc *et al*., 2002). Primary and secondary wastewater treatment and disinfection may reduce the virus level to 0.6 PFU/L, and tertiary treatment may effectively eliminate virus altogether. However, tertiary treatment is uncommon (Azadpour-Keeley *et al*., 2003). In drinking water treatment facilities, researchers have demonstrated that detectable virus persists in finished waters (Payment and Armon, 1989).

Although no virus is capable of replication outside of a host cell, infectious enteric virus particles may persist in the environment for long periods. Once excreted, enteric viruses have been found to remain infectious for 130 days in seawater, 120 days in fresh water and sewage, and 100 days in ambient soil (Fong and Lipp, 2005). Whereas many

waterborne pathogens are endemic among cattle herds and poultry flocks and can be transmitted to humans as zoonotic infections (*e.g.*, *Cryptosporidium*, *Campylobacter*, *Salmonella*, *Listeria*, *E. coli* O157:H7, and *Giardia*) (Gerba and Smith, 2005), enteric viruses are specialized to exist in human hosts and, in most cases, only originate from sources of sewage (Reynolds *et al.*, 2008).

5.3. Detection of Viruses in Environmental Waters

Viruses were first isolated from water sources in the late 1930s. Scientists measured enteroviruses, specifically poliovirus, in feces and wastewater (Bosch, 1998; Griffin *et al.*, 2003). Viruses were gathered and concentrated by passing water through a gauze pad. The crude sample subsequently was inoculated onto a culture of monkey kidney cells. As interest in environmental virology blossomed and virus recovery methods improved between the 1960s and the 1980s, researchers discovered that rotavirus and norovirus also contaminate water sources (Metcalf *et al.*, 1995; Griffin *et al.*, 2003).

In 1970, the American Society of Civil Engineers concluded that the methods for identifying and quantifying viruses in water were inadequate (Hill *et al.*, 1971). Cell culture was the only assay for virus detection in environmental waters, despite the existence of immunological assays used routinely to identify viruses in a clinical setting. The extremely dilute concentration of virus in environmental samples precluded less sensitive immunological detection methods. The only alternative was electron microscopy, which required much more technical expertise than cell culture (Griffin *et al.*, 2003), or assays employing nucleotide hybridization or antibody-based

detection. For these reasons, cell culture was the most widely used assay for virus detection until the 1990s (Abbaszadegan *et al*., 1993) when molecular methods such as polymerase chain reaction (PCR) were introduced (See Section 7.2).

5.4. Correlation Among Indicator Bacteria and Enteric Viruses

For bacterial indicators and viral pathogens to correlate in the environment and in treatment systems, the indicators would have to occur exclusively with pathogenic viruses in feces and exhibit identical resistance and susceptibility responses to environmental stressors, filtration, and disinfection. Although a number of researchers have reported correlations among bacterial indicator density and enteric viruses (Bergeisen *et al*., 1985; Lawson *et al*., 1991; Gersberg *et al*., 2006), many others have failed to find a strong association between bacteria and viruses in surface water, ground water, and treatment systems (Gerba *et al*., 1979; Labelle *et al*., 1980; Payment and Armon, 1989; Havelaar *et al*., 1993; Nasser *et al*., 1993; Scandura and Sobsey, 1997; Nasser and Oman, 1999; Borchardt *et al*., 2004; Jiang and Chu, 2004; Skraber *et al*., 2004a).

It is now widely recognized that traditional indicators such as total coliforms, fecal coliforms, and *E. coli* do not respond to environmental conditions or treatment processes in the same manner as human enteric viruses (Gerba *et al*., 1979; Griffin *et al*., 1999; Jiang *et al*., 2007; Borchardt *et al*., 2004; Francy *et al*., 2004). Coliform bacteria are more susceptible than enteric viruses to conventional disinfection chemicals such as chlorine, coagulants such as alum, and extremes in pH, salinity, and temperature

(Metcalf *et al*., 1995; Bosch, 1998; Fong and Lipp, 2005). Bacteria, which are 2.5- to more than 100-fold larger than viruses, are more easily filtered in engineered filtration systems and some natural aquifers (Macler and Merkle, 2000; Azadpour-Keeley *et al*., 2003; Reynolds *et al*., 2008). Ultimately, bacterial indicators may expire or be removed from water sources at different rates than viral pathogens, giving rise to viral outbreaks in water supplies that had been deemed acceptable by fecal coliform counts (Fong *et al*., 2005; See Section 5.5) or inappropriately indicating a public health risk in the absence of pathogenic viruses. This lack of correlation has led researchers such as Nwachuku and colleagues (2002) to conclude that monitoring as regulated by the TCR is an inadequate rubric to protect public health. However, alternative indicators for enteric viruses have not been adopted.

5.4.1. Surface Water

Enteric viruses in wastewater may contaminate surface waters used for drinking, recreation, and fish harvesting. Ninety percent of treated wastewater is discharged to marine surface waters in the United States as a result of coastal development. This translates to 10^{10} gallons of treated wastewater entering coastal waters daily (NRC, 1993), some of which is not disinfected (Griffin *et al*., 2003). Analyses of virus concentrations measured by cell culture have detected 1.82×10^2 to 9.2×10^4 particles/L in untreated wastewater and 1.0×10^{-3} to 1.0×10^2 particles/L in treated wastewater or polluted surface water (Rose, 1986; NRC, 1993; Rose *et al*., 1996). Because enteric viruses have been found to remain infectious for 130 days in seawater and 120 days in

fresh water and sewage (Fong and Lipp, 2005), the impact of this virus load must be monitored accurately.

Griffin *et al.* (1999) ranked 19 water samples obtained from the canals of the Florida Keys in terms of the presence of bacterial indicators such as *Enterococcus* spp. At the same sites, these researchers assayed for enterovirus, hepatitis A virus, and Norwalk virus and compared the water quality rankings to the presence or absence of virus. Only three sites exceeded guidance levels set by the USEPA for enterococci; however, at 18 of 19 sites, enterovirus, hepatitis A virus, and/or Norwalk virus genomes were detected by reverse-transcriptase (RT)-PCR (See Section 7.2) and dot blot hybridization. In coastal waters off western Florida, six watersheds were sampled for fecal coliforms, enterococci, and *Clostridium perfringens* and compared to the presence of infectious enteroviruses (Lipp *et al.*, 2001). Bacterial indicator densities varied (from less than 10 to 4488 cfu/100 mL) and at some sample sites were elevated significantly over others as determined by one-way ANOVA. In contrast, infectious enteroviruses were detected at low levels between 0.17 and 0.59 infectious units/100 L in five of six watersheds. No significant difference in virus concentration was detected among the watersheds. Cluster analysis demonstrated that no indicator density threshold existed above which virus was always detected; instead, viruses were detected at both "low risk" and "high risk" sites.

Total and fecal coliforms incorrectly estimated viral pollution in source river water used for drinking water production. Using regression analysis, Skraber *et al.* (2004b) showed that, in water samples collected during the winter (held at 4, 18, or 25°C), infectious

poliovirus survived up to 1.5-fold longer in river water than fecal coliforms. The opposite was observed in summer waters (held at 4, 18, or 25°C) when infectious poliovirus exhibited almost 2-fold faster die-off than fecal coliforms. These authors reported that although the seasonal composition of river water samples significantly affected survival rates, the temperatures at which the samples were held did not. In the urban rivers and creeks of southern California, investigators observed that fecal indicator bacteria and other water quality indicators did not correlate with adenovirus, enterovirus, or hepatitis A virus genetic material (Jiang and Chu, 2004). When researchers ranked the sampling sites in terms of bacterial water quality indicators, the lowest-quality site was free of virus, whereas the second highest-quality site was positive for virus.

Virus decay is defined by: (1) virus removal through loss of infectivity; (2) virus removal (*i.e.*, filtering) by irreversible attachment to sediments and settling; and (3) virus retardation by reversible attachment to sediments and release (Schijven and Hassanizadeh, 2000). Total decay rates (k_D) for enteric viruses and coliform bacteria have been measured and reported in the primary literature (reviewed in Azadpour-Keeley *et al.*, 2003). Decay rates vary based on environmental conditions and experimental methods, but similar measurement methods generally indicate that bacteria degrade with different kinetics than viruses. For instance, a comparison of the survival of indicator bacteria, MS2 male-specific coliphages (*i.e.*, the bacteriophage subgroup that infects coliform bacteria via the "male" F-pilus), and enteric human viruses in river water demonstrated that coliphage survival was similar to that of poliovirus 1 (Springthorpe *et*

al., 1993); however, *E. coli* had variable survival rates and a potential for regrowth, making it unsuitable as an indicator of virus presence or absence.

5.4.2. Ground Water

Until recently, ground water was considered a more protected source of drinking water, often requiring no treatment (Azadpour-Keeley *et al*., 2003). Experts believed that ground water was free of pathogens because of the natural filtration capacity of the subsurface and the extended distance a microorganism would have to cross to reach the water table (Amundson *et al*., 1988). Moreover, even if it became contaminated, experts hypothesized that ground water would be purified as it flowed through the aquifer because pathogens would be subject to microbial protease and nuclease activity and stagnation (Cliver and Herrmann, 1972; Dizer *et al*., 1984; Azadpour-Keeley and Ward, 2005). Current research has challenged this assumption, and the USEPA now estimates that 168,000 viral illnesses occur each year because of improperly treated ground water sources of drinking water (USEPA, 1996). In addition, the current regulatory approach of treating GWUDI as more likely to be contaminated than ground water that does not interact with surface water (*i.e.*, the SWTR) may be incorrect. Researchers have observed viruses in ground water wells regardless of the level of surface water contribution (Borchardt *et al*., 2004).

Depending on the hydrogeological settings and climate, viruses can be introduced into ground water via failed septic systems, underground storage tank and sewer line leaks, sewage lagoons, pit latrines, and landfill leachates (Macler and Merkle, 2000; Azadpour-

Keeley *et al*., 2003; Gessel *et al*., 2004). Viruses also can infiltrate ground water when partially treated and untreated wastewater and solids are discharged to the land (Azadpour-Keeley and Ward, 2005). Sludge generated during wastewater treatment may contain viruses associated with suspended solids; these may adsorb to crops or percolate to ground water when sludge is land-applied (Metcalf *et al*., 1995; Gerba and Smith, 2005). Viruses have been reported to persist 100 days in soil (Fong and Lipp, 2005) so percolation of infectious virus to ground water is possible even if a precipitation event does not happen immediately. In contrast, in dry soil that had been land-applied with manure, the bacteria *Salmonella anatum* persisted only 27 days (Johnston *et al*., 1996). This bacterial species as well as fecal coliforms persisted only 6–10 days in the soil-runoff mixing zone after manure application (Gessel *et al*., 2004). Others have reported that although bacteria may persist for up to 1 year in soil, they generally survive no longer than 2 months. In contrast, viruses commonly survive for 3 months but may persist for up to 6 months (Gerba and Smith, 2005). Primary and secondary treatment followed by disinfection can lower the concentration of viral contamination to negligible levels. However, wastes destined for land application rarely undergo these processes. As with drinking waters, viral pathogens in treated sewage are not measured directly; rather, virus risk is monitored by bacterial indicators (Griffin *et al*., 2003; Gerba and Smith, 2005).

Bacteria are more easily filtered than viruses through some natural aquifers (Macler and Merkle, 2000; Azadpour-Keeley *et al*., 2003). Research has demonstrated that in ground water systems, viruses are generally more mobile and persistent than indicator bacteria (Bitton *et al*., 1983; Scheuerman *et al*., 1987; Nasser and Oman, 1999; Pang *et al*., 2004).

In particular, sand, sand/gravel mixtures, and fissured limestone with larger pore sizes are likely to facilitate virus transport but retard bacterial transport. (Metcalf *et al.*, 1995; Woessner *et al.*, 2001; Abbaszadegan *et al.*, 2003; Azadpour-Keeley *et al.*, 2003). Scandura and Sobsey (1997) found that norovirus is poorly filtered by many soil types. Thus, virus may pass to water intakes despite coliform bacteria being filtered out. Conversely, *E. coli* exhibited a larger velocity than MS2 male-specific coliphage through alluvial aquifers (Sinton *et al.*, 2000). MS2 was used as a model for enteric viruses because it approximates Norwalk virus in size (Havelaar *et al.*, 1993). In this case, the extremely small pores in the aquifer size-excluded *E. coli*, but MS2 were small enough to be trapped in the pores, thus slowing their relative velocity. Nasser and Oman (1999) demonstrated that hepatitis A virus and poliovirus 1 exhibited similar inactivation patterns in ground water and wastewater effluents at various temperatures. In constrast, *E. coli* were inactivated at significantly faster rates in ground water at 4 and 37°C and at lower temperatures were inactivated faster regardless of water type.

The interaction of viruses with substrates governs the persistence and extent of virus transport in the subsurface. Viruses adsorb to and detach from aquifer sediments via electrostatic interactions and hydrophobic effects. Exterior proteins projecting from the viral capsid or envelope are amphoteric, which allows the virus to interact with substances in the subsurface over a range of conditions (Flint *et al.*, 2004; Azadpour-Keeley and Ward, 2005). For most viruses, exterior proteins carry a net surface charge of zero between pH 3 and 7. Between pH 4 and 9, most sediments are negatively charged. If a particular virus has zero net charge at pH 6, then at pH 5, it would have a net positive

charge and would adsorb to sediments in the aquifer. In this situation, transport would be diminished (Azadpour-Keeley *et al*., 2003). The buffering capacity and high organic content of clays and sandy loams contribute to virus retention by providing hydrophobic surfaces to which viruses adsorb (Dizer *et al*., 1984; Kinoshita *et al*., 1993; Azadpour-Keeley *et al*., 2003). Consequently, these substances are likely to hinder virus transport.

Adsorption of viruses to soils and retardation of transport should not be equated with virus inactivation. Rather, adsorption to solids may increase virus persistence by shielding infectious particles from degradation via microbial proteases and nucleases (Bosch, 1998; Fong and Lipp, 2005). The cool temperatures of soil also are favorable to virus persistence because they aid in maintaining capsid protein integrity. Virus sorption to soils is reversible under certain ionic and pH conditions, and the actions of percolating virus-free water can promote virus release (Bales *et al*., 1993). In the case of poliovirus, reversible sorption from clay resulted in the release of infectious particles (Carlson *et al*., 1968). Under some pH and hydrophobicity conditions, sandy soils can release virus for days to weeks into virus-free water (Kinoshita *et al*., 1993). *E. coli*, by comparison, is inactivated 10 times faster than poliovirus in ground water (Bitton *et al*., 1983).

Locas *et al*. (2007) sampled ground water from wells across Quebec, Canada, and reported that total coliforms were always present in conjunction with culturable enteric viruses but that in two instances, infectious enteric viruses were present in the absence of the fecal indicators *E. coli* and enterococci. Of four well sites that were negative for all fecal indicators and total coliforms during a year of sampling, norovirus RNA was

detected twice by RT-PCR. The infectivity of this virus could not be ascertained because a cell culture system was not available. These investigators also reported that total coliforms and enterococci were regularly isolated from sites that were absent of culturable enteric viruses and norovirus RNA, suggesting that these bacterial indicators were inadequate at correlating with either the presence or the absence of virus contamination.

In a Wisconsin study of municipal ground water well contamination, enteric viruses such as enteroviruses, rotavirus, hepatitis A virus, and norovirus were detected by RT-PCR in 50% of wells prior to chlorination at the wellhead (Borchardt *et al.*, 2004). Infectious hepatitis A virus also was confirmed by cell culture in 3 of the 48 wells analyzed. Total coliform bacteria, *E. coli*, and fecal enterococci were absent from all 48 wells. A survey of 448 ground water utility wells across 35 states reported that enterovirus, rotavirus, and/or hepatitis A virus genetic material was present in 32% of ground water wells, suggesting that whether or not the viruses were viable, the well waters were exposed to virus contamination (Abbaszadegan *et al.*, 2003). Fout *et al.* (2003) observed that 21 of 29 U.S. utility wells were positive for enterovirus, reovirus, norovirus, or hepatitis A genetic material.

5.4.3. Water Treatment Systems

In a treatment system, positive coliform results may be obtained as a result of a variety of water system deficiencies. Treatment processes may be inadequate for removal or inactivation of coliform bacteria, which may include inadequate primary disinfection,

filter breakthrough, or loss of disinfectant residual within the distribution system (McFeters *et al.*, 1986; Hrudey *et al.*, 2003). Even when a treatment system is operating properly, however, contaminated water may enter a distribution system through leaks, water main breaks, or repairs conducted without disinfectant flushing (Geldreich *et al.*, 1992).

The passage of coliforms through drinking water treatment plants does not correlate to the passage of viral pathogens. Several researchers have concluded that the differential susceptibilities of viruses and bacteria to water treatment processes make the sole use of bacterial indicators inappropriate (Azadpour-Keeley *et al.*, 2003; Fong and Lipp, 2005; Yates, 2007). The ability of disinfection, coagulation, clarification, and filtration to eliminate bacteria was well established in the earlier part of the twentieth century (Logsdon, 1990). However, inactivation and removal of viruses through treatment systems is more challenging (Nasser *et al.*, 1995).

In terms of disinfection, Sobsey (1989) reviewed the characteristics that make traditional indicators more sensitive to disinfection than enteric viruses. Free chlorine is known to inactivate coliforms and pathogenic bacteria (USEPA, 1989b; Johnson *et al.*, 1997; Rice *et al.*, 1999). However, doses of chlorine that inactivate bacteria are inadequate for hepatitis A virus, enteroviruses, rotavirus, and noroviruses (Melnick *et al.*, 1978; Keswick *et al.*, 1985; Bosch *et al.*, 1991), possibly because these viruses are protected by aggregation and association with submicron-sized particles in wastewater (Hejkal *et al.*, 1981). For instance, in 1984, residents of Braun Station, Texas, who were served by a

chlorinated artesian well experienced a Norwalk virus outbreak. Although the untreated well water had coliform levels up to 2,600 cfu/100 mL, tap water samples were negative for coliforms (D'Antonio *et al.*, 1985). This outbreak highlights the fact that chlorine, while effective for inactivating coliforms, may not be adequate for viruses. UV disinfection also does not inactivate bacteria and viruses identically. Chang *et al.* (1985) observed that poliovirus and rotavirus require approximately 3 and 4 times greater fluence than *E. coli* for 99.9% inactivation. Adenoviruses, which have stable, dsDNA genomes, also have demonstrated resistance to UV disinfection (Reynolds *et al.*, 2008).

Under the SWTR, direct filtration receives less log credit for removal of viruses than for removal of protozoa. Only 1–2 log reduction of enteric viruses through conventional treatment would be expected for properly operating filters (Havelaar *et al.*, 1995) compared to 5-log reduction of protozoa (Nieminski and Ongerth, 1995). Ultrafiltration and microfiltration membranes can provide an absolute barrier to bacteria and protozoa (Jacangelo *et al.*, 1991). In contrast, virus removal through membranes varies depending on the type of membrane used. For instance, whereas an ultrafiltration membrane with a nominal pore size of 0.01 μm can provide more than 6.5-log removal of MS2 coliphage, less than 1-log removal of MS2 was demonstrated for three different microfiltration filters with nominal pore sizes ranging from 0.1 to 0.2 μm (Jacangelo *et al.*, 1995).

Payment and coworkers (1985) studied the decreases in concentrations of various fecal indicator bacteria—including total coliforms, fecal coliforms, and fecal streptococci—and viruses throughout sequential treatment processes at seven water treatment systems

across Montreal, Canada. These authors reported that raw water contained 10^5 to 10^6 cfu/L of total coliforms and 3.3 most probable number of cytopathogenic units (MPNCU) of virus per liter. In finished water, indicator bacteria were uniformly absent; however, infectious viruses were detected in 11 of the 155 finished water samples (7%). Throughout treatment steps, these investigators detected infectious poliovirus, coxsackie virus, echovirus, and poliovirus. Whereas sedimentation followed by filtration removed 95.15% and 99.97% virus, respectively, ozonation and final chlorination were not effective at removing the remaining infectious virions. Moreover, the 6-log reduction of indicator bacteria did not correlate with the 4–5 log reduction of virus. Instead, the density of virus particles in finished waters tended to correlate with the virus density in raw source waters. Source waters containing high concentrations of virus tended to give rise to finished water containing residual virus despite being deemed potable by the absence of indicator bacteria (Payment *et al*., 1985). Similarly, Keswick *et al.* (1984) tested concentrated raw, clarified, filtered, and chlorinated finished drinking water samples derived from heavily polluted source water for viruses and total coliform bacteria. They reported that during dry conditions, four of nine finished water samples were positive for infectious viruses. In contrast, none of these samples contained detectable coliforms.

Rather than underestimating the presence of viral pathogens, coliforms sometimes may overestimate pathogenic contamination by occurring independently of fecal pollution. Drinking water distribution systems may test positive for coliforms because of regrowth of microorganisms that were injured through treatment but remained viable

(LeChevallier, 1990). Alternatively, sloughing of biofilms in a distribution system can reintroduce microorganisms, including coliforms. In these cases, a coliform assay would give a positive result in the absence of recent fecal pollution. Such a result may require a water treatment system to incur unnecessary costs.

5.5. Coliform Prediction of Waterborne Disease Outbreaks of Viral Etiology

Since 1971, the Centers for Disease Control and Prevention (CDC), the USEPA, and the Council of State and Territorial Epidemiologists have consolidated and maintained the Waterborne Disease Outbreak Surveillance System, which compiles voluntarily reported data on waterborne disease outbreaks. For an event to be classified as a waterborne disease outbreak, two or more people must experience the same or similar symptoms of illness (Blackburn *et al.*, 2004). This stipulation can be waived and a single case can be considered an outbreak if the case is laboratory-confirmed primary amebic meningoencephalitis or if it is a case of chemical poisoning confirmed by water quality monitoring.

Public health departments across the nation are expected to detect and investigate outbreaks in their localities and report outbreaks to the surveillance system. In addition to reporting an outbreak, public health departments may provide water quality data in which the water supply implicated in the outbreak is analyzed for total coliforms, fecal coliforms, *E. coli*, and/or a suspected infectious agent of bacterial, parasitic, or viral etiology. Because surveillance is passive and reporting is not mandated, the waterborne disease outbreak incidence compiled by the surveillance system is predicted to be a gross

underestimate (Craun *et al*., 2002; Craun *et al*., 2006). Craun (1990) suggested that as little as one-tenth to one-half of waterborne disease outbreaks in the United States are reported.

Public health breakdowns take place even when coliforms are monitored. Disease outbreaks have occurred in water supplies for which bacterial indicator levels were within regulatory compliance. For instance, enteric viruses were isolated from water samples during a hepatitis outbreak at a military camp, even though the samples were consistently free of indicator bacteria (Bosch *et al*., 1991). Conversely, cases of indicator counts exceeding the public health risk threshold are not always associated with subsequent disease outbreaks (Craun *et al*., 1997; Hrudey and Hrudey, 2007).

The implementation of a more accurate indicator of virus presence or absence is becoming even more crucial as sensitive subpopulations are growing larger (Reynolds *et al*., 2008). Sensitive subpopulations include those who are more susceptible to severe illness or death from enteric virus infections, such as the elderly, organ transplant patients, and HIV/AIDS patients. This demographic currently totals 20–25% of the U.S. population. Consequently, future waterborne disease outbreaks are expected to have an even greater impact on public health (Craun *et al*., 2006).

From 1971–2002, viral pathogens were confirmed to cause 8% of the 764 reported waterborne disease outbreaks in the United States (Reynolds *et al*., 2008). However, 47% of these outbreaks were reported as unknown acute gastrointestinal illness, and many of

these mimicked a viral etiology (Leclerc *et al*., 2002). It is likely that greater than 8% of outbreaks result from viral agents because (1) nonculturable or slow-replicating viruses may have been unrecognized as the causative agents, and (2) public health utilities often omit virus testing even if implicated water supplies are negative for pathogenic bacteria and protozoa (Craun *et al*., 2002; Reynolds *et al*., 2008). Some researchers have suggested an upper bound of 19.5 million cases/year of viral illness associated with drinking water in the United States (Reynolds *et al*., 2008).

From 1991 to 1998, 35 reports of outbreaks of viral or unknown etiology included water quality data. These data indicated that 81% of viral or unknown outbreaks among all water systems co-occurred with elevated coliform counts (Craun *et al*., 2002). Of the two outbreaks in CWS in which a viral etiology was determined, coliforms were not detected in the finished water (0% correlation). Of the five CWS outbreaks of unknown etiology, coliforms were only detected for three (60% correlation). Notably, total coliforms were detected in 100% of the outbreaks in which a bacterial pathogen was the causative factor. This suggests that putative indicators may be best suited for use with pathogens of similar sizes and infectious cycles. That is, bacterial indicators may be most likely to colocate with bacterial pathogens, viral indicators with viral pathogens, and protozoan indicators with protozoan pathogens.

Whereas the proportion of waterborne disease outbreaks related to contaminated, untreated surface waters has decreased since 1971, the proportion related to untreated ground water has remained relatively constant (Craun *et al*., 2006). Aquifers act to

naturally filter bacterial pathogens but are more susceptible to virus infiltration (See Section 5.4.2). For instance, Scandura and Sobsey (1997) reported that norovirus is poorly filtered by most soil types, which could allow passage of this virus species to the water table. Indeed, of the five outbreaks from 2001 to 2002 in which norovirus was confirmed to be the causative agent, all were associated with ground water systems (Yoder *et al.*, 2004).

Deficiencies related to treatment systems have decreased since the late 1990s. However, distribution system contamination (both microbial and chemical) has become a greater concern (Reynolds *et al.*, 2008). Of the one million miles of distribution networks in the United States, 20% are considered to be in poor working order (American Water Works Service Company, 2002) leading to an increase in water main breaks. Lower levels of disinfectant residuals are maintained in the distribution system to limit DBP production, and pressure fluctuations in the systems can cause back siphonage and cross-contamination of nonpotable and potable water. For instance, a cross-connection from a nonpotable, untreated pond led to a norovirus outbreak in the reporting period 2003–2004 (Liang *et al.*, 2006). Moreover, low or negative pressure could draw in untreated ground water if leaky distribution piping exists below the water table. Disease outbreak statistics indicate that current water treatment regulations have not been effective at reducing the number of distribution system-related outbreaks (Reynolds *et al.*, 2008; Craun and Calderon, 2001; Levy *et al.*, 1998).

During the summer of 2004, a waterborne disease outbreak occurred in Ohio, affecting approximately 1,450 people with gastroenteritis (Fong *et al*., 2007). A mixture of bacterial, viral, and parasitic pathogens were isolated from patients. A subsequent investigation of total coliforms, *E. coli*, enterococci, and *Arcobacter* indicated substantial contamination of untreated well water. The contamination likely was caused by interactions between ground water and surface water resulting from overflow from wastewater treatment plants and septic tanks during an extreme precipitation event. Notably, the wells containing the highest densities of total coliforms (90 and 38 cfu/100 mL) were not associated with virus contamination. Similarly, the wells containing the highest *E. coli* densities (4 and 2.6 cfu/100 mL) were negative for enteric viruses. Norovirus was cultured from infected patients, but was not isolated from the well water implicated as heavily contaminated by coliforms. This led Fong *et al*. (2007) to conclude that bacterial indicators may colocate with viral pathogens in some instances, but candidates from their suite of bacterial indicators often occurred in the absence of viral pathogens (14 of 16 samples) leading to a high proportion of false-positives.

Figure 5.1 depicts the etiologies of reported waterborne disease outbreaks between 1930 and 1996 (Leclerc *et al*., 2002). The earliest data suggest that bacterial outbreaks were by far the most commonly reported, possibly because viral and parasitic disease courses were less well understood at the time. Consequently, a bacterial indicator was most reasonable to signify the presence of pathogens. Since 1930, however, outbreaks of bacterial etiologies have plummeted as a result of filtration and disinfection treatments. In contrast, outbreaks of unknown etiologies—which often are suspected to be virus-

caused—and protozoan outbreaks both exhibit more erratic patterns. Detection of coliform bacteria is effective at indicating bacterial contamination (Craun *et al.*, 2002), and Figure 5.1 supports the presumption that controlling coliform levels has, in turn, controlled levels of pathogenic bacteria. However, evidence that pathogenic outbreaks of nonbacterial etiologies can be predicted or controlled by monitoring coliforms is much less clear. The results instead support the development and use of indicators specific to nonbacterial pathogens. Regarding viral outbreaks, the most logical indicator would be a ubiquitous, nonpathogenic, seasonally consistent virus that exhibits the same transport and resistance characteristics as pathogenic enteric viruses.
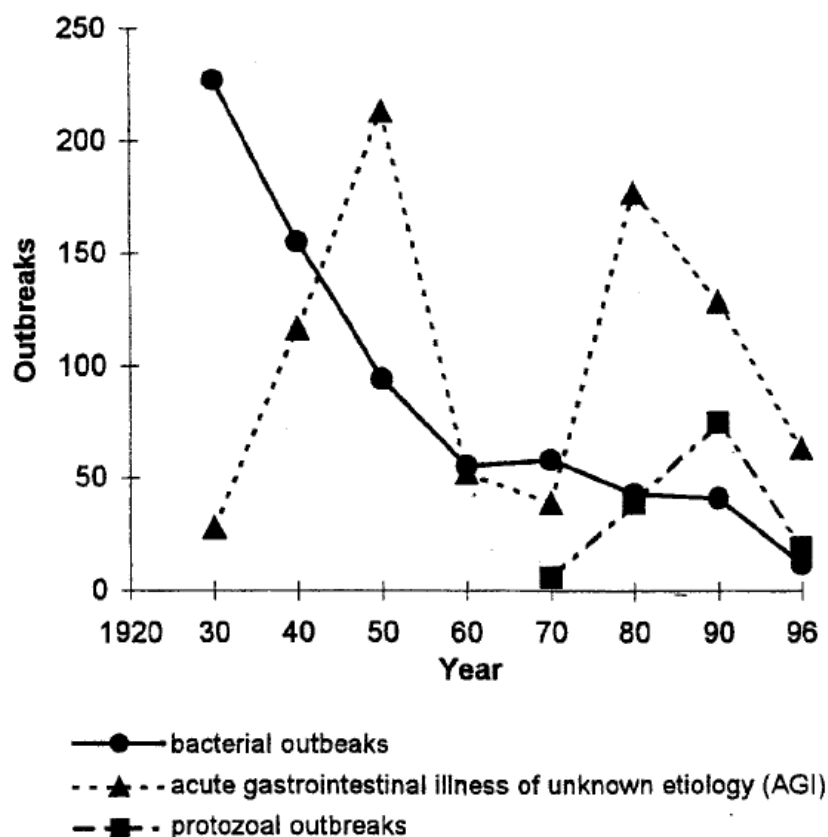
Figure 5.1. Historical depiction of the etiologies of waterborne disease outbreaks in the United States. Reproduced with permission from Leclerc *et al.*, 2002.

CHAPTER 6 – ALTERNATIVES TO COLIFORMS: INDICATOR VIRUSES

Reports finding a lack of correlation between bacterial indicators and viral pathogens as well as the occurrence of waterborne disease outbreaks without concomitant coliform elevation support the argument that bacteria are incapable of always indicating virus contamination in source waters, treatment systems, and finished waters. As an alternative, coliphages and representative enteric virus species have been proposed as putative indicators of pathogenic viruses.

6.1. Coliphages

The coliform viruses, coliphages, have been investigated as possible fecal indicators since the 1980s (Osawa, 1981; Furuse, 1987). In particular, coliphages appeared to be well suited to indicate enteric viruses in ground water systems based on similarities in environmental survival (Donnison and Ross, 1995; Long and Sobsey, 2004; USEPA, 2006) and size (Abbaszadegan *et al.*, 2003). The size similarity between enteric viruses and coliphages is particularly important when transport through an aquifer is considered. However, there are shortcomings associated with using coliphages as an indicator organism, and some researchers have recommended coliphage monitoring in conjunction with, but not in place of, coliform monitoring (Long and Dewar, 2008). Unlike enteric viruses, coliphages may continue to replicate in surviving bacterial hosts after being shed in feces (Havelaar and Pot-Hogeboom, 1988; Nasser and Oman, 1999). Indeed, researchers have observed a proliferation of coliphages in sewage water (Snowdon and Cliver, 1989; Borrego and Cornax, 1990; Armon and Kott, 1995). Consequently, coliphage quantities and persistence in environmental waters may significantly exceed

the quantities of human enteric viruses and incorrectly suggest contamination of water sources (Nasser and Oman, 1999; Pang *et al*., 2004). Alternatively, coliphages associated with bacterial hosts may be removed when bacteria are filtered during water treatment or passage through an aquifer. Consequently, coliphages may be absent despite the presence of enteric viruses.

A number of publications have supported the argument that coliphages alone are not ideal indicators of enteric viruses. For instance:

(1) Following a massive outbreak of viral, bacterial, and parasitic etiologies affecting 1,450 people in Ohio during 2004, coliphages were isolated from untreated well water, but neither total coliphages nor male-specific coliphages colocated exclusively with human adenovirus (Fong *et al*., 2007). Contaminated ground water samples were obtained that were either negative for total or male-specific coliphage and positive for adenovirus (two well sites) or were positive for total or male-specific coliphage but negative for adenovirus (four well sites). At nine well sites, both adenoviruses and coliphages were undetected.

(2) A ground water study in Canada reported that culturable human enteric viruses occurred in 2 of 12 untreated well sites that were free of male-specific coliphages (Locas *et al*., 2007).

(3) In Wisconsin, researchers sampled four GWUDI wells monthly for 1 year to detect viral genetic material or culturable, infectious viruses. They reported virus presence in 24 of 48 samples obtained prior to chlorination at the wellhead. In contrast, neither male-specific nor somatic coliphages (*i.e.*, the subset of

coliphages that infects bacteria via the cell membrane) were enumerated in any of the well samples (Borchardt *et al.*, 2004).

(4) Coliphage survival in soil after manure application varied by coliphage species. Whereas male-specific coliphages exhibited similar die-off to fecal coliforms (6–10 days), somatic coliphages persisted as long as 143 days (Gessel *et al.*, 2004).

(5) In Barcelona, Spain, a study of bacteriophage survival through drinking water treatment processes reported that somatic coliphages, male-specific coliphages, and bacteriophages infecting the intestinal bacterium *Bacteriodes fragilis* were removed at different treatment stages (Jofre *et al.*, 1995). Enteric viruses existed in the treatment plants at extremely low numbers and were not correlated with bacteriophage survival.

6.2. Human Enteric Viruses

Some researchers have suggested using one enteric virus species to indicate other enteric pathogens (Kopecka *et al.*, 1993; Metcalf *et al.*, 1995). Adenovirus has been proposed as an indicator because of its remarkable resistance characteristics and lack of seasonal variability. However, this virus did not correlate with hepatitis A virus or enteroviruses in urban waterways (Jiang, 2002). Other pathogenic enteric viruses exhibit epidemiological patterns, occurring epidemically in short bursts or with seasonal fluctuations. For instance, enterovirus infections peak in summer or fall (Skraber *et al.*, 2004a; Tani *et al.*, 1995) and noroviruses and reoviruses in winter (Tani *et al.*, 1995; Haramoto *et al.*, 2006). This makes it difficult to assign a single pathogenic indicator to the global, year-round enteric virus population (Diniz-Mendes *et al.*, 2008). In addition, Skraber *et al.* (2004a)

were unable to culture enteroviruses, and a facile culture system is not available for norovirus. Thus, infectivity of these proposed indicators cannot be assessed, and therefore the utility of such an indicator in treatment systems would be questionable. Other enteric viruses are highly correlated with socioeconomic status (*e.g.*, hepatitis A virus), giving rise to endemic conditions in regions with poor sanitation regardless of the degree of drinking water contamination (Fernandez-Molina *et al.*, 2004).

Given these caveats, an enterically transmitted virus that is neither seasonally nor demographically distributed and that is shed in large quantities without pathological consequences or immune clearance may best serve as an indicator of enteric viruses.

CHAPTER 7 – METHODS FOR DETECTING VIRUSES IN ENVIRONMENTAL WATERS

Ideally, indicator bacteria inform water quality officials about breaches in water treatment systems or contamination of water sources before a public health crisis occurs. However, total and fecal coliforms and *E. coli* can be misleading indicators of viral pollution. If a virus species is to be used as an indicator of other viruses, then sensitive, specific, and robust tools must be available to assess the presence or absence of the indicator.

The two primary methods in practice today for virus identification are cell culture and PCR. Susceptible and permissive cells in culture, when inoculated with infectious virus, may manifest signs of cell dysfunction or death and may release replicated virus progeny into the culture supernatant. The lack of morphological changes in the culture suggests that the virus preparation was not infectious. When compared to molecular methods, cell culture is time-consuming, costly, less sensitive (sensitivity is a function of the particle-to-PFU ratio; See Section 5.2), and requires highly developed skills and sterile technique. It generally takes 1–3 weeks to assay for infectious virus by this method.

PCR involves the enzyme-catalyzed amplification of a specific region of a DNA template. PCR is rapid, sensitive, specific, cost-effective, and simple to perform. Results are obtained within hours, and in some cases, the resolution of this technique approaches a single molecule of template DNA. Recently, PCR has been adapted to detect viral nucleic acid from environmental water samples (Abbaszadegan *et al*., 1999; Cho *et al*., 2000; Taylor *et al*., 2001; Fout *et al*., 2003). However, the fundamental drawback of PCR is that virus infectivity cannot be ascertained.

7.1. Cell Culture

Environmental water samples often need to be collected in large volumes (*e.g.*, hundreds of liters) because virions in environmental waters are very dilute. Consequently, the first step in cell culture (and similarly for PCR) is concentration and purification, by as much as four orders of magnitude (Griffin *et al*., 2003). This can be accomplished by passing water samples through positively or negatively charged filters, ultracentrifugation, ultrafiltration, or precipitation with polyethylene glycol (PEG). Viruses can be eluted from filters with a beef extract solution. Additional concentration may be accomplished by flocculation (USEPA, 1994). The percent recovery during concentration/purification can be determined by processing a known concentration of virus stock (*e.g.*, poliovirus) in parallel with the experimental samples (Abbaszadegan *et al*., 1999; Fuhrman *et al*., 2005).

The next step in cell culture is to inoculate the concentrated virus sample onto a culture of cells. For enteric viruses, the BGMK cell line often is used (USEPA, 1987); this cell line is capable of replicating adenoviruses and some enteroviruses. (No cell line is capable of replicating all enteric virus species.) Cells are grown in a buffered medium containing antibiotics and a nutrient cocktail that mimics conditions in the intact host organism. If the cells are capable of being infected by the virus(es) present in the sample, they will respond in a dose-dependent way—they may lyse, change in morphology, or fuse into syncytia. All of these ramifications are classified as cytopathic effects (CPE). CPE may be visible under a dissecting microscope or even by the naked eye within days, although some slow-growing viruses may take weeks to elicit CPE. Notably, some virus species do

not produce CPE (*e.g.*, parainfluenza), and would give a false-negative result in culture. Alternatively, coconcentrated nonviral toxins in the sample may be lethal to the cells and mimic CPE, leading to false-positive results. Positive CPE results suggest virus presence but do not necessarily identify the virus species because some viruses elicit the same CPEs.

In a water sample in which the contaminating viruses are not known, serum neutralization tests can be used. In this technique, the virus sample is combined with serum containing antibodies against known viral antigens (*e.g.*, against poliovirus antigens). Subsequent loss of infectivity (*i.e.*, absence of CPE) indicates that the antibody recognized and neutralized the viral antigens and thus identifies the virus species. Alternatively, a cell line that amplifies only a single virus species may be selected. The most important limitation of cell culture is that host cell lines have not been identified for some enteric viruses. In these cases, cell culture techniques cannot be used (Noble *et al.*, 2003; Fong and Lipp, 2005).

7.2. PCR

All cellular organisms use DNA polymerase to replicate their DNA in preparation for cell division. PCR harnesses DNA polymerase to amplify target nucleic acid sequences to detectable levels. In 1983, Kary Mullis recognized that DNA could be duplicated by intentionally heat-denaturing the double helix and adding short nucleotide segments (*i.e.*, primers), free nucleotides, and DNA polymerase to restore each denatured strand to a double helix consisting of one strand of original template and one strand of newly

synthesized DNA (Mullis *et al.*, 1986). By designing primers complementary to the DNA sequences upstream and downstream of the target, the target DNA sequence can be preferentially amplified. After about 30 rounds of heat denaturation and polymerization, the target DNA sequence is so abundant—generally reaching a 10^6-fold amplification of the original template concentration—that the post-reaction sample is effectively pure target DNA.

Originally, PCR was conducted using *E. coli*-derived DNA polymerase. This procedure required that the researcher "recharge" the reaction with fresh polymerase after each cycle because the heating step denatured the enzyme irreversibly (Saiki *et al.*, 1985). The process of PCR became much simpler and of higher fidelity when *E. coli* polymerase was replaced with *Thermus aquaticus* DNA polymerase (a.k.a. Taq polymerase) (Saiki *et al.*, 1988). *T. aquaticus* inhabits hot springs, and its proteins have evolved extreme thermostability. The Taq polymerase protein is capable of maintaining its conformation throughout each heat denaturation step.

PCR of virus DNA from environmental samples requires liberation of viral nucleic acid from the capsid. This traditionally is accomplished by extracting with guanidium thiocyanate and passing the sample through a silica column to remove the dissociated capsid proteins (Griffin *et al.*, 2003). This method purifies both RNA and DNA, which is particularly pertinent to the isolation of enteric viruses, most of which have RNA genomes. PCR can only detect DNA sequences, so detection of enteric viruses with RNA genomes must be preceded by a process called reverse transcription, in which purified

retroviral reverse transcriptase (RT)—an RNA-dependent DNA polymerase—is incubated with an RNA template and free nucleotides to generate double-stranded, complementary DNA (cDNA).

Environmental waters accumulate humic and fulvic compounds and metal ions as a result of biosynthetic and biodegradative processes (Abbaszadegan *et al*., 1993). The process of sample concentration in preparation for PCR (or cell culture) concomitantly concentrates organic compounds that may inhibit active enzymes needed for RT and/or PCR (Wilson, 1997). To remove organic acids and metals, a number of methods such as phenol-chloroform extraction, precipitation, chelation, biotinylation, chromatographic separation, ultracentrifugation through a sucrose gradient, or immunomagnetic separation are available (Schwab *et al*., 1995; Ijzerman *et al*., 1997; Fout *et al*., 2003). Alternatively, it may be sufficient to dilute the PCR sample slightly (*e.g.*, 1:10), thus lessening the inhibition effect enough that amplification can be detected.

One method to check for false-negative PCR results is to test half of a water sample as normal and seed the other half with the virus of interest (Schwab *et al*., 1997; Borchardt *et al*., 2003; Fout *et al*., 2003; Borchardt *et al*., 2004). If neither sample amplifies, then inhibitors are present. Alternatively, false-positives may occur as a result of laboratory contamination. In this case, the DNA template is volume-replaced by water and used as a negative-control sample to ensure that contamination did not occur (Borchardt *et al*., 2004; Fuhrman *et al*., 2005).

PCR can identify any pathogen in a water sample as long as some of the pathogen's genetic sequence is known. In the case of virus identification, primers can be designed complementary to conserved or variable regions of the genome to amplify entire virus orders or specific virus species. However, PCR cannot determine whether the pathogen was active or infectious at the time of sampling (Scott *et al*., 2002; Griffin *et al*., 2003; Fong and Lipp, 2005). Because PCR only indicates the presence or absence of a target sequence, it would yield a positive result for a noninfectious virus if the virus particle's genetic material was intact. In some cases, viral nucleic acid, particularly DNA, may persist even after the viral envelope or capsid is disrupted and infectivity is lost (Straub *et al*., 1995; De Serres *et al*., 1999). For this reason, PCR detection is limited in the information it can provide, for example, it may underestimate the inactivation of viruses through a treatment system (Sobsey *et al*., 1998; Yates, 2007).

In ground water, PCR detection of viral genetic material confirms that a "path of contamination" exists and that virus—whether viable or not—is capable of reaching the water table (Yates, 2007). In addition, surface water studies have reported similar results for virus detection by RT-PCR and tissue culture in the Florida Keys (Griffin *et al*., 1999) and the Sarasota Bay estuary (Lipp *et al*., 2001), respectively, suggesting that although PCR may overestimate virus presence in some cases, it can be a valid and useful technique for virus monitoring.

The inability of PCR to determine infectivity has led to debates about whether a positive PCR result is sufficient to cause public health alarm. Some investigators contend that

RNA genomes degrade rapidly and thus would not be detected by PCR as free nucleic acid from an inactivated virus (Kopecka *et al*., 1993). Others caution that even an intact virus with a preserved genome may be damaged and incapable of entry into a host cell, thus precluding infection but remaining detectable by PCR (Nuanualsuwan and Cliver, 2003). Gassilloud *et al*. (2003) monitored RNA viruses for infectivity and positive PCR detection in mineral ground water and reported that temperature had a marked effect on virus infectivity but not on RNA genome persistence. For instance, poliovirus was inactivated linearly at 35°C, whereas its genome persisted much more robustly and degraded according to logarithmic kinetics at this temperature. At 10°C, however, poliovirus infectivity and genome integrity persisted to a similar extent. This research studied water samples similar to commercial bottled water, which is quite different from raw source water, and likely different from finished drinking water. In river water, Skraber *et al*. (2004b) observed that poliovirus genomic RNA persisted two-fold longer than infectious poliovirus. Enriquez *et al*. (1993) reported that infectious poliovirus and its genome declined in parallel in well water and dechlorinated tap water at 15°C and 37°C. However, poliovirus and its genome did not decline in parallel in autoclaved well water or phosphate buffer. The presence of proteases, RNases, and DNases likely plays a large role in the persistence of genomes from nonintact virus. These enzymes are copious in environmental waters but would be denatured during the autoclaving process.

Some researchers have proposed methods to preselect for infectious virus before a PCR analysis is performed. Nuanualsuwan and Cliver (2002) demonstrated that hepatitis A virus, poliovirus, and a feline calicivirus (a model for norovirus) that were inactivated

with UV radiation, heat (72°C), or hypochlorite would not trigger a positive PCR result if the virus sample was pretreated with proteinase K (to degrade partially denatured coat proteins) and nuclease (to degrade an exposed genome). In contrast, intact, infectious viruses were not susceptible to enzyme pretreatment and positive PCR results were obtained. The inactivation methods used by these researchers were akin to common disinfection processes at water treatment plants and were expected to render the virus species noninfectious but not fully degraded or physically removed, much like disinfection would accomplish in a treatment system. The researchers suggest that their pretreatment methods could be applied to other virus species and other disinfection procedures. Other investigators have reported that isolating viral particles on a positively charged Sephadex filter and eluting with high ionic strength beef extract selected for intact, infectious viral capsids, rather than free viral RNA or damaged particles (Abbaszadegan *et al.*, 1999).

7.3. Variations in Cell Culture and PCR

Neither cell culture nor PCR is without shortcomings. Frontiers in PCR have allowed researchers to obtain quantitative results, higher resolution, and simultaneous detection of different pathogens. In addition, integration of cell culture and PCR may maximize the utility of both procedures while minimizing the drawbacks. Currently implemented modifications to cell culture and PCR are discussed below.

(1) Real-time PCR involves the detection of a fluorescent signal emitted during the amplification reaction. The signal intensity is proportional to the amount of the target DNA amplicon. By amplifying a known concentration of control DNA in parallel, the ratio of the fluorescent signals allows for quantification of the experimental target sample. Results from this type of PCR inform researchers about viral concentration and in the future, may be compared to minimum infectious doses to estimate health risks (Scott *et al.*, 2002; Griffin *et al.*, 2003).

(2) Nested PCR is a more sensitive version of PCR in which a target sequence is amplified and the sample undergoes a second round of PCR to amplify a sequence nested within the initial amplicon (Metcalf *et al.*, 1995; Abbaszadegan *et al.*, 1999). This approach is taken when an extremely low concentration of template DNA (*e.g.*, a single molecule of template in the sample) is expected or when negative results are obtained using conventional PCR despite other evidence suggesting presence of template DNA in the sample. A variation on the same concept is to follow PCR with Southern hybridization, in which radioactively labeled DNA segments (*i.e.*, oligonucleotides) are hybridized to the amplicon to confirm its integrity and to intensify the positive signal (Abbaszadegan *et al.*, 1999; Noble *et al.*, 2003; Fong *et al.*, 2005). For both nested PCR and PCR/Southern, the resolution approaches one molecule of template DNA.

(3) Multiplex PCR allows different target DNAs to be detected in the same reaction vessel. For instance, if a number of enteric virus species are hypothesized to exist in a

water sample, they can be assayed simultaneously in the same sample vial (Formiga-Cruz *et al.*, 2005). This technique can save time if many samples are to be processed. However, it may require a great deal of parameter optimization in order to create conditions that are favorable for each template to denature and for each primer to anneal specifically and efficiently. For instance, if the primers being used have significantly different guanine/cytosine contents, it may be difficult to optimize the heating steps, as guanine-cytosine bonds are more heat-stable than adenine-thymine bonds. A number of other reaction components also must be optimized for consistent replication of each template in the multiplex reaction. This may include varying the concentrations of magnesium cations, primers, free deoxynucleotides, and enzymes. In some cases, the characteristics of different templates are so diverse that adequate optimization is not possible.

(4) Integrated cell culture-PCR (ICC-PCR) combines the best qualities of both techniques (Bosch, 1998; Griffin *et al.*, 2003; Fong and Lipp, 2005). In this method, cells are inoculated with an environmental water sample, the culture is incubated for 1–3 days and cells are harvested before CPE is apparent. The cells are mechanically lysed, nucleic acid is isolated, and PCR is performed. This procedure avoids the 1–3 weeks of culture maintenance often required for full CPE to occur, yet it also detects infectious virus. Theoretically, cells are harvested when virus is actively replicating, but noninfectious virus particles already have degraded. This technique also is useful in the detection of infectious viruses that may be slow growing or that do not produce CPE.

CHAPTER 8 – TORQUE TENO VIRUS: A PUTATIVE INDICATOR OF ENTERIC VIRUSES

Torque Teno virus (TTV) is a small, unenveloped DNA virus that is ubiquitous and seemingly innocuous in humans worldwide and may exhibit similar transport and survival characteristics to pathogenic enteric viruses. In the following discussion, the biological characteristics, isolation techniques, and potential utility of TTV as an indicator of enteric viruses is assessed.

8.1. Biology of TTV

TTV was first identified in 1997 in the serum of a Japanese patient who developed hepatitis of unknown etiology following a blood transfusion (Nishizawa *et al*., 1997). The virus was detected by a modified PCR technique called representational difference analysis (RDA), in which differences between two DNA samples can be compared by restriction endonuclease digestion and subtractive hybridization to enrich for genetic sequences that are unique to the experimental sample (Lisitsyn *et al*., 1993). By this method, a viral genome sequence can be sorted from all the genetic material in a human cell. Using RDA, researchers obtained a 500 base-pair clone deemed N22 that was absent before the patient's blood transfusion and lacked homology to sequences already reported in DNA databases (Nishizawa *et al*., 1997). The N22 sequence floated at a density of 1.26 g/cm^3 when centrifuged through a sucrose gradient and was resistant to treatment with DNaseI. These observations suggested that the sequence was encapsidated within a proteinaceous particle and likely was a virus (Nishizawa *et al*., 1997). The putative virus was named "TT" virus after the index patient's initials.

Filtration studies indicate that TTV is 30–50 nm in diameter (Mushahwar *et al*., 1999). Itoh *et al*. (2000) reported a diameter of 30–32 nm when TTV isolated from fecal supernatant was visualized by immunoelectron microscopy (Figure 8.1).
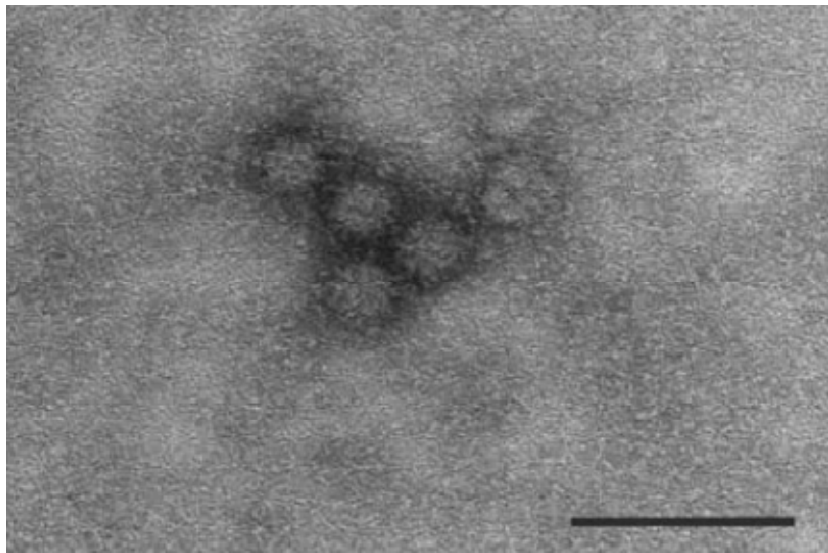
Figure 8.1. Micrograph of TTV. Icosahedral virus-like particles of 30–32 nm were found to aggregate after human fecal supernatant containing TTV genotype 1a was incubated with human anti-TTV-1a-specific antibody. Scale bar represents 100 nm. Reproduced with permission from Itoh *et al*., 2000.

Other researchers reported that detergent exposure did not change the density of the particle, suggesting that the virus is not enclosed in a host-derived lipid envelope (Okamoto *et al*., 1998b). Moreover, the unencapsidated genome was sensitive to DNaseI and mung bean nuclease but was resistant to RNaseA and some restriction endonucleases, suggesting that the structure was single-stranded DNA (ssDNA; Okamoto *et al*., 1998b). Genome sequencing, specifically of the GC-rich region, indicated that the genome was a covalently closed circle (Miyata *et al*., 1999). These findings led to the full

name of TTV being changed to reflect its genome structure. The "TT" now stands for "Torque Teno," which is derived from the Latin for "thin necklace" (Biagini *et al*., 2004).

The average genome length of a TTV isolate is 3.8 kilobases (kb); of that, 1.2 kb do not appear to code for viral proteins. Hybridization and nuclease protection studies indicate that the virus encapsidates its negative strand (Mushahwar *et al*., 1999), meaning that an infected cell must synthesize the complementary strand of the TTV genome before viral messenger RNA (mRNA) and proteins can be produced. Three mRNAs are transcribed from open reading frames (ORFs) in the viral genome (Kamahora *et al*., 2000), and six proteins with distinct subcellular localizations are translated via alternative splicing and alternative translation initiation mechanisms (Qiu *et al*., 2005; Kakkola *et al*., 2008).

TTV is classified into the genus *Anellovirus* but is not yet assigned a virus family (Biagini *et al*., 2004). Phylogenetic analyses of TTV isolates further classify these viruses into 5 genogroups differing by more than 50% and 39 genotypes differing by more than 30% (Peng *et al*., 2002; Todd *et al*., 2005). Genogroups 1 and 2 are most prevalent worldwide (Abe *et al*., 1999). Reports of the TTV genome sequence have described it as extremely variable across TTV isolates (Tanaka *et al*., 1998; Viazov *et al*., 1998; Mushahwar *et al*., 1999). Divergences of 47–70% have been reported at the amino acid level (Biagini *et al*., 1999; Luo *et al*., 2002). However, the high degree of divergence is not distributed evenly over the genome. In all isolates, a GC-rich region of 108–160 nucleotides is present in the untranslated region (UTR) (Hallett *et al*., 2000; Heller *et al*., 2001; Peng *et al*., 2002). Also conserved are the poly-A sequence downstream and the

TATA box upstream of the coding regions (Erker *et al*., 1999; Hijikata *et al*., 1999; Hallett *et al*., 2000; Heller *et al*., 2001).

Interestingly, the coding regions of TTV are less conserved than the UTR. For instance, the coding region of ORF 1 contains three hypervariable regions (HVRs) in tandem (Mushahwar *et al*., 1999; Nishizawa *et al*., 1999). Variability within ORF 1, which is believed to code for the TTV capsid protein, may be crucial to evasion of the host immune system (Takahashi *et al*., 1998b). If the capsid protein varies with each infectious cycle, then cellular receptors would be unable to recognize and remove circulating TTV particles. The UTR contains conserved stem loop structures (Hijikata *et al*., 1999; Okamoto *et al*., 2002). The stem loops are the sites of transcription factor binding sites, promoters, and enhancer elements that may be crucial for efficient replication and transcription (Miyata *et al*., 1999; Kamada *et al*., 2004; Suzuki *et al*., 2004; See Figure 8.2 for a basic TTV genetic map; See Figure 8.3 for more detail regarding stem loops and hypervariable regions of TTV).
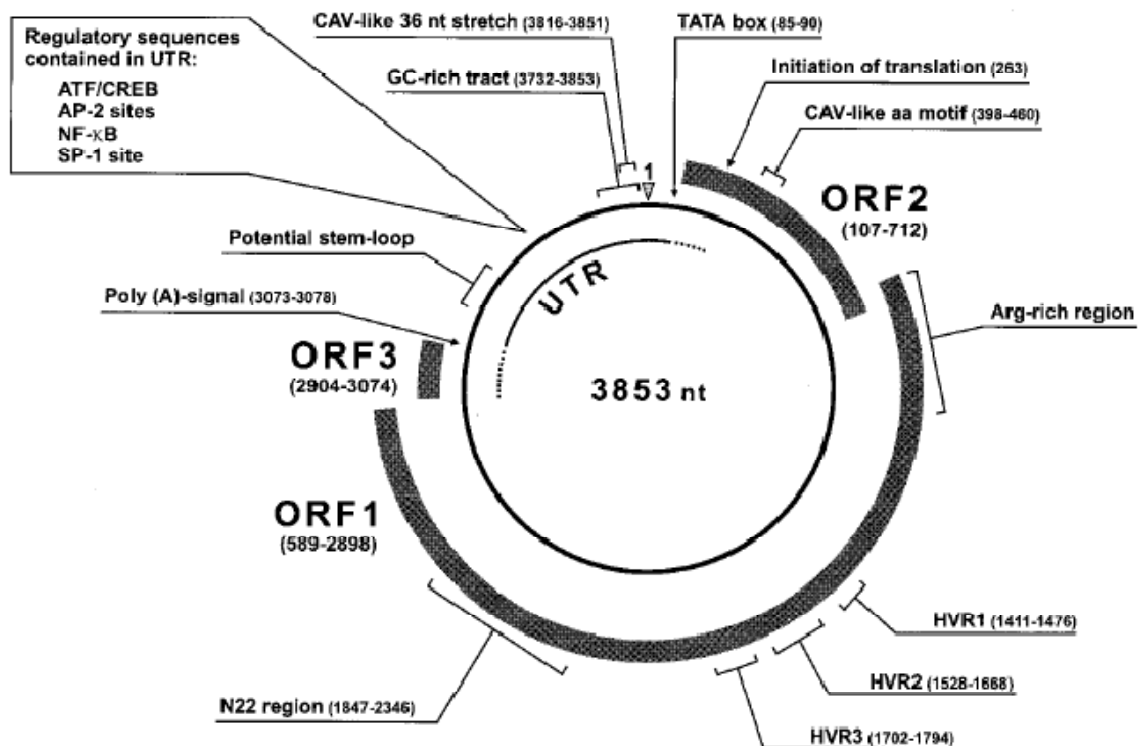
Figure 8.2. TTV genome map. Depicted are regulatory sequences and structures, open reading frames (ORFs), hypervariable regions (HVRs), Chicken anemia virus (CAV)-like motifs, and the N22 region, which was used to identify the TTV genome as viral. Position of ORF 3 is according to Erker *et al*., 1999, but compare to Figure 8.4. Reproduced with permission from Bendinelli *et al*., 2001.
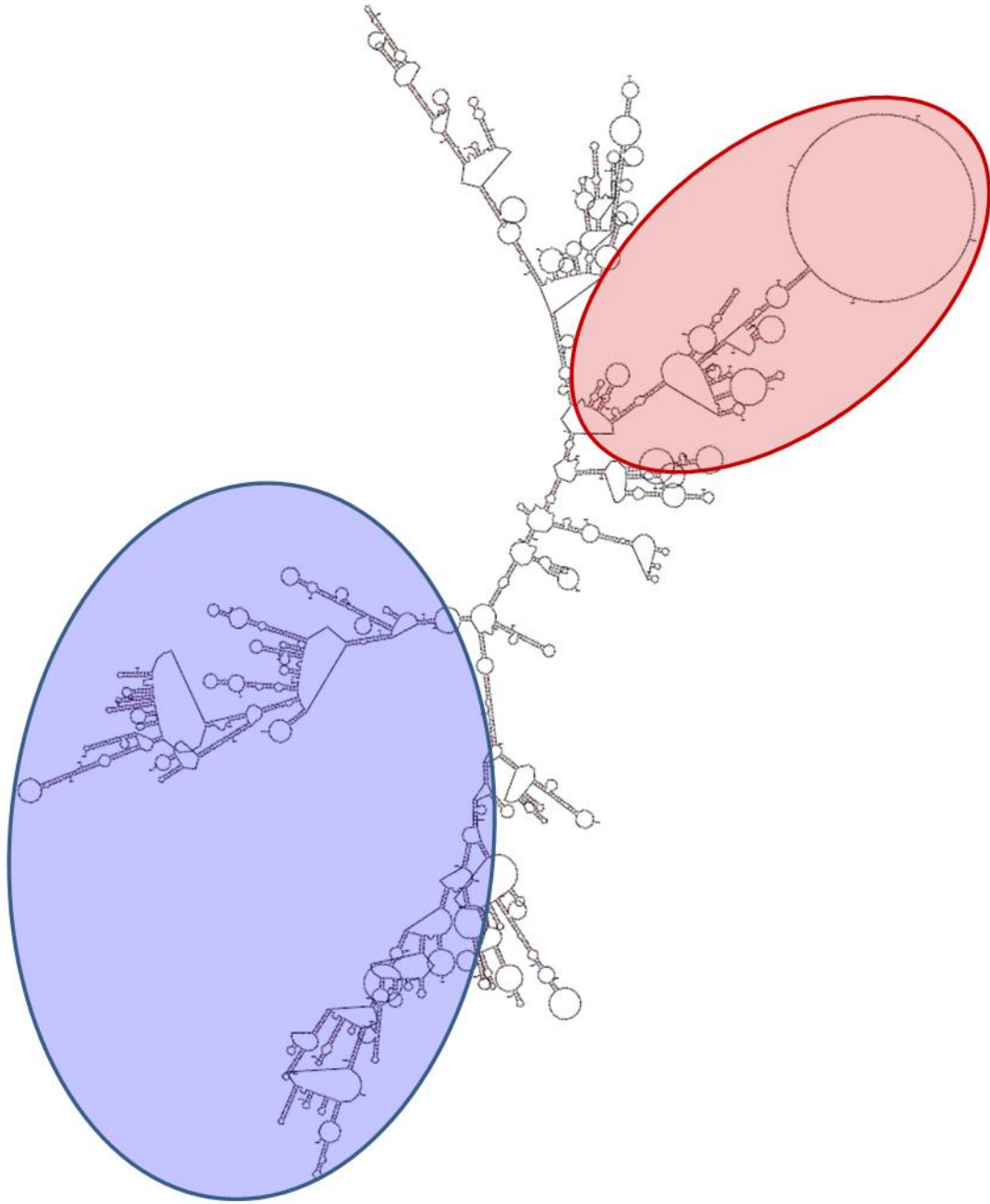
Figure 8.3. **Predicted, energetically stable structure of the TTV genome.** Created using the Mfold Web Server (http://mfold.bioinfo.rpi.edu/) developed by Zuker, 2003. Notice the preponderance of stem loop structures among the conserved regulatory region (blue) and the relative lack of stem loop hybridization among the hypervariable regions (red).

In humans, TTV is detected in bone marrow where lymphoid cells of hematopoietic origin are immunologically activated. TTV also is detected in adenoids and tonsils, saliva, nasal secretions, breast milk, cord blood, plasma/serum, spleen, lung, pancreas, kidney, skin, skeletal muscle, thyroid gland, lymph nodes, liver, bile, and stool (Ross *et al.*, 1999; Okamoto *et al.*, 2000a; Okamoto *et al.*, 2000b; Okamoto *et al.*, 2001; Pollicino *et al.*, 2003; Kekarainen and Segales, 2008). Okamoto *et al.* (2001) suggest that TTV load and genogroup distributions are heterogeneously represented in infected human tissues, although these distributions differ by individual.

TTV infections may be acute or persistent (Nishizawa *et al.*, 1997). Persistent infections with TTV appear to be lifelong and are the only virus infections described to date in which mature virions circulate indefinitely in the blood of infected individuals. In both acute and persistent cases, TTV is described as very dynamic with over 90% of virions cleared each day and generation of 3.8×10^{10} progeny virions per day in patients treated with interferon for concurrent hepatitis C infections (Maggi *et al.*, 2001b).

The method by which TTV establishes persistent infections in otherwise healthy individuals is not understood. In some cases, nucleotide sequences of TTV isolates from persistently infected individuals have demonstrated stability for years, even within the variable coding region (Biagini *et al.*, 1999). However, others have conducted the same experiment and reported rapid mutability and sequence evolution over time (Ball *et al.*, 1999; Gallian *et al.*, 1999; Irving *et al.*, 1999; Leppik *et al.*, 2007). If a cellular DNA polymerase is used to replicate the TTV genome (Kakkola *et al.*, 2007), stability would

be expected because of the polymerase's "proofreading" capacity. Alternatively, the single-stranded nature of the TTV genome may contribute to elevated mutability; this is observed in the single-stranded, linear DNA virus B19 (Shackelton and Holmes, 2006). Healthy individuals frequently are infected with multiple genogroups simultaneously. Worobey (2000) suggests that extensive homologous recombination among different coinfecting genogroups likely maintains variability among TTV isolates.

8.2. Worldwide Prevalence of TTV

Researchers estimate the occurrence of TTV in national populations by obtaining blood or fecal samples from residents and performing PCR analysis to detect the presence of TTV genetic material. This method is rapid and simple to perform, but differences in sample preparation, primer selection, and reaction conditions combine to significantly affect the prevalence data obtained worldwide. The identification of TTV phylogenetic groups that the original TTV primer sets did not amplify (Nishizawa *et al*., 1997; Okamoto *et al*., 1998a) have led to highly variable estimates of TTV DNA seroprevalence in the primary literature (Bendinelli *et al*., 2001; Pollicino *et al*., 2003). The design of primers against ORF 1 led to discrepancies across reports because this ORF contains highly divergent regions (Mushahwar *et al*., 1999), and consequently, certain ORF 1 primers gave negative PCR results whereas other ORF 1 primers and some primers outside of ORF 1 amplified TTV DNA from the same specimens (Leary *et al*., 1999; Springfeld *et al*., 2000). Primers designed against the UTR and within ORF 2 resulted in higher prevalence estimates (92% versus 23% with other primers) among Japanese subjects and 10–100 fold greater viral titers (Takahashi *et al*., 1998a; Springfeld

et al., 2000). UTR primers currently are believed to give the true prevalence of TTV infection in a population (Bendinelli *et al.*, 2001), and a recent study reported that PCR of the TTV genome using 3' and 5' UTR primers is highly consistent as analyzed statistically using the Cronbach alpha coefficient (Ergunay *et al.*, 2008). However, others have suggested that UTR primers are nonspecific (Springfeld *et al.*, unpublished observations) or that the UTR primers do not detect all virus genogroups (Erker and Leary, unpublished observations). Exhaustive comparisons of PCR conditions and results have not been published and prevalence data for some regions, such as North America, have only been collected using ORF 1 primers. Although new TTV primer sequences are published frequently, a standardized TTV PCR protocol has not yet been described.

Charlton *et al.* (1998) collected blood samples from North American blood donors, patients with liver disorders, and individuals with or without exposure to blood products. Using a seminested PCR amplification technique with primers against sequences in ORF 1, these researchers reported a 1% prevalence among healthy blood donors and a 4% prevalence among those without exposure to blood products but with liver disease. They observed that liver disease and exposure to blood products were associated with incidences of TTV infection ranging from 15–27%. In addition to using primers against a potentially divergent genome region, Charlton *et al.* (1998) did not perform Southern hybridization to identify false-negatives in their PCR results. Also using primers directed against ORF 1 but confirming their amplified PCR products using Southern hybridization, Desai *et al.* (1999) reported that 10% of healthy, volunteer blood donors and 13% of commercial blood donors in the United States were infected with TTV. The

prevalence was slightly higher among intravenous drug abusers (17%) and lower among patients with non-A-E hepatitis (2%).

Current estimates suggest that TTV prevalence is moderate in the North America and northern Europe, intermediate in Asia, and high in Africa and South America, with an average prevalence of approximately 80% worldwide (Springfeld *et al.*, 2000; Bendinelli *et al.*, 2001; Table 8.1).

Table 8.1. Worldwide prevalence of TTV determined using primer sets against variable and conserved genomic regions. ORF 1 is divergent and may not provide reliable information on TTV prevalence. The UTR is conserved and currently is regarded as providing the true prevalence in a population. Reproduced with permission from Bendinelli *et al.*, 2001.

Country	% Positive by:	
	ORF1 PCR	UTR PCR
Africa		
Congo	43–44	
Egypt	29	85
Gambia	86	
Kenya	Unknown	
Nigeria	52	
Sudan	7	
Asia		
China	5–11	
Japan	10–58	70–93
Korea	14	
Mongolia	43	
Myanmar		96
Nepal		82
Pakistan	16	
Saudi Arabia	19	100
Singapore		98
Taiwan	10–53	
Thailand	7–36	
Europe		
Finland	17	73
France	5	
Germany	7–14	
Italy	9–50	
Spain	14	
The Netherlands		72
United Kingdom	2–10	47–57
North America		
United States	1–11	
Oceania		
Papua New Guinea	75	
South America		
Bolivia		82
Brazil	20–62	
Colombia	16	
Ecuador	71	

TTV viremia (*i.e.*, circulation in blood) appears to be common in the early months of life, and virus load may peak during middle age or later (Abe *et al.*, 1999; Saback *et al.*, 1999; Bendinelli *et al.*, 2001), which suggests that TTV primarily is spread by environmental exposure (See Section 8.3). Christensen and colleagues (2000) used dilution PCR to determine the number of TTV genomes in healthy Danish blood donors and immunocompromised patients. They reported that TTV circulated in healthy blood donors at magnitudes ranging from 1×10^3 to 7×10^4 TTV genome copies/mL serum. In HIV-infected patients, a higher TTV load was observed, ranging from 1×10^3 to 9×10^6 copies/mL serum, although this result could be an effect of a severely weakened immune system (Christensen *et al.*, 2000). Indeed, HIV-infected patients with worse prognoses (*i.e.*, ~15% of patients surviving after 1,600 days as compared to ~40% of patients surviving with better prognoses) exhibited higher TTV loads in their serum (3.5×10^5 TTV/mL serum or more).

Preliminary results suggest that TTV is present in the blood sera of farm animals (mammalian and avian) and nonhuman primates (Leary *et al.*, 1999). Amplified sequences from TTV-positive swine, dogs, and cats were similar, but not identical, to TTV sequences amplified from humans (Leary *et al.*, 1999) and range between 2.1 and 2.9 kb in length (Okamoto *et al.*, 2002). Sequences within the UTR are conserved in animals and humans. These results indicate that TTV is not strictly a human virus, but transmission characteristics, dynamics of nonhuman TTV infections, and the worldwide TTV prevalence in most animals have not been described to date (Leary *et al.*, 1999; Kekarainen and Segales, 2008). Recent work suggests that TTV may be common in

swine but may be sequestered to fewer tissues than in humans (Kekarainen and Segales, 2008).

8.3. Modes of TTV Transmission

TTV is known to circulate in the blood of infected individuals, and populations with histories of exposure to blood products (*e.g.*, via blood transfusion or hemodialysis) or who abuse intravenous drugs tend to have higher frequencies of TTV infection and higher virus loads. However, parenteral routes of transmission (*i.e.*, via injection) do not explain the global prevalence and ubiquity of TTV. Moreover, the increase in TTV prevalence with age supports environmental, rather than parenteral, exposure (Ergunay *et al.*, 2008). This suggests that the fecal-oral route is the most common pathway of spread (Bendinelli *et al.*, 2001). Individuals with TTV viremia also test positive for fecal TTV (Okamoto *et al.*, 1998a; Luo *et al.*, 1999; Ross *et al.*, 1999; Ukita *et al.*, 1999; Romeo *et al.*, 2000), and TTV isolated from feces is capable of infecting sensitive and permissive cells in the laboratory (Maggi *et al.*, 2001a). TTV transmission by the fecal-oral route is likely through secretion of bile from infected liver cells into feces (Okamoto *et al.*, 1998a; Ukita *et al.*, 1999). Indeed, TTV is detected in liver tissue and bile at 10–100-fold greater titers than in plasma (Okamoto *et al.*, 1998a; Ross *et al.*, 1999; Ukita *et al.*, 1999; Nakagawa *et al.*, 2000). The prevalence of TTV among individuals worldwide suggests that even if TTV is shed in feces intermittently or at low levels (Okamoto *et al.*, 1998a; Ross *et al.*, 1999) the density of TTV in the environment is expected to be high (Bendinelli *et al.*, 2001).

Alternative modes of TTV transmission have been proposed, including transplacental or via umbilical cord blood (Saback *et al.*, 1999; Morrica *et al.*, 2000); contact with hair, skin, or saliva of infected individuals (Osiowy and Sauder, 2000); and nosocomial infection (Matsumoto *et al.*, 1999). These modes are likely to be tertiary to fecal-oral and parenteral transmission (Saback *et al.*, 1999; Bendinelli *et al.*, 2001).

8.4. Pathogenicity of TTV

Initially, it was believed that TTV was a novel viral agent that could induce hepatitis (Nishizawa *et al.*, 1997), but subsequent studies of TTV prevalence indicated that TTV circulates in a large proportion of healthy individuals. Moreover, TTV does not appear to exhibit seasonal variance or epidemic bursts of infection (Vaidya *et al.*, 2002; Haramoto *et al.*, 2005b; Diniz-Mendes *et al.*, 2008).

Currently, the pathogenicity of TTV is unclear, although studies have been published that investigate the relationship between TTV and hepatic disorders, acute respiratory disorder, progression to AIDS, various cancers, autoimmune disorders, and kidney disease (reviewed by Bendinelli *et al.*, 2001; Irshad *et al.*, 2006; Hino and Miyata, 2007). Disease associations have not been substantiated, and elevated TTV levels in diseased patients likely reflect the compromised immune status of the individual. In rare cases, TTV appears to induce transient and mild liver abnormalities, but temporary liver dysfunction is an effect of many viral infections, including those caused by enteric viruses (Bendinelli *et al.*, 2001). Given the failure of attempts to assign a pathology, Griffiths (1999) and Simmonds *et al.* (1999) have suggested that TTV may constitute one

of the estimated 500 species of commensal intestinal microorganisms in humans. To date, no other commensal viruses have been described (Bendinelli *et al.*, 2001).

8.5. Preliminary Support for the Indicator Potential of TTV

Given its worldwide ubiquity, fecal-oral mode of transmission, lack of seasonal variance, and similar size and composition to pathogenic enteric viruses, TTV may be useful as an indicator of virus contamination. Currently, little is known about the environmental stability of TTV, although Takayama *et al.* (1999) demonstrated that TTV infectivity was not lost after 95 hours of dry heat treatment (65°C). Investigators suspect that the TTV virus particle is highly stable (Verani *et al.*, 2006). As discussed below, several investigators have tracked TTV in the environment or in treatment systems. Their results suggest that TTV is not correlated with coliform indicators, but may colocate with various enteric viruses.

In Manaus County of the Brazilian Amazon, more than 90% of the 1.7 million residents lack sewage collection, and waters of various small, contaminated streams empty into the Negro River. Diniz-Mendes *et al.* (2008) collected 52 water samples from 13 locations across this region four times (August, November, February, and June) during a 1-year period. Levels of TTV were determined by real-time PCR and compared to total and fecal coliform densities and other water quality parameters. TTV was detected in 92.3% of surface water samples, ranging from 1,300 to 746,000 TTV genomes per 100 mL water. TTV presence did not fluctuate by season or geographic area, and the TTV load did not correlate with coliform density or physicochemical parameters. However, the

TTV positivity rate of 92.3% paralleled the positivity rate reported by De Paula *et al*. (2007) for hepatitis A virus in the same geographic region.

To assess the TTV positivity rate in Italy, researchers collected samples of river water receiving treatment plant effluent monthly for 1 year (Verani *et al*., 2006). They reported that TTV was present in 3 of 12 samples (25% positivity rate). Interestingly, TTV and rotavirus (33% positivity rate) occurred either simultaneously or within 1 month's sampling period of each other. In addition, TTV occurred 1–2 months after enterovirus was detected, and simultaneously or within 2 months of noroviruses g1 and g2 in all but one case (3-month difference). Whereas the pathogenic viruses were observed in seasonal clusters, TTV positivity was distributed rather evenly throughout the year in June, September, and March.

TTV is found in 5% of surface water samples in Japan without seasonal variance (Haramoto *et al*., 2005a). When TTV was monitored through eight activated sludge wastewater treatment plants in Japan monthly for 1 year, researchers reported that TTV genetic material was detected with 97% frequency in influent, 18% in secondary effluent after activated sludge treatment but before chlorination, 24% in final effluent after chlorination, and 0% in effluent for reuse following filtration and ozonation (Haramoto *et al*., 2005b). In contrast, coliforms decreased sequentially with each step in the treatment process, and the concentration of coliforms did not correlate with the number of positive TTV samples collected at any step. These results indicate that chlorination did not affect the ability of PCR to detect TTV genetic material, although chlorination may have

rendered the virus noninfectious without affecting the amplified genome region (Nuanualsuwan and Cliver, 2002).

Hepatitis viruses A and E (both enterically transmitted) and TTV are common in India. Hepatitis A infects nearly all residents early in childhood, and while symptomatic infection is rare in adults, subclinical shedding is common. Hepatitis E is implicated in epidemics of disease following spikes of fecal contamination. Vaidya *et al*. (2002) compared sewage treatment plant influent and effluent concentrations of these viruses via PCR and observed that raw sewage prevalence of TTV DNA (12.7% positive rate) was statistically similar to the prevalence of hepatitis E virus RNA (11.0%) and hepatitis A virus RNA (24.4%), although hepatitis A virus was significantly more prevalent than hepatitis E virus. Following treatment, hepatitis A virus was significantly reduced in PCR detectability (to 4.1%), but the reductions in TTV (to 2%) and hepatitis E virus (to 10.8%) were not statistically significant. Others have described hepatitis A virus as being highly sensitive to chlorination (Azadpour-Keeley *et al*., 2003) so the results described by Vaidya *et al*. (2002) are reasonable. Notably, the sample size for effluent prevalence detection was very small owing to treatment system failure during the study. The true change in TTV prevalence, if any, would be better assessed with a larger sample size. Similar influent prevalence rates between TTV and hepatitis A virus or hepatitis E virus indicated that the viruses were detected to the same frequency, but not every TTV-positive sample contained hepatitis A virus or hepatitis E virus simultaneously. These results were not confirmed by cell culture, so the infectivity of each virus species

following treatment could not be determined. Moreover, ORF 1 primers were used to detect TTV, so influent and effluent magnitudes may be underestimates.

As a putative indicator, TTV should be abundant where water is not adequately treated and diarrheal disease is common and should exist at low or undetectable levels where water treatment leads to clean, potable water. Poor sanitation may increase TTV transmission by the fecal-oral route, as indigenous rural populations of Nigeria, Gambia, Brazil, and Ecuador had incidence up to 74% (Prescott and Simmonds, 1998). Similarly, the countries of Bolivia and Burma—both with high risks of waterborne disease—had incidences of 82% and 96%, respectively, among otherwise healthy individuals (Abe *et al.*, 1999).

More research must be done to assess the utility of TTV as an indicator of enteric viruses. PCR detection of the co-occurrence of TTV DNA with the genetic material of other viruses is limited in its interpretation by:

(1) the need to concentrate water samples, thereby potentially concentrating PCR inhibitors, and the different concentration methods available;

(2) the choice of primers, some of which give rise to unstable or insensitive PCR outputs; and

(3) the inability to discern whether the presence of viral nucleic acid equates to the presence of infectious virus.

A key experiment will be to track TTV in drinking water before, during, and after a waterborne disease outbreak (*e.g.*, in a region where seasonal outbreaks can be predicted) to determine whether TTV levels rise and fall in parallel with culturable viral pathogens and viral nucleic acid.

8.6. TTV Detection by PCR

The main shortcoming of PCR is that a positive result does not provide information about infectivity. A very stable virus genome (*e.g.*, dsDNA) may persist even if the virus particle is rendered noninfectious. Alternatively, very unstable virus genomes (*e.g.* ssRNA) likely degrade concurrent with virus inactivation. The stability of the circular, ssDNA genome of TTV has not been studied in environmental waters, but some researchers have reported that TTV DNA from fecal extracts degrades by approximately 3 log within 1 week when monitored by real-time PCR at 37°C (Desai *et al.*, 2005).

As described above, TTV's genetic hypervariability makes the choice of primers a crucial undertaking. Several of the primer sets described to date are mapped to the TTV genome in Figure 8.4. If primers are designed against a divergent region of the TTV genome, the sensitivity and stability of the amplification reaction will be compromised. Indeed, Desai *et al.* (1999) used overlapping primer sets to detect TTV in infected individuals and demonstrated that in many cases only one of the sets successfully amplified the virus genome. They suggested that the use of a single primer pair may lead to an underestimation of TTV prevalence and highlighted the need for primers that detect all

TTV variants to maximize sensitivity. Current knowledge maintains that the conserved

UTR is superior to other genetic regions for determining prevalence.
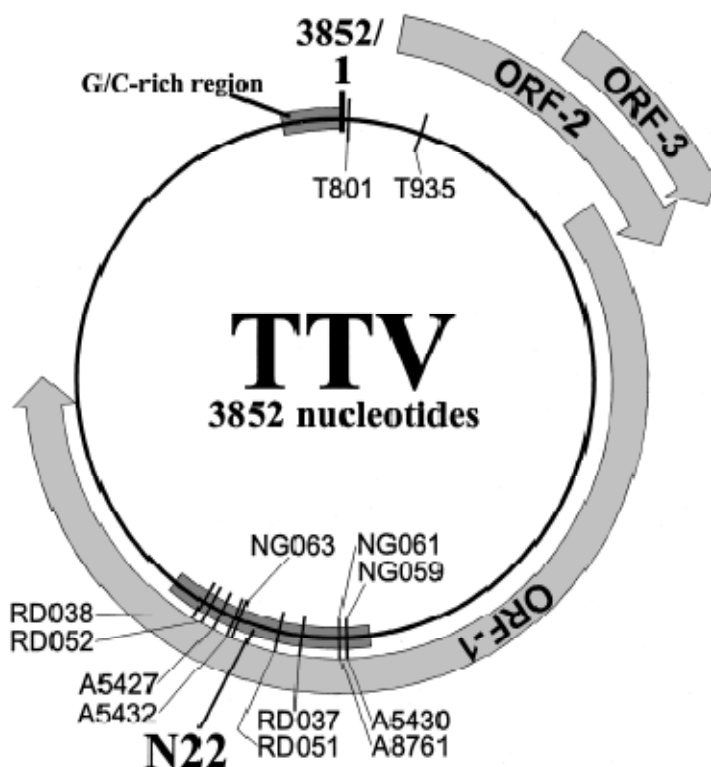
Figure 8.4. **TTV genome map showing the location of various published primer sets within the N22 segment of ORF 1 and within the UTR and ORF2.** Takahashi (1998a) demonstrated that when UTR/ORF 2 primers T801 and T935 are used, an increase in prevalence and virus load is observed over the results obtained with ORF 1 primers. Note that Springfeld *et al.*, 2000, cite Mushahwar *et al.*, 1999, for ORF positions; however, the cited report only maps ORFs 1 and 2. The basis for this ORF3 position and the reason for the discrepancy with the map in Figure 8.2 is unknown. Reproduced with permission from Springfeld *et al.*, 2000.

8.7. TTV Detection by Cell Culture

If TTV is to be used as an indicator—particularly in a treatment system in which virus particles may be inactivated but not removed—a cell culture system must be available to determine TTV infectivity. Whereas all human viruses are capable of infecting one or more human cell types *in situ*, the infectious cycle may be difficult or impossible to replicate *in vitro*. TTV is detected in lymphoid cells and hepatocytes; the former are thought to contribute to circulating TTV in individuals with viremia, and the latter likely contribute to fecal excretion of TTV (Bendinelli *et al*., 2001).

Peripheral blood mononuclear cells (PBMCs) include B-lymphocytes, T-lymphocytes, monocytes, polymorphonuclear leukocytes, granulocytes, and natural killer cells. PBMCs stimulated with phytohemagglutinin (PHA) can be productively infected *in vitro* with TTV isolated from fecal extracts to release progeny virions into the culture supernatant (Maggi *et al*., 2001a). Maggi *et al*. (2001a) observed that peak titers ranging from 4.2×10^4 to 6.2×10^5 DNA copies/mL supernatant were reached approximately 2 weeks following infection. TTV infections of PHA-stimulated PBMCs lacked cytopathic effect and were self-limiting; release of progeny viruses ended after 21–28 days. Notably, stimulated PBMCs cultured from TTV-infected donors appeared to release TTV continuously at titers of 10^4 to 10^5 DNA copies/mL supernatant.

Mariscal *et al*. (2002) demonstrated that when PBMCs were stimulated by PHA, lipopolysaccharide, and interleukin-2, the cells could be infected with serum from a TTV-infected individual to produce TTV genomic ssDNA, mRNA, and dsDNA (Figure 8.5).

TTV dsDNA is believed to be an intermediate form of TTV genome replication (Mushahwar *et al*., 1999). This same dsDNA species is detected in liver tissue samples and bone marrow cells from infected individuals (Okamoto *et al*., 2000a; Okamoto *et al*., 2000b). In contrast, only TTV ssDNA could be recovered from unstimulated PBMCs (Mariscal *et al*., 2002). When supernatant was collected from stimulated, infected PBMCs and applied to stimulated PBMCs collected from TTV-negative donors, TTV DNA and mRNA were isolated after an incubation period. These signs of a productive infection were absent when infectious supernatant was transferred to unstimulated PBMCs.
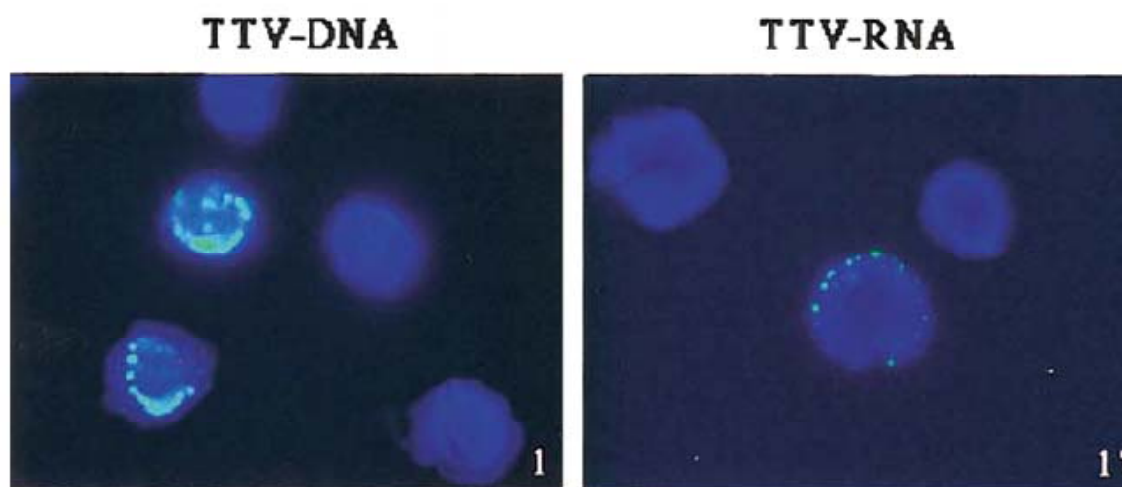
Figure 8.5. TTV infection of PBMCs. TTV DNA and RNA are observed by *in situ* hybridization after stimulated PBMCs are infected with TTV. Reproduced with permission from Mariscal *et al*., 2002.

Desai *et al*. (2005) confirmed that activated PBMCs will replicate TTV isolated from fecal extracts or plasma of infected individuals. These researchers also suggested that the

Chang liver cell line, derived from nonmalignant human liver tissue, and the Raji β-lymphoblast cell line support TTV infection. A productive infection in activated PBMCs peaks at approximately 2 weeks postinoculation, reaching a 2–3 log increase in TTV genome copies/mL over the original inoculum. Replication in PBMCs was self-limiting within 21–28 days postinoculation, supporting the results obtained by Maggi *et al*. (2001a). In Chang liver cells, TTV titers peak within 1–5 days, but only reach 1/100 of the titers observed from infected, activated PBMCs (Desai *et al*., 2005).

Interestingly, PBMCs exhibit no decrease in cell viability upon infection with TTV (Maggi *et al*., 2001a; Mariscal *et al*., 2002), whereas Chang liver cells lose adherence to the substratum and form rounded, granulated cell clumps in the supernatant within 48–72 hours of inoculation (Desai *et al*., 2005). This observation suggests that Chang liver cells may be a useful model to readily and visually determine the infectivity of TTV. However, others have reported that they could not replicate the CPE observed by Desai and coworkers using a different, less common TTV genotype (Kakkola *et al*., 2007).

To date, no animal model of TTV infection has been described, although some investigators have proposed the use of a swine model (Kekarainen and Segales, 2008). An animal model of TTV infection could complement the information gleaned from *in vitro* studies by demonstrating transmission characteristics, infection dynamics, and persistence. In addition, an animal model of infection would allow for the collection of TTV-specific antibodies and the design of immunohistochemical and *in situ* tissue

hybridization experiments. Both cell culture and animal models are crucial next steps to provide insight into the molecular biology of TTV.

CHAPTER 9 – ASSESSING TTV AS A VIRAL INDICATOR

The unique characteristics of TTV and support from preliminary studies suggest that this virus may be useful as an indicator of enteric viral pathogens. Below, methodology is discussed to assess the potential of TTV as an indicator. Once methods are available to detect TTV reliably, research should focus on the following:

(1) Assessment of the density and occurrence of TTV in source waters;

(2) Evaluation of TTV persistence through drinking water treatment processes (coagulation, clarification, filtration, and disinfection); and

(3) Comparison of these data to those for coliforms, coliphages and enteric viruses.

9.1. Proposed Method for PCR Detection of TTV

Full-length TTV genomic sequences, collected worldwide, have been deposited in sequence databases. These sequences have confirmed that the TTV genome has regions of enormous variability; however, conserved regions also exist and appear to be localized to the UTR (Leary *et al*., 1999; Pollicino *et al*., 2003). PCR primers against variable and conserved regions of the TTV genome are available in the literature (Leary *et al*., 1999; Biagini *et al*., 2001; Pollicino *et al*., 2003), and primer sets have been characterized for specificity, sensitivity and ability to detect single genotypes of TTV or the entire virus genus. In water and serum samples, TTV prevalence publications typically use seminested PCR; this technique approaches a resolution of one molecule (Okamoto *et al*., 1998a; Okamoto *et al*., 1998b; Springfeld *et al*., 2000).

It is anticipated that TTV may be present at low levels in source wasters because of dilution, decay, and other environmental factors. Concentration of low levels of viruses from source waters may be achieved using hollow fiber ultrafiltration (HFUF) (Hill *et al.*, 2005; Olstadt *et al.*, 2008). This system is based on a 30,000 Dalton (Da) molecular weight cutoff and has been demonstrated to be effective for MS2 male-specific coliphage, noroviruses, and adenoviruses (Hill *et al.*, 2007; Sibley, 2008). It is expected to perform adequately for TTV as well. The recovery efficiencies may be validated using HFUF concentration with spiked PBS and/or dechlorinated and autoclaved tap water prior to use on source water samples. Concentrated eluates would be passaged through positively charged Sephadex and/or Chelex columns to remove inhibitory compounds. This method has been shown to filter humic compounds from a prepared solution of poliovirus (Abbaszadegan *et al.*, 1993). Virus particles then would be eluted from the columns with high ionic strength beef extract and precipitated with PEG. Viral nucleic acid would be liberated from capsids by extracting with guanidium thiocyanate and passing the sample through a silica column (Griffin *et al.*, 2003).

Leary and colleagues (1999) have developed nested primer sets to TTV genome regions 3087–3392 and 3293–3641 (GenBank Accession Number: AB008394). These primers are designed against the UTR of TTV; this region has been suggested by others to most likely detect all TTV genotypes (Itoh *et al.*, 1999; Mizokami *et al.*, 2000; Pollicino *et al.*, 2003). According to the genome organization described by Bendinelli *et al.* (2001), these primers exist within a region of regulatory sequences and stem loops, both of which are well conserved. Indeed, Leary *et al.* (1999) chose the primer sets based on conserved

nucleotide alignments among the most divergent TTV isolates. The specificity of the PCR products was verified using Southern hybridization and sequencing. Primer sensitivity was established by running the PCR system using serum solutions known to contain TTV nucleic acid as the templates. These primer sets together yielded a positive result in nearly 95 percent of known positive samples (Leary *et al*., 1999). This detection capacity is superior to many other primer sets described to date. These nested primer sets could be used in combination to detect conserved sequences of TTV in environmental water samples.

To measure the sensitivity of the PCR system, a region of the TTV sequence could be cloned into a plasmid. The clone could be amplified in competent *E. coli* cells, plasmid DNA could be isolated, and the cloned fragment sequence could be confirmed. Serial dilutions of the plasmid clones then could be spiked into concentrated water samples as the positive control. Pure water could be used as the negative control. Following PCR, gel electrophoresis with ethidium bromide staining would assess whether the positive control amplicon is the correct size and whether any species are amplified in the negative control. Subsequent sequencing of the gel-isolated, positive control amplicon would verify that the primers replicate the target sequence reliably. If inhibitors in the concentrated water samples preclude detection by PCR despite attempts to remove inhibitors, the water samples could be diluted 1:10 or 1:100 prior to PCR (Brooks *et al*., 2005). Dilution has been shown to remove inhibition sufficiently to allow for TTV detection in contaminated river water (Diniz-Mendes *et al*., 2008).

Determination of TTV infectivity currently is not possible as a facile *in vitro* culture system for this virus is unavailable. However, researchers culturing PBMCs and Chang liver cells suggest that a TTV-permissive and susceptive cell line may soon be in place for infectivity assessment (Maggi *et al*., 2001a; Mariscal *et al*., 2002; Desai *et al*., 2005). A culture method would be an extremely important complement to PCR analyses and would demonstrate: (1) whether TTV prevalence estimates in source waters correlate with infectious virus; and (2) the survival of infectious TTV particles through treatment system processes.

9.2. Proposed Evaluation of TTV in Source and Drinking Waters

The occurrence and density of TTV in feces, wastewater, and environmental source waters can be evaluated. In addition to monitoring for TTV, fecal and water samples can be analyzed for total coliforms using Colilert® in the quantitray format (Standard Method 9223, APHA *et al.*, 2005). Representative TTV-positive and TTV-negative samples also can be assayed for enteric viruses using the USEPA total culturable virus method and for coliphages using USEPA Method 1602 (USEPA, 2001b). These data can be used to evaluate whether TTV colocates with other enteric viruses and/or other indicators.

After demonstrating the ubiquitous nature of TTV in source waters, its fate through drinking water treatment processes can be evaluated. Prior research on the fate of TTV through wastewater treatment has demonstrated the ability of various processes to remove TTV. In particular, Haramoto *et al*. (2005b) found a positive TTV signal in 97% of wastewater influent samples over a 1-year period. Secondary and final effluent were

positive for TTV 18% and 24% of the time, respectively. Subsequent research should focus on TTV fate through drinking water treatment processes in comparison to currently used indicator organisms.

Numerous samples in geographically distinct areas of the United States can be evaluated, allowing for a diverse sampling of waters and treatment scenarios. A minimum of three treatment plants should be included in such a study. Samples at the plant influent and after each treatment step could be collected monthly and tested for TTV, *E. coli*, total coliforms, fecal coliforms, and turbidity. (The latter three represent required testing parameters under the SWTR.) Accepted methodologies from Standard Methods (APHA *et al.*, 2005) could be used to detect bacterial indicators and turbidity. Results from all measurements could be analyzed statistically to identify whether correlations exist.

CHAPTER 10 – CONCLUSIONS AND RECOMMENDATIONS

Among the enteric pathogens, viruses have the lowest infectious dose, are shed in the highest numbers, resist environmental stressors and treatment methods, and are specialized to infect only humans (Reynolds *et al*., 2008). For these reasons, it is critical to select an indicator that precisely colocates with enteric viruses. Traditional bacterial indicators colocate with viruses under some conditions, but the correlation is unreliable. The passage of the SWTR and subsequent amendments to the SDWA (*e.g.*, IESWTR and LT2) highlight the realization that viral pathogens do not always behave similarly to bacterial indicators. In fact, the sole use of bacterial indicators has led to instances of virus presence in the absence of indicators as well as indicator replication in receiving waters and false-positive predictions of health risks.

Bacterial indicators such as coliforms are useful for predicting the presence of bacterial pathogens. In an investigation of waterborne disease outbreaks from 1991–1998, total coliforms were detected in 100% of the outbreaks in which an enteric bacterial pathogen was the causative factor (Craun *et al*., 2002). This suggests that the most suitable indicator for a given pathogen group is one with similar size, transport, and survival characteristics. Consequently, an indicator of pathogenic enteric viruses should be a representative virus that demonstrates such similarities.

Traditional coliform monitoring takes about 1–2 days before results are obtained, and subsequent detection of fecal coliforms or *E. coli* may increase the testing duration. Virus detection by PCR is well established and results can be obtained from a concentrated

water sample within hours. Cell culture can be used to assess the infectivity of virus particles but requires 1–2 weeks for results. ICC-PCR, which compounds the benefits of cell culture and PCR, can rapidly and sensitively detect infectious virus in 2–3 days.

The start-up costs of molecular and *in vitro* methods to detect viruses are substantial, and some water utilities may lack the capability to perform these techniques. However, the accurate detection of virus presence and absence would somewhat balance these costs. The implementation of virus detection would eliminate false-positive results related to coliform growth and natural occurrence in source waters. Such false-positive results may cause a water utility to incur unnecessary costs in enhanced disinfection and filtration measures. Alternatively, more accurate virus detection would reduce the number of waterborne disease outbreaks of a virus etiology and likely would prevent many of the outbreaks of unknown etiologies.

An accepted viral indicator of enteric viruses is lacking. A virus that is representative of enteric viruses and is consistently detectable in the environment is hypothesized to perform as a useful indicator. TTV is unique among viruses because it is innocuous and ubiquitous in the human population and lacks any seasonal fluctuations, demographic selectivity, or geographical distribution. In this sense, TTV appears to be viral analog to coliform bacteria. However, like other viruses, TTV cannot replicate outside of a host cell and demonstrates the fate and transport characteristics of a colloidal particle rather than a living bacterial cell.

More research is needed to assess the indicator potential of TTV. A reliable PCR protocol must be established for this virus so that comparisons can be made in the literature regarding prevalence and colocation of TTV with other viruses and with traditional water quality indicators. A cell culture system capable of demonstrating CPE in response to infectious TTV also should be developed. The Chang liver cell line is a possible candidate. If the indicator capacity of TTV is substantiated, TTV detection could be performed routinely as a complement to bacterial indicators. If cost to water utilities is prohibitive, it may be possible for TTV to be tracked on a triggered basis. For instance, precipitation events often correlate with waterborne disease outbreaks (Curriero *et al.*, 2001). Selectively monitoring TTV during precipitation may be nearly as effective as routine monitoring. Alternatively, outbreaks of viral etiologies often are associated with contaminated ground water and distribution system failures. TTV monitoring could be limited to these water supplies.

Preliminary research suggests that TTV may serve as a reliable indicator of viral pathogens. Development of TTV detection methods and a concerted monitoring effort in surface water, ground water, and through treatment systems are needed to assess the indicator potential of TTV. Such work is expected to significantly advance the field of water quality indicators and lead to more efficient protection of the public health.

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