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Identifying septic pollution exposure routes during a waterborne norovirus outbreak - A new application for human-associated microbial source tracking qPCR

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Abstract

In June 2017, the Pennsylvania Department of Health (PADOH) was notified of multiple norovirus outbreaks associated with 179 ill individuals who attended separate events held at an outdoor venue and campground over a month period. Epidemiologic investigations were unable to identify a single exposure route and therefore unable to determine whether there was a persistent contamination source to target for exposure mitigation. Norovirus was detected in a fresh recreational water designated swimming area and a drinking water well. A hydrogeological site evaluation suggested a nearby septic leach field as a potential contamination source via ground water infiltration. Geological characterization revealed a steep dip of the bedrock beneath the septic leach field toward the well, providing a viral transport pathway in a geologic medium not

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previously documented as high risk for viral ground water contamination. The human-associated microbial source tracking (MST) genetic marker, HF183, was used as a microbial tracer to demonstrate the hydrogeological connection between the malfunctioning septic system, drinking water well, and recreational water area. Based on environmental investigation findings, venue management and local public health officials implemented a series of outbreak prevention strategies including discontinuing the use of the contaminated well, issuing a permit for a new drinking water well, increasing portable toilet and handwashing station availability, and promoting proper hand hygiene. Despite the outbreaks at the venue and evidence of ground water contamination impacting nearby recreational water and the drinking water well, no new norovirus cases were reported during a large event one week after implementing prevention practices. This investigation highlights a new application for human-associated MST methods to trace hydrological connections between multiple fecal pollutant exposure routes in an outbreak scenario. In turn, pollutant source information can be used to develop effective intervention practices to mitigate exposure and prevent future outbreaks associated with human fecal contaminated waters.

Keywords

HF183; microbial source tracking; ground water; septic; norovirus; outbreak

1. Introduction

Norovirus is the most common cause of acute gastroenteritis (AGE) outbreaks and AGE illness across the age spectrum in the United States (US), accounting for between 19 and 21 million total illnesses per year, primarily causing vomiting and diarrhea (Hall et al., 2013). This highly infectious virus is spread typically via the fecal-oral route through either direct person-to-person transmission, consumption of contaminated food or water, or through contact with contaminated environments (e.g., contaminated surfaces) (Lopman et al., 2012, Messner et al., 2014). Human noroviruses are classified into five genogroups (GI, GII, GIV, GIII, and GIX) (Chhabra et al., 2019), among which the GII.4 genotype is responsible for most illnesses and associated with elevated rates of severe outcomes (Burke et al., 2019). Because of their small size and surface properties, these viruses are capable of infiltrating through subsurface matrices to contaminate ground water (Berger, 2008, Bradford et al., 2015, Fout et al., 2017). Norovirus is believed to remain infectious in ground water for months and remain detectable in the environment for years if left untreated (Kauppinen et al., 2017, Seitz et al., 2011). Contaminated ground water accounted for 422 (52.7%) of known drinking-water outbreaks in the US from 1971 to 2006, and of the US drinking water outbreaks attributed to norovirus, approximately 32% were credited to undisinfected ground water sources within roughly the same time period (Craun et al., 2010, Wallender et al., 2014). Norovirus was also the cause of 47% of untreated recreational water outbreaks in the US from 2000 to 2014 (Graciaa et al., 2018, Sinclair et al., 2009).

Onsite septic systems are used worldwide to treat domestic wastewater from individual or small groups of dwellings in areas that are not connected to a main sewage etwork, with an estimated 25% of US households relying on septic systems (USEPA, 2002). Recent reports indicate that septic systems are a common contributor of fecal pollution to surface waters

(USEPA, 2013, USEPA, 2014) and were the primary contributor to outbreaks associated with undisinfected ground water sources used for drinking in the US from 1971 to 2008 (Wallender, et al., 2014). Several studies have shown septic system influences on the microbial quality of surface waters (Atoyan et al., 2011, Lipp et al., 2001, Lipp et al., 2001, Young et al., 1999), and septic system density associated with both higher fecal contamination levels in surface waters (Lipp, et al., 2001, Mallin et al., 2012, Sowah et al., 2014, Sowah et al., 2017, Verhougstraete et al., 2015), as well as with endemic diarrheal illness (Borchardt et al., 2003). Unsurprisingly, failing, poorly designed, or overloaded septic systems are often associated with surface water fecal contamination (Ahmed et al., 2005, Habteselassie et al., 2011). Failing septic systems have also been a factor in norovirus outbreaks worldwide associated with contaminated surface water (Yoder et al., 2004) and norovirus outbreaks in the US associated with contaminated well water (Anderson et al., 2003, Gunnarsdottir et al., 2013). Surprisingly, newly constructed and inspected septic systems operating below capacity have also been implicated as a source of well water contamination linked to norovirus outbreaks (Borchardt et al., 2011, Jack et al., 2013, Kauppinen et al., 2017).

The hydrogeological features contributing to septic contamination leading to viral outbreaks are not commonly described. For those reported outbreaks with geological information, karst or fractured bedrock aquifers and unconsolidated alluvial or glacial sand and gravel are the most often reported hydrogeological risk factors, likely because those features can allow for efficient microbial transport to the subsurface (Berger, 2008). Conversely, certain hydrogeological features are not commonly found at sites where septic contamination of the subsurface has led to viral outbreaks, such as horizontally layered, cemented geologic strata, (*i.e.,* non-karst sandstone, siltstone and shale), likely because the layers sufficiently prevent septage infiltration into the aquifer. Understanding human fecal waste movement through the subsurface environment is critical to help characterize how these potential transport routes can channel pathogens such as norovirus to surrounding drinking water sources and recreational water areas.

The presence and concentration of human fecal contamination in the environment can be measured using molecular-based tools that target genetic sequences harbored by microbes closely associated with the human gut microbiome (Boehm et al., 2013). Human-associated microbial source tracking (MST) PCR-based methods are available that can track human waste in environmental settings from point or non-point sources, such as combined sewer overflows, sewage line breaks, or malfunctioning septic systems (Shanks et al., 2020). In particular, the human-associated MST genetic marker, HF183, is reported to effectively detect human waste in US environmental waters, even when multiple non-human fecal sources are present (Layton et al., 2013). A nationally validated and standardized HF183 quantitative real-time PCR (qPCR) protocol was recently released by the US Environmental Protection Agency (USEPA) for characterizing human fecal waste in recreational waters (USEPA, 2019). In addition, the HF183 genetic marker was recently utilized to identify septic contamination in household drinking water wells (Murphy et al., 2020, Naphtali et al., 2019).

Because of the diverse modes of norovirus transmission and persistence in the environment, it can be difficult to determine a single environmental contamination source or exposure during a norovirus outbreak. However, since the most prevalent strains of human norovirus (GI and GII) are thought to be exclusively shed in human waste and not in other animal feces (Villabruna et al., 2019), HF183 could be a useful tool during an outbreak response to characterize human fecal contamination exposure routes and develop effective mitigation strategies to prevent future outbreaks (Paruch et al., 2019). Here we describe a norovirus outbreak at an outdoor event venue with a campground where inconclusive epidemiologic information necessitated an environmental investigation to determine the contamination exposure route(s) and pollutant source to guide targeted mitigation and prevention practices. Based on a site evaluation, the campground septic system was suspected as the pollutant source for both a recreational creek and drinking water well via contaminated ground water. Because the site did not contain any of the common hydrogeological risk factors for virus transport to ground water, the HF183 genetic marker and general fecal indicator viruses (somatic and f-specific coliphage) were used as microbial tracers to demonstrate any hydrogeological connections between the malfunctioning septic system, drinking water well, and recreational water area. Findings from the investigation highlight a new application for human-associated MST methods to help trace hydrological connections between multiple fecal pollutant exposure routes in an outbreak scenario and illustrate how source information can be used to develop effective mitigation strategies to prevent future outbreaks.

1.1. Background Outbreak and Epidemiology Information

This section describes available outbreak information and epidemiological activities available prior to the on-site environmental investigation reported in this study. On June 13, 2017, the Pennsylvania Department of Health (PADOH) was notified of AGE, defined as vomiting or diarrhea (≥ 3 loose stools in 24 hours period), among attendees at an event held June 8–11 at an outdoor venue and campground (Figure 1; Event 2). On June 18, PADOH was again notified of AGE among attendees at a second event (Figure 1; Event 3) held at the same venue the following week. Upon epidemiological investigation (see Supplementary Material (SM)), a total of 179 AGE cases were self-reported to PADOH by event attendees between May 31 and June 18. Norovirus was detected in seven of eight clinical specimens submitted by event attendees with AGE; GI was detected in three specimens and GII was detected in the other four specimens (data not shown). Four of these samples were further typed as norovirus GI.7[P7] in two GI clinical samples and GII.13[P16] in the two other GII clinical samples. Standard interventions such as practicing proper hand hygiene and disinfecting surfaces were recommended and implemented to prevent any additional norovirus outbreaks. However, concern remained that other transmission routes beyond person-to-person contact could be contributing to AGE associated with the venue.

On June 21, PADOH partnered with the U.S. Centers for Disease Control and Prevention (CDC) to further test for the presence of norovirus and fecal contamination in venue waters. Norovirus GI and GII, as well as *Escherichia coli*, were found in the primary drinking water well and the recreational creek (Table S1). On June 27, the PA Department of Environmental Protection (PADEP) also found evidence of fecal contamination (*Escherichia coli* presence) in the drinking water well consistent with PADOH results and issued a

boil water advisory requiring the venue to supply safe drinking water and discontinue the use of the contaminated kitchen well (data not shown). These initial water quality testing and standard epidemiological efforts could not alone identify a primary mode of transmission responsible for the norovirus outbreaks (see Supplemntary Material for details on epidemiological methods curve). Further, field observations raised suspicions about the overuse of an on-site septic system which could contribute to environmental persistence of fecal pathogens in venue waters. As a result, CDC and PADOH decided to conduct an on-site environmental investigation (July 18–19; this study) to determine the source of norovirus contamination and to provide evidence-based recommendations for mitigation strategies and prevention practices prior to an upcoming scheduled event with an anticipated 2000 to 3000 attendees scheduled for July 26–30 (Figure 1, Event 4).

2. Materials and Methods

2.1. On-Site Environmental Investigation Site Characterization

The on-site environmental investigation was conducted at a local outdoor venue and campground situated in Pennsylvania, USA consisting of a creek with multiple recreational swimming areas, two groundwater wells, community kitchen and shower/toilet facilities, a camp services building, multiple camp sites, and a nearby farmhouse (Figure 2). This section describes venue water and sanitation facilities, as well as site ground water flow hydrogeology.

Water and Sanitation.—Community facilities for water and sanitation management consisted of a kitchen well connected to a distribution network supplying water across the venue and a septic system. The kitchen well (Figure 2 – site G) served as the primary drinking water source for the venue's public water system, intermittently serving a variable population of event attendees but continuously serving a small population of site staff. A liquid-hypochlorite in-line disinfection system was attached to the kitchen well with a 53 liters per minute maximum flow rate and a 1,211-liter total retention tank capacity. The kitchen well was attached to three distribution lines serving the venue kitchen, two shower houses, and 25 spigots located across the property. The kitchen well depth is 65 m below ground surface with ground water inflow to the well occurring between 64 and 65 m. However, some influx into the pipe annulus could occur from sandstone intervals within the depths 15 to 64 m below ground surface. Assuming such influx, the well likely pulled ground water from NE-SW trending, inclined bedding partings that intersected the kitchen well bore at depth. The well was cased to 64 m below ground surface, and the upper 15 m (surface casing) was pressure grouted, likely limiting any surface and near-surface water intrusion into the well. Moreover, the well was located on an upland plateau further preventing surface water intrusion from flooding. The well completion report stated there was an 8 m depth to bedrock, indicating the 15 m deep surface casing was grouted into bedrock (unpublished PADEP report 2008).

The septic tank leach field was located on a river terrace about 50 m from and about 3 m above the adjacent creek (Figure 3). Upon inspection, the river terrace surface soil appeared predominantly fine grained (fine sand, silt, clay) slackwater deposits with very few visible

gravel clasts. In contrast, the stream appeared to be incised into bedrock with intermittent sandstone outcrops at the water edge consisting of cobble to boulder-sized fields with patchy sediment. Given the large number of transient site inhabitants at the venue (events up to 3000 people) and the single septic system serving the majority of visitors, there may have been periods that the leach field received greater volumes than designed (13,628 liters per day for a permitted population served of 1200), and it is likely the leach field would continue to discharge leachate as the site was continuously inhabited by a smaller number of individuals throughout the year.

Ground Water Flow.—To investigate potential hydrological connections between the septic system leachate and both the drinking water well and recreational creek, site hydrogeology was evaluated to predict the ground water flow pattern from the septic leach field. Figure 3 shows images of the site and an interpretive geological cross section along line A-A' showing the dip of the geological beds on the west side of the syncline, the surface location of the septic tank leach field, and the 65 m deep well, approximately to scale. Figure 3 (top panel) is an aerial photograph of the site. Figure 3 (middle panel) is a Light Detecting and Ranging (LIDAR) image of the site showing the topography by minimizing vegetation. The LIDAR image shows the flat terraced floodplain at the tip of the creek horseshoe bend where the septic leach field is located. The kitchen drinking well (Figure 2 – site G) was located on the upland, elevated 18–25 m above the adjacent flood plain, and 100 m east of the of the septic tank leach field. The Hampshire Formation is shown in Figure 3 (bottom panel) with bedding dipping 50 degrees to the east (Brezinski et al., 2013). The arrows show the interpreted flow path for the leachate traveling down bedding dips to the kitchen well intake. At the 50-degree dip, the beds approximately connect the near surface leach field subsurface discharge to the 65 m deep intake at the bottom of the well. The leachate flow path to the recreational creek likely passed through the soil and surficial materials as it flowed downhill to the recreational creek. See SM for further details on site-specific geological information.

2.2. Sampling Plan and Collection Methods

Because of the rapid nature of an outbreak response and the upcoming large event (Figure 1, Event 4), access to the property was limited to two days (July 18–19) for environmental sampling and site evaluation. Therefore, a strategic sampling plan was developed to assess a possible hydrological connection between the septic leach field, well, and recreational creek (Figure 2).

Well samples were collected from: 1) the kitchen well (Figure 2 - site G) because it was reportedly used by the majority of exposed individuals and had previously yielded detectable norovirus GI and GII types in the initial response (June), and 2) the farmhouse well (Figure 2 - site H) used only by property staff and served as a ground water control unlikely to be impacted by septic leachate. Creek samples were collected at the following locations: 1) immediately upstream of a designated swimming area reportedly utilized by exposed individuals and downstream of the projected septic leach field surface discharge (Figure 2 – site A), 2) perpendicular to the septic leach field slightly upstream from where norovirus was detected in the creek in June (Figure 2 – site B), 3) upstream of a second designated

swimming area and downstream of sites A and B (Figure 2 – site C), and 4) upstream of the property serving as a surface water control site not impacted by property activities (Figure 2 – site I). An exploratory shallow ground water sample was collected in closest proximity to the camp site on the septic leach field about 1 m from the creek water edge and 50 m from the edge of the septic field (Figure 2 – site F). Soil samples were collected from two locations in the camping area located immediately above the septic leach field (Figure 3 – sites D and E) to evaluate potential exposure to attendees via direct contact with surface level septic seepage.

Large volume water samples were collected from the well, creek, and ground water utilizing dead-end ultrafiltration (DEUF) for the concentration of low concentration microbes (Smith et al., 2009). Briefly, water sample (ranging from 197 to 335 L for well water, 46 to 200 L for creek water, and 39 L for ground water) were passed through a 30 kilodalton hollow-fiber ultrafilter (REXEED 25S, Asahi Kasei Medical Co., Ltd, Tokyo, Japan) to capture all microorganisms, including viruses (Table 1). The ground water sample was collected by digging an ~ 0.3 m hole into the creek bank, which quickly filled to a depth of approximately 10 cm. Approximately 40 L of water was pumped out of the hole into sterile containers and stored on-site overnight in shade to allow for sediment to settle followed by DEUF the following day. In addition to higher volume DEUF samples, paired grab water samples for FIB measurements (*E. coli* and enterococci) were collected in sterile 250 ml bottles following Standard Method 9060 A (Rice et al., 2017). Soil samples were collected by gathering 500 ml of surface soil into a sterile 1-liter bottle using a sterile sampling shovel. All samples were shipped on ice to CDC (Atlanta, GA) for processing within 24 hours of collection.

2.3. Sample Processing

DEUF ultrafilters were processed following methods described by Hill et al. (2010). Briefly, ultrafilters were backflushed with a 500-mL solution containing 0.5% Tween 80, 0.01% sodium polyphosphate, and 0.001% Antifoam Y-30 Emulsion. A portion of the backflush was set aside for fecal indicator culture assays (~ 50 ml): *E. coli* (10 ml), somatic coliphage (20 ml), and F-specific coliphage (20 ml). The remaining backflush was subjected to secondary concentration using polyethylene glycol (PEG) precipitation to precipitate viruses, followed by centrifugation to pellet the precipitate. A portion of the PEG pellet (750 μl) was subjected to total nucleic acid extraction via bead-beating followed by purification using silica and polyvinyl polypyrrolidone (PVPP) spin columns following methods described by Hill, et al. (2010). Briefly, total nucleic acids were eluted from columns with 80 μl Tris-EDTA buffer. One extraction method blank, with purified water substituted for test sample, was performed with each extraction event (n = 9 samples per event).

Soil samples were processed following methods by Mull et al. (2013). Briefly, an elution buffer of phosphate-buffered saline with 0.01% Tween 80 was added to the ~500 ml volume of soil up to 100 ml of eluent; the solution was shaken for 1 minute, then allowed to settle for 15 minutes. The procedure was repeated for a total of two elution steps, and the eluates were combined (~200 ml). Eluates then underwent secondary concentration using

PEG precipitation as described above. Soil concentrates were subject to total nucleic acid extraction for detection of norovirus GI and GII and the human-associated MST genetic marker following methods described above for water concentrates.

2.4. Microbiological Assays

Selection of microbial targets.—Five microbial targets were selected for fecal pollutant characterization including: the human-associatedHF183/BFDrev *Bacteroides* MST genetic marker (Haugland et al., 2010), F-specific and somatic coliphage, which have been reported as successful tracers of septic contamination to ground water (Verstraeten et al., 2005) and more closely mimic the fate and transport of pathogenic viruses in ground water than bacterial indicators (Flynn et al., 2015), and finally, general indicators of fecal contamination, fecal coliforms, *E. coli*, and enterococci.

Culture-Based Fecal Indicators.—Culturable *E. coli*, total coliforms, and enterococci were enumerated using IDEXX® Colilert®−18 and Enterolert, respectively (IDEXX, Westbrook, ME). Soil eluate, all grab water, and DEUF backflush samples were tested for culturable total coliforms and *E. coli*. Creek and ground water grab samples were also assayed for enterococci. The soil eluate and grab water volumes assayed were 10 and 100 ml, respectively. In addition, two DEUF backflush volumes (10 and 0.1 ml) were assayed. Somatic and F-specific coliphages were enumerated in separate 20 ml volumes of DEUF backflush using a modified version EPA Method 1602 (USEPA, 2001). Briefly, the method was modified so that each plate contained 5 ml of sample in 30 ml of 1X media with the same concentrations of appropriate antibiotic, host, and $MgCl_2$ in the final volume as Method 1602. Culture positive controls and media blanks were run in parallel to all samples. The following control strains were used: 30 colony forming unit (CFU) BioBalls® (BTF Pty. Ltd., USA) for *E. coli* (National Collection of Type Cultures 9001) and *Enterococcus faecalis* (NCTC 12697); *E. coli* CN-13 (American Type Culture Collection 700609) for F-specific coliphage; and bacteriophage-ϕX174 (ATCC 13706-B1) for somatic coliphage (ATCC, Manassas, VA).

All F-specific coliphage plaques were picked and typed to determine the species-specific genogroup following methods previously described with minor modifications (Friedman et al., 2011). Briefly, for each sample, plaques were picked for typing using 1-mL serological pipette and combined into a microcentrifuge tube containing PBS with separate tubes for each sample and up to 10 plaques per tube. Tubes were frozen prior to overnight enrichment in 1x tryptic soy broth, 0.15% streptomycin/ampicillin, and log phase *Escherichia coli* HS(pFamp)R host. Five milliliters of enrichment were spun at 210 x g for 30 minutes, followed by 4,000 x g for 10 min. A portion of the supernatant was then extracted (750 μl) following the previously described method for water samples without the following steps: addition of proteinase K, bead-beating, and final PVPP column purification. F-specific coliphage genogroups I-IV were detected using molecular methods according to Friedman, et al. (2011) and chemistry described below.

Molecular-Based Pathogen and MST Genetic Marker Characterization.—DEUF processed total nucleic acid extracts were assayed using reverse transcription (RT)-qPCR

for norovirus GI (Hill, et al., 2010), norovirus GII (Jothikumar et al., 2005), F-specific coliphage (Friedman, et al., 2011) and qPCR for the human-associated *Bacteroides* genetic marker (HF183/BFDrev) (Haugland, et al., 2010). Primer and probe sequences for each (RT-) qPCR assay are listed in the SM (Table S2). Briefly, each sample was analyzed in duplicate using 5 µl of extract in 50-µl reactions using either Taqman™ Fast Virus 1-Step Master Mix (ThermoFisher Scientific, Waltham, MA) (RT-qPCR) or Environmental MasterMix 2.0 (Life Technologies, Grand Island, NY) (qPCR) on an Applied Biosystems (ABI) 7500 thermocycler (ABI, Carlsbad, CA). To test for amplification inhibition, each (RT-)qPCR was run with an internal amplification control (Taqman™ Exogenous Internal Positive Control Reagents, ABI, Carlsbad, CA) according to manufacturer's instructions, and inhibition presence was defined as a difference of more than 2.3 between sample and NTC control internal control Cq values (Boehm, et al., 2013). For each instrument run, the amplification threshold was set to 0.03. To monitor for extraneous DNA contaminants, three no template controls were included with each instrument run.

A RT-qPCR or qPCR result was considered a positive detection if duplicate reactions amplified at a quantification cycle (C_q) value < 40, if only one reaction amplified at a C_q < 36, or if any amplified product was confirmed to be the target via sequencing (norovirus only). Otherwise, the result was considered a non-detect. To enumerate HF183/BFDrev genetic markers, a linearized synthetic DNA plasmid was used as a reference standard (Integrated DNA Technologies, Coralville, IA) (Haugland, et al., 2010) consisting of six 10-fold serial dilutions (10^0 to 10^5 copies per reaction). HF183/BFDrev copies per reaction were estimated using the previously described master calibration model (Sivaganesan et al., 2010). The lower limit of quantification (LLOQ) was defined as the average C_q from repeated testing of the lowest standard dilution. Any positive detections below the LLOQ were assigned a concentration of 1 copy per reaction for data reporting. The number of norovirus GI and GII genomic equivalents (GE) were estimated as previously published (Hill, et al., 2010, Jothikumar, et al., 2005) and reported as GE per 100 ml water. The following positive control F-specific coliphage strains were used for each RT-qPCR typing assay: MS2 for I (ATCC 15597-B1), GA for II (provided by Stephanie Friedman at USEPA), Q-B for III-Q like (ATCC 23631-B1), MX1 for III-M like (provided by Brian McMinn at USEPA), and NL95 for IV (provided by Stephanie Friedman at USEPA).

Norovirus Genome Sequencing and Typing.—RT-qPCR was performed targeting a segment of the norovirus genome that includes partial regions of the polymerase and capsid following methods previously described by (Cannon et al., 2017). Briefly, viral nucleic acid was added to a mixture of Qiagen One-Step RT-qPCR (Qiagen, Germantown, MD) reagents, RNase Inhibitor (20 U), and GI or GII specific primers and probes at concentrations of 400 nM and 200 nM, respectively. RT-qPCR was conducted under following cycling conditions: 30 minutes at 42°C, 15 minutes at 95°C, and 40 cycles of 95°C, 50°C, and 72°C for 1 minute each, followed by 10 minutes at 72°C. Expected PCR product of either 579 bp for GI viruses or 570 bp for GII viruses was visualized on a 2% agarose gel (Seakem-ME, Lonza, Rockland, ME) containing Gel Green (Biotium, Hayward, CA) and PCR purified by ExoSAP-IT (Affymetrix, Santa Clara, CA) or gel purified by QIAquick Gel Extraction Kit (Qiagen) and then outsourced for sequencing by Sanger sequencing as

described by Cannon, et al. (2017) (Eurofins Genomics LLC, Louisville, KY). Sequence editing and typing was performed in BioNumerics v7.6 using default settings (Applied Maths, Sint-Martens-Latem, Belgium). Phylogenetic analysis was performed using pairwise similarity matrix and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis in BioNumerics v7.6 (Applied Maths) for comparison of environmental sequences to four outbreak clinical sample sequences (GI.7[P7] GenBank Accession #: MT357897, MT357898, and MT357899; GII.13[P16] GenBank Accession # MT364380).

3. Results

3.1. Norovirus

During the environmental investigation (July), norovirus GI and GII were again detected in the large volume water samples collected from the kitchen well (Figure 2 – site G) (Table 1). The norovirus GI and GII detected in the kitchen well was typed as GI.7[P7] and GII.13[P16], and phylogenetic analysis demonstrated that genotypes detected in clinical and water samples were 100% identical (Figure 4). Norovirus GI was not detected in the creek adjacent to the septic leach field (Figure 2 – site B) in July, which is slightly upstream from where it was detected in the prior epidemiology effort (June) (Figure 2 – site E). Norovirus GI and GII were not detected in the soil, ground water, or farmhouse well samples (Figure 2 – sites E, D, F, H).

3.2. Fecal Pollution Characterization

FIB, fecal indicator viruses, norovirus, and HF183/BFDrev concentrations for all environmental investigation samples (July) are presented in Table 1. *E. coli* and enterococci were observed in the kitchen well samples (Figure 2 – site H). Total coliforms, but not *E. coli*, were found in the farmhouse well (Figure 2 – site H). *E. coli* and enterococci were detected in the creek, soil, and shallow ground water samples (Figure 2 – sites I, B, A, C, J, D, E, and F). Somatic coliphages were detected in the kitchen well (Figure 2 – site G), all creek samples (Figure 2 - sites I, B, A, C), and the ground water sample (Figure 2 – site F). The kitchen well (Figure 2 – site G) was positive for F-specific coliphages, and all kitchen well samples yielded G2 type RNA F-specific coliphages, which are associated with human feces.

The human-associated *Bacteroides* HF183/BFDrev genetic marker was detected in the kitchen well (Figure 2 – site G) and in all creek samples (sites I, A, B, and C) (Table 1). While HF183/BFDrev was detected in the upstream control sample (Figure 2 – site I), the concentrations in the creek adjacent to the camping site on top of the septic leach field (Figure 2 – site B) and at the downstream swimming area (Figure 2 – site A) were ten-fold higher than the upstream control (Figure 2 – site I) and most distant downstream samples (Figure 2 – site C). No human-associated *Bacteroides* were detected in the soil (Figure 2 – sites D, E) or the lower campsite tap water (Figure 2 – site L), and no human-associated *Bacteroides* was detected in the soil, shallow ground water, or farmhouse well samples (Figure 2 – sites D, E, F, and H).

3.3. Quality Assurance/Quality Control

Amplification inhibition was absent in all samples (data not shown). No contamination was observed in any culture-based indicator assays, nucleic extractions blanks, or no template controls. Positive controls were detected as expected for all assays. The HF183/BFDrev master standard curve ($y = -3.32\ x + 38.3$) had an amplification efficiency *(E*) of 1.00 ($E = (10^{-1/slope} - 1)$) and R^2 of 0.981. LLOQ of the HF183/BFDrev assay had an average Cq of 37.1 (0.16 $\log_{10}$ copies per reaction). The standard curve used to estimate norovirus GI concentration was $y = -3.23x + 37.7$ ($R^2 = 0.942$, $E = 1.04$) and to estimate GII concentration was $y = -3.46x + 37.0$ ($R^2 = 1.00$, $E = 0.95$).

4. Discussion

Waterborne disease outbreak response environmental investigations are conducted to provide supporting data to epidemiological evidence that suggests a specific water source and environmental exposure route. However, data generated from environmental investigations can be limited for several reasons. Environmental investigations tend to occur on a swift timeline due the need to generate information rapidly to inform a management response, and therefore, are designed based off minimal *a priori* epidemiological and site information. Moreover, environmental investigations are often initiated several weeks or longer after the initial outbreak and conducted under restricted site access due permission requirements (e.g., local public health officials and/or landowners, governing body such as Department of Natural Resources), limiting options for a comprehensive sampling plan and accurate assessment of outbreak-associated environmental conditions. Despite these limitations, detecting the outbreak pathogen, evidence of fecal contamination from the suspected pathogen host, and characterization of potential exposure routes (e.g., contaminated drinking water or recreational waters) provides valuable information necessary to develop a weight of evidence explanation that can link site specific environmental factors to outbreak illness trends. This information is critical to characterize contaminant mode of transmission, and in turn, design environmental mitigation or remediation strategies to prevent future outbreaks.

Norovirus GI and GII were detected in a drinking water kitchen well over a two-month span (initial investigation, see SM and this study) following outbreaks associated with multiple events at an outdoor venue and campground, suggesting persistent contamination of the ground water serving this well. Norovirus sequencing results from well cases reported drinking matched identically to the clinical samples, confirming the well as a source of norovirus transmission during the outbreaks (Figure 4). Based on observed norovirus concentrations in water samples (Table 1), the kitchen well sample was 10-fold lower during the environmental investigation compared to the initial epidemiology response (see Table S1). This suggests that the kitchen well norovirus contamination was persistent, but decreasing over time, which is consistent with research reporting that viruses can be discharged from septic tanks for up to 60 days following the last detectable virus in the stool of system users (Anderson et al., 1991). Adenovirus and enterovirus, both viral pathogens used as fecal indicators (Hewitt et al., 2013), were also detected in the kitchen well by molecular methods from archived samples collected during the initial investigation (methods and data available in SI). The detection of multiple enteric viruses suggests fecal

contamination may have originated from multiple infected individuals and is consistent with a septage contamination mixture scenario.

Based on preliminary geological evaluation, viral ground water contamination at this site was unexpected as the location does not contain common hydrogeological risk factors found at sites associated with viral outbreaks due to septic contamination (Berger, 2008). Specifically, this outbreak provides evidence suggesting viral ground water contamination through cemented fine porous media from a properly sited septic tank meeting the requirements of the State sanitary setback distance (PA Code Title 25; §73.13). A possible explanation for viral contamination in this scenario is an overloaded septic system with weakly developed bedding plane partings that steeply dip from the surface toward the well intake (Figure 3). Steeply dipping planar fabrics are an important component of some non-porous media such as fractured bedrock, but porous media like the outbreak site is not generally considered a hydrogeologic risk factor for viral contamination when the beds are flat-lying. In this instance, the normal protections provided by the relatively slow flow in the outbreak site porous bedrock medium (*i.e.,* Hampshire Formation) were potentially subverted by the combination of likely high volume of septic discharge based on the number of reported users and steep (50-degree dip) eastward bedding dip in the direction from the septic tank leach field toward the well intake. A norovirus outbreak in flat-lying protective sandstone beds in Arizona was similarly overwhelmed by high volume discharge from a poorly performing package sewage treatment plant (Lawson et al., 1991). However, the outbreak described here is evidence that structural fabric other than orthogonal fractures can be a hydrogeological risk factor for viral ground water contamination.

FIB and viruses were detected in the kitchen well samples immediately following the outbreaks (initial investigation, Table S1), as well as a month later during the environmental investigation, confirming a continued presence of fecal contamination in the ground water at this location. Total coliforms were also detected in the farmhouse well, suggesting a short infiltration time from the soil zone throughout the property. While F-specific coliphages in the kitchen well sample further supports septic contamination, the inability to track F-specific coliphage contamination relative to somatic coliphage and HF183/BFDrev suggests F-specific coliphage may not always be effective for tracking septic contamination of ground water following an outbreak. Additional research is warranted to further characterize the fate and transport of these microbial tracers in groundwater settings. Based on the high levels of human fecal and pathogen contamination detected in the kitchen well, even a chlorination system providing the recommended 4-$\log_{10}$ virus inactivation may not have provided sufficient disinfection for drinking water consumption, likely leading to the transmission of norovirus through tap water at the venue during the outbreaks (Fout, et al., 2017). The inadequate disinfection scenario is further supported by the presence of total coliforms and HF183/BFDrev in the kitchen tap immediately following the outbreaks (initial investigation, Table S1).

While norovirus was no longer detectable in the recreational creek one month after the outbreaks associated with the venue, *E. coli* or enterococci were measured in all creek samples. However, none of the creek samples adjacent to the venue were above the PADOH fresh bathing water single sample maximum value of 235 per 100 ml *E. coli*

(PA Code Title 28; §18.28). Other norovirus outbreaks with suspected septic contamination of drinking water wells and nearby recreational fresh waters have also documented *E. coli* concentrations in nearby recreational waters within acceptable limits despite the presence of norovirus GI and GII in the water (Jack, et al., 2013). The *E. coli* concentration at the upstream site was slightly higher than the single sample maximum value, which may be associated with the low human fecal signal detected in the upstream sample or because of other naturally occurring fecal sources originating from nearby rural activities. Somatic coliphages were also detected in all creek samples, but there are currently no recommended recreational water quality criteria available based on coliphage. Together, the FIB and viral indicator data confirm the presence of fecal contamination in the creek but, in comparison to the control site, are unable to confirm whether fecal pollution is septic contamination from the adjacent leach field or another local source.

The 2012 EPA Recreational Water Criteria recommends using MST methods when fecal contamination is suspected to "verify the results of the sanitary survey by confirming the presumed sources of fecal contamination in the watershed" (section 6.1.1) (USEPA, 2012). The presence of the human-associated HF183/BFDRev genetic marker in the creek sample adjacent to the septic leach field suggests that ground water septic contamination may be a contributor to fecal contamination in the creek. While the human-associated MST genetic marker was also detected in the control upstream creek sample, the creek samples collected in the ground water flow path adjacent to the septic leach field (Figure 3) yielded approximately 10-fold higher concentrations compared to the upstream and furthest downstream samples, suggesting human fecal input into the creek from the leach field area. Moreover, low concentrations of the HF183 marker in surface waters is not surprising as the HF183 maker has been demonstrated to cross-react with deer feces, which is expected to present in rural environments such as these (Nguyen et al., 2018). Finally, fecal input into the creek from the leach field is further supported by the presence of both FIB and fecal indicator viruses in the ground water samples, suggesting subsurface flow of fecal contamination into the creek.

Recently, a linear relationship was reported between the concentration of the human-associated *Bacteroides* genetic marker (HF183/BFDrev) in surface waters polluted with raw sewage and GI illness rates for recreational swimmers (Boehm et al., 2015). The modeled relationship predicts a 1-$\log_{10}$ increase in risk of GI illness from swimming with every 1-$\log_{10}$ increase in the human-associated marker concentration. Therefore, the risk of illness from swimming by the campsite located above the septic leach field may have been at least 10-fold higher than the risk of illness from swimming upstream or downstream of the camp during the outbreaks. Together with the hydrogeological site evaluation connecting ground water flow patterns between the septic leach field and the creek, the HF183/BFDrev results strongly suggest that recreational water contact, along with consuming water from the kitchen well, were likely routes of norovirus transmission during the outbreaks.

E. coli and enterococci were detected in the soil above the septic leach field, indicating the presence of fecal contamination of unknown origin. However, considering the proximity of the soil samples to the likely norovirus contamination source and the lack of any human-associated MST genetic marker or other microbial tracers in the soil, transmission

of norovirus through surface intrusion of overflowing septic leachate was considered to be unlikely.

While the inclusion of the human-associated HF183/BFDrev method in this waterborne disease outbreak response provided new and useful information on potential norovirus exposure routes, this environmental investigation was subject to multiple limitations. First, the environmental samples collected represent the microbial water quality at a single point in time and may not represent the same environmental conditions present during the outbreak exposure events (environmental investigation conducted ~3 weeks later). Therefore, the results may not reflect the water quality conditions during times of high attendance at the venue (both in terms of septic system usage and recreational use of the creek). Second, the ground water sample was extracted and held in a container for 24 hours prior to ultrafiltration at ambient temperature. Microorganisms could have decayed during this time period; therefore, the results could represent a conservative estimate of the true contamination levels present in ground water. Finally, the septic tank was not directly sampled as part of the environmental investigation allowing for direct confirmation of norovirus in the suspected contamination source, as the septic tank was drained by the property owner immediately prior to the start of the environmental investigation.

5. Conclusion

Together, the epidemiological evidence and environmental investigation results support the response conclusion that an overloaded septic system was the likely source of human fecal and norovirus contamination in the drinking water kitchen well and the recreational creek adjacent to the septic leach field. In response, public health recommendations and communication materials were developed for the venue in preparation for a subsequent large event (Figure 1; event 4) with approximately 3000 anticipated attendees. The public health interventions implemented by the state health department and venue staff during this event (Event 4) included: distributing communication materials regarding proper hand hygiene and norovirus transmission, installing temporary handwashing stations, limiting access to shower and toilet facilities, providing adequate sanitation capacity via portable toilets, and provided access to a new well. Posting of health alert notices of potential risks from swimming near recreational creek areas was also advised by public health officials. While venue staff chose not to implement this recommendation, access to swimming areas was limited due to flooding from heavy rains during the first day. Because of the short-term nature of events held at the venue, Event 4 offered a unique opportunity to measure the impact of recommended prevention measures implemented using active surveillance for AGE. No credible norovirus cases or subsequent outbreaks were identified during or following Event 4 (see SM for further detail), highlighting the real-world impact of implementing MST methods to enhance epidemiological data for developing effective environmental interventions to prevent fecal-transmitted outbreaks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Waterborne norovirus outbreaks can have multiple exposure routes
- Microbial source tracking (MST) can trace fecal pollution in outbreak environments
- Human-Specific MST markers can track septic contamination through ground water
- HF183 can indicate septic-associated norovirus contamination of water
- Human-Specific MST can identify norovirus exposure routes to prevent future cases

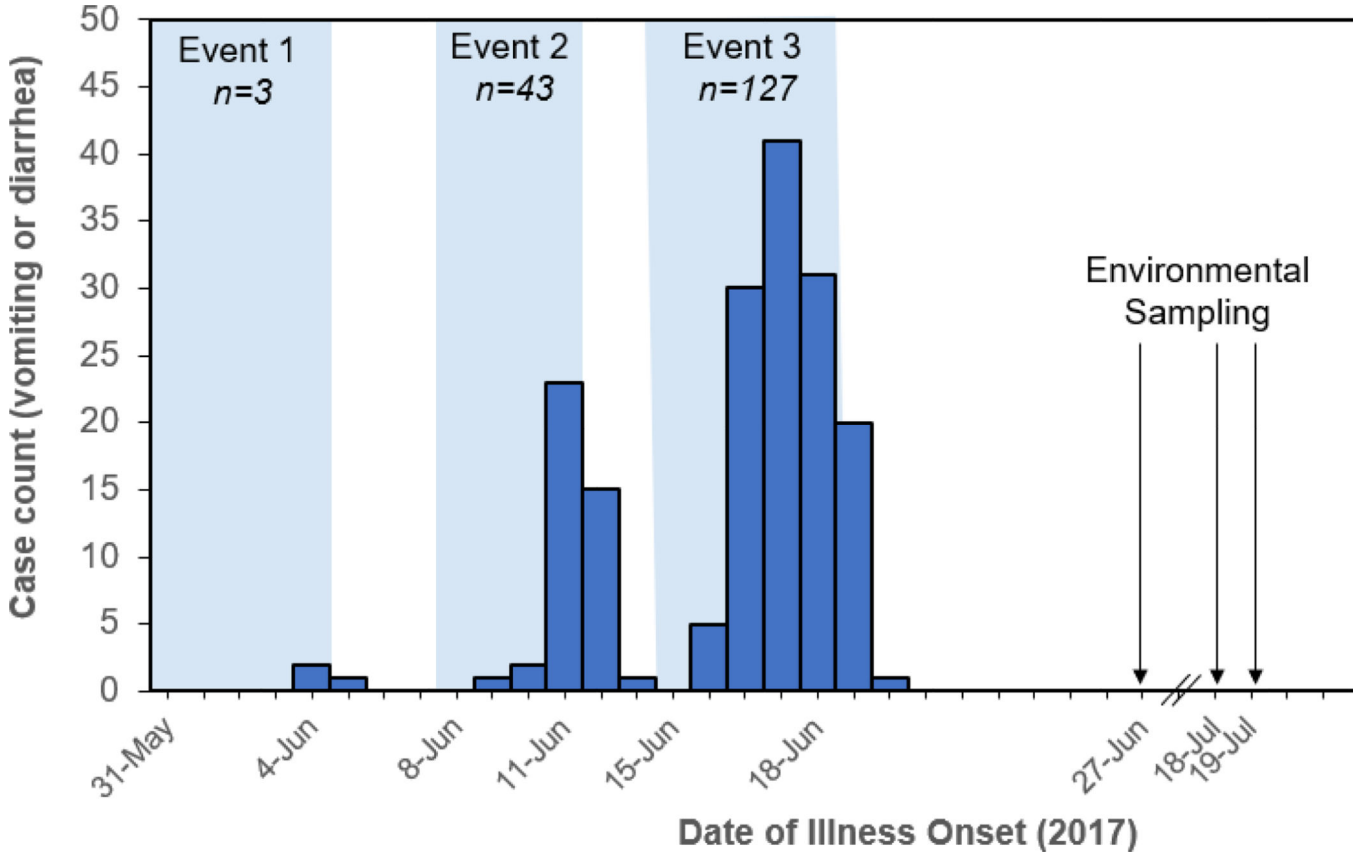

Figure 1. Cases of acute gastroenteritis (AGE) (N=183) at a campground in Pennsylvania from May to June, 2017, by date of illness onset.

A total of 179 cases during events 1–3 were identified through telephone reports. Six cases not shown above could not be linked to a specific event because multiple events were attended. Through active surveillance in event 4, four cases were identified. One ill person from event 4 submitted a stool sample that tested negative for norovirus.

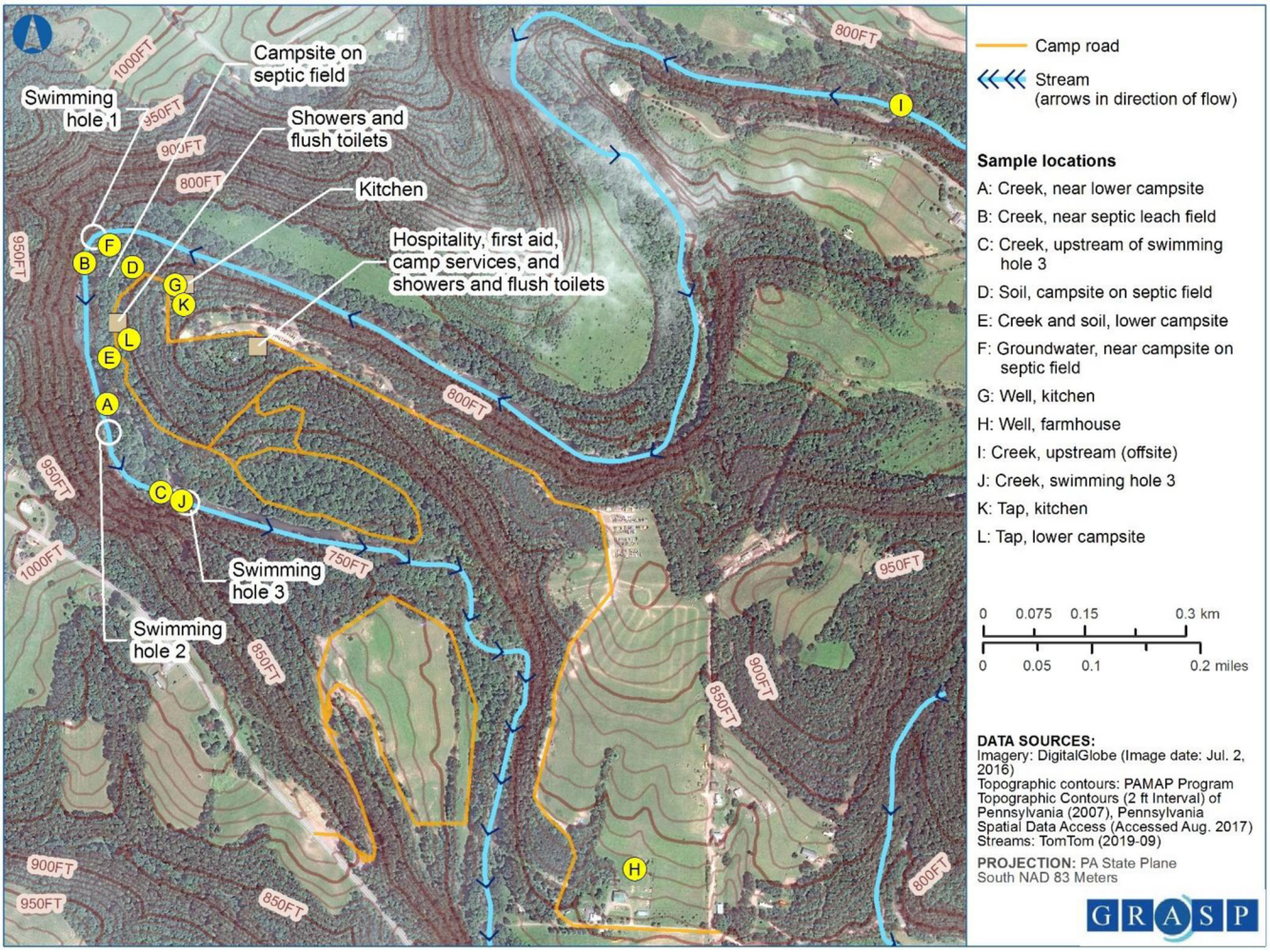

Figure 2.

Map of the environmental sampling sites at the Pennsylvania campground collected by PADOH and CDC.

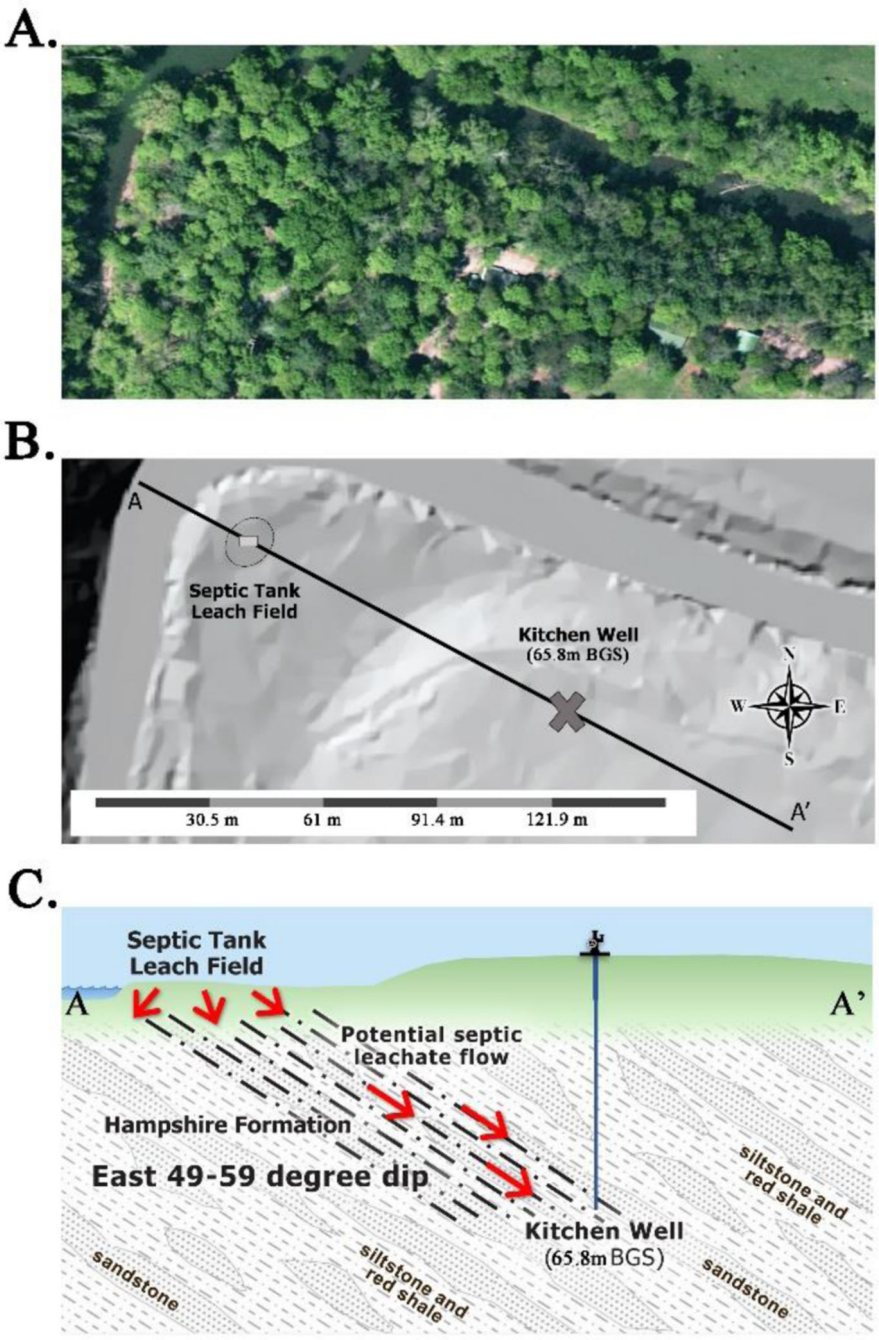

Figure 3.

Aerial photo, light detection and ranging (LIDAR) image and cross-section view of the outbreak site drinking water well, recreational creek, septic tank, and leach field. Panel A: Aerial photograph centered on the kitchen well showing the well location and stream geometry. Panel B: LIDAR image showing the location of the kitchen well, the site topography, the location of the septic tank leach field and the geological cross-section line (A-A'). Panel C: Geological cross-section along the cross-section line (A-A') showing the topography, the well depth, the stream and septic leach field locations and the approximately

50-degree dip of the geological strata in the direction from the septic leach field toward the drinking water well.

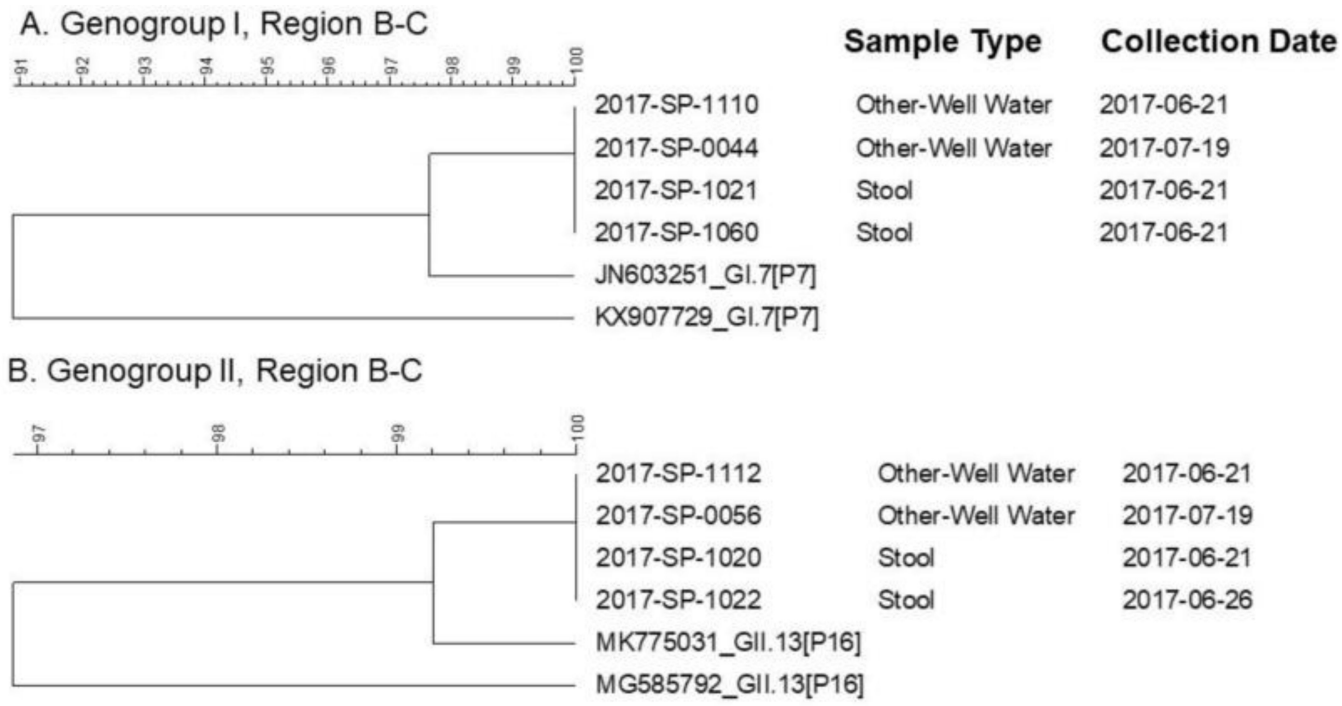

Figure 4.

Phylogenetic analysis of GI.7[P7] (Panel A) and GII.13[P16] (Panel B) detected in outbreak clinical and the kitchen well water samples from June and July collection dates (Figure 2 – site K) using pairwise similarity matrix and unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. The norovirus GI concentration in the creek from the initial investigation (June) was too low for sequence typing.

Table 1.

Microbial results for creek, well, soil, drinking water, and ground water samples. Samples were collected during the environmental investigation (July) in response to three norovirus outbreaks at a campground in Pennsylvania. Creek sites are listed from upstream to downstream.

Site ID	Site description	Sample type	DEUF volume (L)	Norovirus log10 GE/100 ml	HF183 log10 Copies/ 100 ml	Somatic coliphages log10 PFU/100 ml	F-specific coliphage log10 PFU/100 ml	Total Coliforms[a] log10 MPN/100 ml (DEUF)	*E.coli*[a] log10 MPN/100 ml (DEUF)	Enterococci[a] log10 MPN/100 ml (DEUF)
G	Kitchen	Well	335	GI 1.83 GII 0.47	3.07	1.05	1.2	NA [b] (2.35)	NA [b] (2.04)	NA [b] (0.35)
H	Farmhouse	Well	197	ND	ND	<−1.8	<−1.8	1.13 (−0.48)	<1.13 (<−1.5)	NA [b] (<−1.5)
I	Upstream	Creek	111.3	ND	−0.57	1.42	<−1.6	>3.38 (3.38)	2.81 (2.31)	2.00 (2.10)
B	Near septic leach field	Creek	200	ND	0.33	0.13	<−1.8	>3.38 (>3.90)	1.44 (1.44)	0.87 (0.89)
A	Near lower campsite	Creek	142.4	ND	0.45	0.01	<−1.6	>3.38 (>4.05)	1.54 (1.54)	1.16 (1.70)
C	Upstream of swimming hole	Creek	46	ND	−0.62	0.68	<−1.2	>3.38 (3.63)	1.26 (1.14)	1.88 (2.19)
J	Swimming hole	Creek	0.2	NA	NA	NA	NA	>3.38 (NA)c	1.08 (NA)[c]	1.84 (NA)[c]
F	Near campsite on septic field	Ground Water	39.4	ND	ND	−0.26	<−1.2	>3.38 (4.38)	1.90 (1.93)	2.17 (1.86)
E	Lower campsite	Soil	NA[d]	ND	ND	ND	ND	Detect[d]	Detect[d]	Detect[d]
D	Campsite on septic field	Soil	NA[d]	ND	ND	ND	ND	Detect[d]	Detect[d]	Detect[d]

a: FIB were measured in DEUF and grab samples. Grab concentrations are reported, and DEUF sample results are in parentheses;

b: Only a DEUF sample was tested (no grab);

c: Only a grab sample was collected in the field (no DEUF);

d: dry weight of soil sample was not determined, results presented as detect/non-detect only; DEUF: Dead-end ultrafiltration; GE: genomic equivalents; MPN: most probable number; PFU: plaque forming units; ND: not-detected; NA: not assayed.