Title

Global Occurrence of Torque Teno Virus in Water Systems

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Abstract

Bacterial indicator organisms are used globally to assess microbiological safety of waters. However, waterborne viral outbreaks have occurred in drinking water systems despite negative bacterial results. Using viral markers may therefore provide more accurate health risk assessment data. In this study, fecal, wastewater, stormwater, surface water (fresh and salt), groundwater and drinking water samples were analyzed for the presence or concentration of traditional indicators, innovative indicators and viral markers. Samples were obtained in the United States, Italy, and Australia and results compared to those reported for studies conducted in Asia and South America as well. Indicators included total coliforms, *E. coli, Enterococci*, male-specific coliphages, somatic coliphages and microviradae. Viral markers included adenovirus, polyomavirus, and a potential new surrogate, Torque Teno virus (TTV). TTV was more frequently found in wastewaters (38 – 100%) and waters influenced by waste discharges (25%) than in surface waters used as drinking water sources (5%). TTV was also specific to human rather than animal feces. While TTV numbers were strongly correlated to other viral markers in wastewaters, suggesting its utility as a fecal contamination marker, data limitations and TTV presence in treated drinking waters demonstrates that additional research is needed on this potential viral indicator.

Keywords

Torque Teno Virus; Fecal indicators; Viral Pathogen Monitoring

Introduction

Indicator organisms are used to establish potential risk from fecal contamination in drinking waters. The United States Environmental Protection Agency, the Council of European Communities, and the World Health Organization all specify bacterial indicators (*e.g.,* fecal coliforms, *E. coli*) in regulations and guidelines. However, waterborne viral outbreaks have occurred in treated drinking water systems where the systems were in compliance with regulations (Craun *et al.* 2006). In fact, many epidemiological studies fail to show a relationship between viral pathogens and bacterial indicators in environmental systems and through treatment processes (Ashbolt *et al.* 2001; Hamza *et al.* 2011; McQuaig and Noble 2011). Viral and protozoan pathogens are known to be more persistent in the environment than indicator bacteria (Wu *et al.,* 2011; Sidhu *et al.* 2012). In treatment systems, correlations between bacteria and viruses are often lacking as a result of differences in physical removal and inactivation kinetics (Payment *et al.* 1985; Blatchley *et al.* 2007; Carducci *et al.* 2008; Shin & Lee 2010; Lee & Sobsey 2011).

Using a virus to predict viral pathogen risk may overcome these limitations (Kopecka *et al.* 1993; Jiang *et al.* 2001; Abbaszadegan *et al.* 2008). Coliphages resemble many enteric viruses in their physical structure and morphology, and can be detected by plaque assay (Ashbolt *et al.* 2001). Some groups are found in high concentrations in wastewaters, are relatively resistant to chlorination, and can be used to distinguish between fecal pollution of human and animal origin (Leclerc *et al.* 2000; Scott *et al.* 2002; Long *et al*. 2005). However, other groups are rarely found in individual human feces and can replicate in the environment (Muniesa & Jofre 2004; Payment & Locas 2011). An alternative to indicators is direct pathogen monitoring which provides

information on actual risk from a particular virus but may not be indicative of risks from viruses in general. Noroviruses are the most common cause of acute nonbacterial gastroenteritis and based on structural similarities, have been presumed to have similar persistence in the environment and through treatment as other viruses (Bae & Schwab 2008; Park & Sobsey 2011). However, noroviruses are found in high concentrations in cold months but typically not in warm months, and correlations between noroviruses and fecal indicator bacteria are often lacking (Haramoto *et al.* 2006; Westrell *et al.* 2006). Human adenoviruses, which cause respiratory or gastrointestinal illness, have been detected in surface and groundwaters, wastewaters and finished drinking waters, though correlations between this pathogen and indicator bacteria or coliphages were often not present (Jiang & Chu 2004; Carducci *et al.* 2008; Ogorzaly *et al.* 2010; Okoh *et al.* 2010). Additionally, a lack of correlation with other pathogenic viruses indicates that data on adenoviruses may only provide risk from this one pathogen rather than viral pathogens in general. A potential viral surrogate that has not been fully examined is Torque Teno Virus (TTV).

TTV is a small, non-enveloped, single stranded DNA virus that was first isolated in Japan from the serum of a patient with post-transfusion hepatitis (Nishizawa *et al.* 1997). It was later detected in blood samples from patients in several other countries, including the United States, France, Italy, and Brazil (Leary *et al.* 1999; Biagini *et al.* 2000; Bendinelli *et al.* 2001; Maggi *et al.* 2001; Bassit *et al.* 2002; Diniz-Mendes *et al.* 2004; Devalle & Niel 2005). TTV appears to be present ubiquitously in humans and elicits seemingly innocuous infections. TTV in humans can be found throughout the body including in blood and feces, and replicates actively in most tissues and organs (Maggi & Bendinelli 2009; Okamoto 2009a). TTV infections have been

identified throughout the world, with highest infection rates in countries with poor sanitation (Maggi *et al.* 2011). Research suggests that TTV may possibly cause chronic lifelong viremias in most people regardless of age, health status, and other variants.

TTV is excreted in the feces and has been detected in wastewater streams in multiple countries. In Japan, TTV DNA was detected in 12 of 12 wastewater influent samples, with a geometric mean concentration of $1.7x10^4$ genomic copies/liter (Haramoto *et al.* 2008). The concentration of TTV DNA in the influent samples showed no clear seasonal pattern, suggesting that TTV infections occur year-round. Vaidya *et al*. (2002) found raw sewage prevalence of TTV DNA was statistically similar to the prevalence of hepatitis E virus RNA and hepatitis A virus RNA. Diniz-Mendes *et al.* (2008) found a TTV positivity rate of 92.3% in polluted streams of Brazil. In contrast, Hamza *et al.* (2011) found that TTV was not a suitable indicator of fecal contamination in river water in Germany resulting from low detection rates. In Brazil, Vecchia (2009) found that TTV was sporadic in surface water samples. For a new water quality indicator to be widely applied as coliforms or enterococci are currently, their relevance to geographical region and water type must be understood. Therefore, the purpose of this study was to evaluate TTV as an indicator of fecal contamination in water systems from three continents. TTV presence and/or concentration were compared to bacterial fecal indicators, coliphages and enteric viruses. Data were collected in the United States, Italy and Australia and compared to those published for Asia and South America to conduct a preliminary assessment of the suitability of this potential viral surrogate.

Methods

Sample Collection

TTV occurrence was evaluated by collecting and analyzing animal feces, wastewaters, stormwaters, surface waters (fresh and salt), groundwaters and finished drinking waters. Samples were collected and analyzed by Worcester Polytechnic Institute (Worcester, MA, USA), the University of Wisconsin-Madison (Madison, WI, USA), the University of Pisa (Pisa, Italy), and the Commonwealth Scientific and Industrial Research Organization (CSIRO) (Brisbane, Australia). Appropriate positive and negative controls were included for all tests. Samples were diluted or concentrated as appropriate to achieve acceptable detection limits. The samples analyzed are summarized in Table 1.

In the United States, fecal, wastewater and drinking water samples were collected from four different regions (Northeast, South, Midwest, and West) in multiple seasons. Fresh fecal samples (n = 75) included five animal groups: chicken, dog, equine (horse and donkey), rabbit, and ruminant (cow, sheep, goat and llama). Animals were monitored by the sampler and feces were collected in sterile containers immediately after defecation. Wastewater, surface water, ground water and finished drinking water samples were collected from municipalities. Wastewater samples (1 L) included influent and effluent samples (n = 25). Water samples (20 L) (n = 39) included raw surface waters, raw groundwaters and treated drinking waters collected from public water supply distribution systems (Plummer *et al.* 2014).

In Italy, wastewater and surface water samples were collected from the greater Pisa area, localized in the Tuscany region. Samples (n = 24, first sampling period; n = 58, second sampling period) were collected from the city of Pisa activated sludge wastewater treatment plant (1 L

influent and 10 L effluent). Surface water samples (10 L) were collected from the river Fiume Morto (n = 12) downstream from the city of Pisa wastewater treatment plant discharge and from a seawater outfall (n = 12) (Carducci *et al.* 2006; Verani *et al.* 2006).

In Australia, wastewater and stormwater samples were collected from the greater Brisbane area. Influent (1 L) and effluent (20 L) wastewaters were collected from the Luggage Point, Oxley Creek and Bundamba wastewater treatment facilities (n = 44). Stormwater samples (n = 40) were collected from two sites (Fitzgibbon and Markerston catchment areas) in Brisbane, Australia during three storm events. Samples were collected using automated sampling infrastructure (ISCO 6700 or equivalent) triggered by automated flow measurement (Doppler flowmeter or weir) (Toze *et al.* 2012; Sidhu *et al.* 2013).

Bacteria Enumerations

Data were collected for three bacterial indicators: total coliforms, *E. coli*, and enterococci. All enumerations were conducted in accordance with accepted methodologies and with appropriate quality control/quality assurance. In the United States, total coliforms and *E. coli* were enumerated using Standard Methods 9223 (APHA *et al.* 2012) with Colilert® (IDEXX, Westbrook, ME) in the multiple well format (Quanti-Tray®, IDEXX, Westbrook, ME) and yielded a Most Probable Number (MPN) of the target organisms per 100 mL. Dilutions and concentrations were performed as needed (Plummer and Long, 2013). In Italy, *E. coli* and enterococci concentrations were determined by Bio-Rad miniaturized methods (Bio-Rad Laboratories, Milan, Italy) using MUG/EC microplates and MUD/SF microplates, respectively. These methods provide a Most Probable Number of the indicators in accordance with ISO 9308-

3 for *E. coli* (ISO 1998a) and ISO 7899-1 for enterococci (ISO 1998b) (Bofill-Mas *et al.* 2010). In Australia, fecal bacteria (*E. coli* and *Enterococci*) were quantified using the membrane filtration technique. Samples (1 and 10 mL) were filtered through 0.45 µm nitrocellulose (Millipore, Billerica, MA) membranes which were placed on Chromocult™ coliform agar (Merck, Munchen, Germany) for *E. coli* and Chromocult™ Enterococci agar (Merck, Munchen, Germany) for enterococci. The plates were incubated overnight at 37°C and then typical colonies were counted providing colony forming unit counts (Sidhu *et al.* 2012).

Coliphage Enumeration

Coliphages were enumerated in accordance with accepted methodologies and with appropriate QA/QC procedures. The United States samples were analyzed for somatic and male-specific coliphages by EPA Method 1602, the single layer agar method (U.S. EPA 2001). *E. coli* CN-13 (ATCC 700609, Manassas, VA; resistant to nalidixic acid) and *E. coli* F-amp (ATCC 700891, Manassas, VA; resistant to streptomycin and ampicillin) were used as hosts for somatic and male-specific coliphages, respectively. Samples were supplemented with magnesium chloride, log phase host bacteria, and agar. Plates were incubated overnight at 36°C and examined for plaque forming units (PFU)/100 mL. In Italy, somatic coliphages were enumerated by the ISO double agar layer plaque assay method using *E. coli* C (ATTC 13706, Manassas, VA) as the host strain (ISO 1999). The sample, host and top layer agar were mixed and added to a plate with a hard layer of agar. The plates were incubated overnight at 36°C and counted for PFU/100 mL. In Australia, somatic coliphages (Microviridae family) were enumerated using quantitative PCR (qPCR) with Bio-Rad iQ^{TM}5 (Bio-Rad Laboratories, California, USA) using iQ^{TM} Supermix real-time PCR kit. Details are provided in Sidhu *et al*. (2012).

Viral Enumeration

In the U.S. drinking water samples were HFUF concentrated through Asahi REXEED-21S filters with a 30 kDa molecular weight cutoff. HFUF concentrates and wastewaters were concentrated using PEG precipitation, and 0.25 g of fecal samples were extracted directly. Multiple PCR methods were used to enumerate viruses. Extraction of nucleic acid was accomplished by bead beating (PEG concentrates and solid samples) and the use of a clean-up kit (PowerSoil® DNA Isolation Kit, MO BIO Laboratories, Carlsbad, CA) to reduce inhibitor concentrations. For TTV, amplification of target ssDNA was conducted using a traditional PCR assay modified from that reported by Carducci *et al.* (2008). All positive TTV samples and a selected number of negative TTV samples were analyzed for the presence of human adenovirus. A qPCR assay was developed with primer/probe sets, master mix conditions, and thermocycler program modified from those described by Jothikumar *et al.* (2005). Full details of the U.S. methods are provided in Plummer and Long (2013).

In Italy, water samples were concentrated using two-stage tangential flow ultrafiltration. After prefiltration on polypropylene membranes, the samples were filtered through a polysulphone membrane with a 10,000 MW exclusion size. The samples were reconcentrated with a mini-ultrasette apparatus and washed using 15 to 20 mL of 3% beef extract at pH 9, obtaining a concentrated sample of 40 mL at pH 7. The concentrated samples were decontaminated with chloroform and the nucleic acids were extracted with QIAamp DNA kit (QIAGEN, Germany). The extracted nucleic acids were assayed with qualitative and quantitative biomolecular tests

(nested PCR and Real Time PCR) according to published protocols to reveal the presence and the titer of adenoviruses and TTV viral genomes (Carducci *et al.* 2009).

In Australia, samples were analyzed by qPCR for TTV, adenovirus, polyomavirus, and microviridae. The samples were concentrated using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA). Samples were pumped with a peristaltic pump in a closed loop with high-performance, platinum-cured L/S 36 silicone tubing. Samples were concentrated to approximately 100 mL and further concentration of sample was carried out by JumboSep with 100 K MWCO filters (Pall, Australia) to a final volume of approximately 10 mL (Sidhu *et al.* 2013). Nucleic acid was extracted from 200 µL of each concentrated sample using the QIAGEN DNeasy Blood and Tissue kit (QIAGEN Inc., Valencia, CA) per manufacturer instructions, and stored at 80°C until processed. Amplifications were performed in 25 µL reaction mixtures using iQ Supermix (Bio-Rad Laboratories, Berkeley, CA). The PCR mixture contained 12.5 µL of Supermix, 400–500 nM each primer, 400–600 nM corresponding probe and 3 µL of template DNA (Sidhu *et al.* 2013).

Statistical Analyses

Statistical analyses were conducted using IBM's Statistical Package for the Social Sciences (SPSS) product, Version 17.0. Analysis of variance (ANOVA) was conducted on the quantitative data sets to evaluate differences based on season and country. Correlations analyses were conducted to determine correlations among the bacterial indicators, coliphages and viruses. The Spearman Rank correlation analysis was used for quantitative data sets, and the point biserial

correlation for binary data sets. Statistical analyses were conducted for data sets with over 20 results. All analyses were conducted at the 95% confidence level ($\alpha = 0.05$).

Results

Bacterial Indicator Results

Total coliforms, *E. coli* and/or enterococci were enumerated in all samples (data not shown). Results showed expected patterns. Fecal samples had a wide range of indicator concentrations, from below detection limits to 10^8 MPN per gram. Among water samples, raw wastewaters had the highest indicator concentrations (up to 10^8 MPN per 100 mL total coliforms and 10^6 MPN per 100 mL *E. coli*). Levels decreased by 2 to 3 orders of magnitude through treatment, with bacteria levels in the hundreds of MPN per 100 mL in final wastewater samples prior to disinfection. Next, stormwater samples from Australia had lower fecal bacterial levels than wastewater, with concentrations in the tens to 10^4 CFU/100 mL. Seawater and surface water samples were variable depending on overall water quality. Samples downstream of a wastewater treatment plant outfall had *E. coli* in the thousands per 100 mL, compared to drinking water sources in the tens per 100 mL. Finished drinking waters were all negative for *E. coli*, as expected.

Coliphage Results

Coliphage concentrations in fecal samples, wastewaters and waters are summarized in Table 2. Male-specific and somatic coliphages were detected in approximately half of the fecal samples with 41 of 75 samples (54.7%) below detection limits for male-specific coliphages and 32 of 75 (42.7%) below detection limits for somatic coliphages. For fecal samples with detectable levels

of coliphages, the maximum male-specific and somatic coliphage concentration were observed in chickens ($2.0x10^6$ PFU/g and $2.5x10^7$ PFU/g, respectively).

The U.S. raw wastewater samples had maximum concentrations of $3.0x10^5$ and $1.6x10^5$ PFU/100 mL for male-specific and somatic coliphages, with median concentrations on the order of 10^4 PFU/100 mL. Coliphage reductions through treatment varied significant, with approximately 4 log reduction for male-specific coliphages and no significant reduction for somatic coliphages. The raw wastewater samples from Italy had a maximum somatic coliphage concentration of $1.0x10^7$ PFU/100 mL, with a median of $2.4x10^6$ PFU/100 mL and a median reduction of 2 orders of magnitude through treatment. Stormwater samples had a median of 90 PFU/100 mL and a maximum of 870 PFU/100 mL for somatic coliphages.

Coliphage concentrations in surface waters from the U.S. were much lower than samples from Italy. The median and maximum concentration of somatic coliphages in the sea water samples (n = 12) were 250 and 700 PFU/100 mL. In surface water samples in Italy, median and maximum concentrations were on the order of 10^5 PFU/100 mL. For the U.S. surface water samples, 80.0% and 46.7% were below detection limits for male-specific and somatic coliphages, and for ground water samples, 75.0% were below detection limits for both male-specific and somatic coliphages. For samples with detectable levels, concentrations were in the tenths to ones of PFU/100 mL. U.S. surface and groundwater samples were all drinking water sources. Similarly, the distribution system samples had a high percentage of non-detects (80.0%) for coliphages.

Virus Results

Quantitative results for viruses are summarized in Table 3. TTV was above detection limits in 36 of 58 (62.0%) wastewater samples in Italy with a maximum concentration of $3.6x10^5$ genomic copies per mL in raw wastewater and a median reduction of an order of magnitude through treatment. Stormwater and wastewater samples from Australia were quantified for TTV (n=22), adenovirus (n=44), polyomavirus (n=44) and microviridae (n=22). All samples had quantifiable virus numbers, with maximum TTV in raw wastewater of $2.4x10^3$ genomic copies per mL which was reduced by approximately two orders of magnitude through treatment. Maximum adenovirus, polyomavirus and microviridae concentrations were all on the order of 10^3 genomic copies per mL in treated effluent with similar removals through treatment. Stormwater samples tested for viral markers were primarily in the one and tens of genomic copies per mL with maximum concentrations of 13, 9.1, and 33 genomic copies per mL for TTV, adenovirus, and polyomavirus, respectively.

In addition to the quantitative virus data, presence/absence testing was conducted using different methodologies for selected samples. TTV was present in 3 of 76 fecal samples (4.0%). In wastewaters, TTV was present in 38 to 49% of samples, depending on sample type (raw versus final) and location (country). Surface water detection was rare, with 3 of 12 river waters in Italy positive for TTV; however, no sea water samples in Italy and no surface water samples in the U.S. had TTV. There was 1 (of 4) groundwater samples and 4 (of 20) drinking water samples positive for TTV in the U.S. Adenovirus was not found in any fecal samples, surface waters, groundwaters or drinking waters in the U.S., but was detected in the majority of wastewater samples (100% of raw samples and 67% of treated samples).

Discussion

TTV is ubiquitous and has been reported in a wide range of fecal samples from warm-blooded animals and humans. It has also been detected in certain animal species, including non-human primates (Verschoor *et al.* 1999; Cong *et al.* 2000; Okamoto & Mayumi 2000), farm animals (pigs, chickens, cows and sheep) (Devalle & Niel 2005; Leary *et al.* 1999; Brassard *et al.* 2010; Martinez Guino *et al.* 2010; Lang *et al.* 2011; Liu *et al.* 2011;; Sibila *et al.* 2009), companion animals (dogs and cats) (Biagini *et al.* 2007; Okamoto 2009b; Zhu *et al.* 2011), and wild animals (wild boar and sea lions) (Martinez *et al.* 2006; Ng 2009). For example, a study of 158 fecal samples collected from dogs younger than 1 year old with diarrhea in a pet clinic in China showed that 20 specimens (20/158, 13%) were positive for Torque Teno canis virus DNA using detection with PCR (Lan *et al.* 2011). While TTV has been identified in a variety of animal fecal samples, this study only had a 4.0% detection rate for TTV in fecal samples. In this study, a human based TTV sequence was utilized and therefore presence of this sequence would not be expected in animal fecal samples. Positive detection of human TTV in 4.0% of non-human animal feces may have been a result of human-animal cross infection. The one TTV positive chicken sample was from a private farm (and was weakly positive) and the two TTV positive dog samples were from companion animals.

TTV has been detected in feces and thus in sewage in prior research (*e.g.*, Haramoto *et al.* 2008). The percentage of raw wastewater samples that are positive for TTV varies greatly in different studies: 97% in Japan (Haramoto *et al.* 2005), 50% in Germany (Hamza *et al.* 2011), and 13% in India (40 mL sample volume, Vaidya *et al.* 2002). In this study, quantitative data showed

detectable levels of TTV in 100% (n=11) and 69% (n=29) of raw wastewaters from Australia and Italy, respectively. Using presence/absence data, 49% (n=41) and 38% (n=13) of raw wastewaters in Italy and the U.S. were positive for TTV, respectively. Although there were differences in samples volumes and analytical methods, these values are comparable to previously published statistics. In this study, maximum concentrations of TTV were on the order of 10^5 genomic copies per mL in raw wastewaters in Italy and 10^3 genomic copies per mL in Australia. Results are comparable to Hamza *et al.* (2011), who found TTV at concentrations on the order of 10^3 genomic copies per mL in raw wastewater in Germany. Haramoto *et al.* (2008) detected TTV in 12 of 12 wastewater samples in Japan with a mean and maximum concentration of $1.7x10^4$ and $4.8x10^4$ genomic copies per liter (for comparison, on the order of 10^7 per mL), respectively. Removal rates of TTV through wastewater treatment in this study were similar to Hamza *et al.* (2011), who found 1.7 – 2.3 and 2.6 – 3.5 $\log_{10}$ removals for adenovirus and TTV, respectively. In India, TTV was isolated in 2% of wastewater effluent samples using 40 mL volumes. In this study, the percentage of treated wastewater samples with TTV (via quantitative or presence/absence testing) ranged from 39 to 100%, with differences based on sample volume, methodology, and treatment stage (before or after final disinfection).

The concentration or presence of viruses in environmental and drinking waters depends on the water source and influence of sewage discharges or other pollution sources on those waters. River water samples (10 L) collected from the Ruhr and Rhine Rivers in Germany in 2008-2009 were positive for adenoviruses in 108 of 111 (97.3%) of samples, and for TTV in 56 of 108 (51.9%) of samples (Hamza *et al.* 2011). These samples were collected in populated regions where wastewater treatment plant discharges were 1.5 to 9 km upstream of each sampling

location. Diniz-Mendes *et al.* (2008) found high prevalence rates (92%) for TTV in samples from polluted streams in Brazil. In contrast, Vecchia *et al.* (2012) quantified TTV and fecal pollution in an urban area in Brazil that was influenced by non-treated sewage. TTV and adenovirus were found in 28.6% and 21.4% of river samples, respectively. A study of the Tamagawa River in Japan found TTV in only 5.6% of samples and adenovirus in 61.1% of samples (Haramoto *et al.*, 2010). The sampling locations included a recreational area, and two sites with significant wastewater influences. In this study, samples were collected in water sources expected to be less influenced by wastewater discharges. While TTV and adenoviruses were found in all stormwater samples in Australia, median values were in the tenths to ones of genomic copies per mL. TTV was not found in seawater samples in Italy, nor in surface waters in the U.S. One groundwater sample was positive for TTV. Vecchia *et al.* (2012) found TTV below detection limits in surface waters in an area in Brazil with 92% sewerage, comparable to sewerage rates in areas of study in the U.S.

Analysis of Variance

Analysis of variance was conducted on quantitative data sets to evaluate seasonal differences. There were no seasonal differences for indicators (bacteria or coliphages) in fecal samples, wastewaters, or fresh surface waters. For drinking water samples, seasonal differences were found for *E. coli* ($n=20$; $p=0.042$) and somatic coliphages ($n=20$; $p = 0.016$). However, *E. coli* was below detection limits in all samples, and thus this variance was based on the detection limit rather than actual differences in samples. For somatic coliphages, only 4 of 20 samples were above detection limits. Blatchley *et al.* (2007) found that coliphages exhibited seasonal effects with concentration higher in the summer than those observed in the winter in wastewaters. In this

study, differences in phage concentrations were not seen in wastewaters (for which many more samples had concentrations above detection limits than in drinking waters). In the literature, adenoviruses have been proposed as indicators of fecal pollution from human sources because of their culturability, resistance characteristics and lack of seasonal variability (Jiang *et al.* 2001; Choi & Jiang 2005; Jiang *et al.* 2007; Simmons & Xagoraraki 2011). There were insufficient data in this study to test the seasonality of adenoviruses. An ANOVA was performed between countries for the wastewater samples, and somatic coliphages varied by country (U.S. and Italy; $n=107$; $p < 0.001$). For TTV in wastewaters ($n=80$), there was no seasonal or geographical variation.

Correlation Analysis

Spearman Rank correlations were calculated for quantitative data sets and point biserial for binary data sets. Table 4 shows correlation analysis results for TTV in various matrices, though correlations were performed for all data sets with $n \geq 20$ (full results not shown). In the fecal samples, there were correlations between the bacterial indicators and somatic coliphages, but no correlations to male-specific coliphages. TTV presence (of which only 3 samples were positive) was not correlated to any other parameter tested.

In wastewater samples, expected correlations between bacterial indicators were found, and similarly, the quantitative virus data sets correlated to one another (TTV, adenovirus, polyomavirus, microviridae). In a similar study of sewage treatment, Vaidya *et al*. (2002) found TTV DNA correlated to enteric viruses in raw sewage where the prevalence of TTV DNA was statistically similar to the prevalence of hepatitis E virus RNA and hepatitis A virus RNA. This

study also included TTV correlations to *E. coli*, enterococci, somatic coliphages, and viruses. TTV and microviridae (a family of bacteriophage which includes somatic phage ΦX-174) may provide useful information based on their correlations to other viruses in wastewaters.

For the stormwater samples, relationships were identified between *E. coli*, enterococci and somatic coliphages, but very few correlations were found with other viral pathogens. In surface waters, TTV presence was correlated to *E. coli* and somatic coliphages; however, with very few samples positive for TTV, these relationships should be considered preliminary. Other data in the literature has shown no statistical correlation between somatic coliphages and enteroviruses, human adenovirus, or Norwalk (I and II) virus in rivers in France (Hot *et al.*, 2003). In drinking waters, the number of TTV positive samples was again low; however in these samples, TTV was not correlated to other indicators.

Conclusions

Currently, bacterial indicators such as coliforms, *E. coli*, and enterococci are applied to waters worldwide to indicate the potential risk for fecal contamination. The strengths and weaknesses of these indicators regarding this universal application are supported by over 100 years of use. Improvements in microbial diagnostics and disease surveillance have demonstrated that bacterial indicators are not always protective when the pathogens in question are viruses. Based on its characteristics and recent discovery, Torque Teno virus has been investigated in multiple geographical locations for its potential as a universal indicator. This study analyzed three environmental monitoring datasets that included TTV from three different continents (North America, Europe and Pacifica). These results were compared to those in the literature from Asia

and South America. The results demonstrate the presence and occurrence of TTV on all five continents; however, there was significant variability in environmental prevalence and concentrations. Comparisons of TTV to bacterial indicators and other viral indicators demonstrated that its occurrence and concentrations do not strongly correlate to either group. Thus, TTV monitoring could potentially provide supplemental information about a water's microbial content than bacterial indicators or other candidate viral indicators. However, the datasets were small and did not utilize the same methodologies. Overall, these results support the need for careful, coordinated investigation of TTV as a water quality viral indicator before it can be adopted or abandoned.

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Table 1 – Summary of Sampling Events

Source	Location	Dates (MM/YY)	Total Samples	Individual Samples	Sample Type
Fecal	United States	06/10 to 04/11	75	10	Chicken
				15	Dog
				22	Equine
				3	Rabbit
				25	Ruminant
Wastewater	United States	06/10 to 04/11	25	13	Influent
				12	Effluent
	Italy	04/04 to 03/05	24	12	Influent
				12	Effluent
		03/07 to 04/08	58	29	Influent
				29	Effluent
	Australia	01/10 to 06/10	44	22	Influent
				22	Effluent
Stormwater	Australia	01/12 to 03/12	40	16	Markerston Catchment
				24	Fitzgibbon Catchment
Surface Water	United States	05/11 to 03/12	15	15	Fresh Surface Water
	Italy	05/04 to 04/05	12	12	River Water
	Italy	05/04 to 04/05	12	12	Sea Water
Ground-water	United States	05/11 to 03/12	4	4	Raw Groundwater
Drinking Water	United States	05/11 to 03/12	20	20	Distribution System

Table 2 – Coliphage Indicator Data

Country	Source	Sample Type	n	Male Specific Coliphage (PFU/g or 100 mL)			Somatic Coliphage (PFU/g or 100 mL)		
				Min	Median	Max	Min	Median	Max
U.S.	Fecal	Chicken	10	BDL	BDL	$2.0x10^{6}$	BDL	$2.0x10^{4}$	$2.5x10^{7}$
U.S.		Dog	15	BDL	BDL	170	BDL	6.1	$1.8x10^{4}$
U.S.		Equine	22	BDL	9.2	$2.9x10^{4}$	BDL	BDL	$1.0x10^{5}$
U.S.		Rabbit	3	BDL	370	$4.9x10^{4}$	BDL	BDL	$3.0x10^{5}$
U.S.		Ruminant	25	BDL	BDL	$5.2x10^{4}$	BDL	180	$8.4x10^{4}$
U.S.	Wastewater	Raw	13	$2.2x10^{3}$	$9.0x10^{4}$	$3.0x10^{5}$	733	$4.0x10^{4}$	$1.6x10^{5}$
U.S.		Final	12	BDL	120	760	170	$1.4x10^{3}$	$5.1x10^{5}$
Italy		Raw	41	NT	NT	NT	$4.0x10^{5}$	$2.4x10^{6}$	$1.0x10^{7}$
Italy		Final	41	NT	NT	NT	$1.0x10^{3}$	$1.9x10^{4}$	$2.0x10^{6}$
Australia	Storm-water	Storm-water	40	NT	NT	NT	1.0	91	870
Italy	Surface Water	Sea	12	NT	NT	NT	0.10	250	700
Italy		River	12	NT	NT	NT	$4.6x10^{4}$	$1.7x10^{5}$	$4.6x10^{5}$
U.S.		Fresh	15	BDL	BDL	1.8	BDL	BDL	5.8
U.S.	Groundwater	Raw	4	BDL	BDL	1.0	BDL	BDL	0.34
U.S.	Drinking water	Distribution	20	BDL	BDL	190	BDL	BDL	0.52

NT – Not Tested, BDL – Below Detection Limit

Table 3 – Virus Data

(a) Quantitative Data														
Country	Source	Sample Type	n	TTV (genomic copy per mL)			Adenovirus (genomic copy per mL)			Polyomavirus (genomic copy per mL)			Microviridae (genomic copy per mL)		
				Min	Median	Max	Min	Median	Max	Min	Median	Max	Min	Median	Max
Italy	Waste-water	Raw	29	BDL	697	$3.6x10^5$	NT	NT	NT	NT	NT	NT	NT	NT	NT
Italy		Final	29	BDL	17	$2.4x10^4$	NT	NT	NT	NT	NT	NT	NT	NT	NT
Australia		Raw	11-22	130	250	$2.4x10^3$	110	510	$9.1x10^3$	410	$1.0x10^3$	$2.2x10^3$	$1.2x10^3$	$2.2x10^3$	$5.5x10^3$
Australia		Final	11-22	0.19	0.90	3.9	0.18	0.83	6.0	0.077	0.26	1.2	0.090	0.43	3.8
Australia	Storm-water	Stormwater	24-40	0.010	2.2	13	0.0040	0.22	9.1	0.010	0.010	33	NT	NT	NT

(b) Presence/Absence Data							
Country	Source	Sample Type	n	TTV Positive		Adenovirus Positive	
				Number	Percentage	Number	Percentage
U.S.	Feces	Chicken	10	1	10%	0	0%
U.S.		Dog	15	2	13%	0	0%
U.S.		Equine	22	0	0%	0	0%
U.S.		Rabbit	3	0	0%	0	0%
U.S.		Ruminant	25	0	0%	0	0%
U.S.	Wastewater	Raw	13	5	38%	12 (of 12)	100%
U.S.		Final	12	5	42%	8	67%
Italy		Raw	41	20	49%	NT	NT
Italy		Final	41	16	39%	NT	NT
Italy	Surface water	Sea	12	0	0%	NT	NT
Italy		River	12	3	25%	NT	NT
U.S.		Fresh	15	0	0%	0	0%
U.S.	Groundwater	Raw	4	1	25%	0	0%
U.S.	Drinking water	Distribution	20	4	20%	0 (of 11)	0%

NT - Not Tested

Table 4. Correlation analysis results (two tailed, 95%) for TTV versus other indicators and viruses. Statistically significant correlations in bold; p-value and n value in parentheses (ID = insufficient data if n < 20; NA = data not collected)

(a) TTV Presence (point biserial correlations)

Matrix	Parameter					
	Coliforms (	*E. coli*	Enterococci	Male-specific coliphage	Somatic coliphage	Adenovirus Presence
Feces	0.156 (0.181, 75)	0.070 (0.553, 75)	NA	-0.025 (0.83, 75)	0.057 (0.624, 75)	*
Wastewater	0.050 (0.814, 25)	0.150 (0.123, 107)	**0.423 (0.001, 58)**	**0.421 (0.036, 25)**	0.138 (0.156, 107)	-0.302 (0.151, 24)
Surface water	ID	**0.396 (0.013, 39)**	NA	ID	**0.421 (0.008, 39)**	ID
Drinking Water	-0.116 (0.627, 20)	-0.130 (0.584, 20)	NA	-0.115 (0.628, 20)	-0.177 (0.455, 20)	ID

* cannot be computed because one variable constant

(b) TTV (gc/mL) (Spearman correlations)

Matrix	Parameter					
	E. coli	Enterococci	Somatic coliphage	Adenovirus (gc/mL)	Polyomavirus	Microviridae
Wastewater	**0.553 (0.000, 58)**	**0.524 (0.000, 58)**	**0.319 (0.015, 58)**	**0.710 (0.000, 22)**	**0.823 (0.000, 22)**	**0.740 (0.000, 22)**
Stormwater	0.102 (0.635, 24)	0.088 (0.683, 24)	-0.354 (0.089, 24)	-0.275 (0.193, 24)	0.144 (0.501, 24)	NA