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FECAL SOURCE TRACKING USING HUMAN AND BOVINE ADENOVIRUS AND POLYOMAVIRUSES

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PROJECT SUMMARY

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Background and Need: Many Wisconsin residents have as their immediate source of water a private well that has a contemporary record of suspected fecal contamination (based on repeated detection of *Escherichia coli* in well water samples). The simple *detection* of commonly targeted fecal indicator bacteria, like fecal coliform bacteria and *E. coli*, provides little information about contamination source(s) (e.g., human vs. livestock), and dedicated resources are typically lacking for more thorough investigations that may elucidate the sources of groundwater contamination. Accordingly, need exists for (i) the investigation of microbial indicators, such as host-specific adenoviruses (AdV) and polyomaviruses (PyV), whose detection in groundwater provides information on contamination sources, and (ii) the exploration of methods for collecting and detecting source-diagnostic microorganisms in groundwater samples from such "problem wells."

Objectives: The objectives of this study were: (1) to ascertain the utility of bovine AdV (BAdV) and bovine PyV (BPyV) as fecal contamination indicators by determining their prevalence in cattle wastes; (2) to quantify the efficiency of hollow fiber ultrafiltration (HFUF) for concentrating viruses from natural groundwater; and (3) to evaluate the likelihood that AdV and PyV will be detected and prove useful for fecal source attribution in private groundwater samples deemed vulnerable to fecal contamination.

Methods: Human and animal waste samples collected in southern Wisconsin were investigated for human and bovine AdV and PyV using both previously published and novel polymerase chain reaction (PCR) assays. Subsequently, selected PCR methods were applied to groundwater samples for fecal source attribution. Between March 2010 and February 2011, groundwater samples (~115 L, each) were collected by HFUF from eleven households with contemporary records of suspected fecal contamination; five of these homes were sampled on three to four separate occasions. The bacteriophage, PDR1, an enteric virus surrogate, was injected into the HFUF system during sample collection. The recovery of this virus was quantified by TaqMan® PCR to indicate the overall method efficiency of virus concentration and analysis. Groundwater samples concentrated by HFUF were analyzed for coliform bacteria, *E. coli,* livestock and human AdV and PyV, and *Rhodococcus coprophilus*, an indicator of grazing herbivore fecal contamination. Twenty-eight additional groundwater samples ($\leq$ 600 mL) were concentrated and assayed by PCR for bovine BAdV-10 and BPyV-1 to evaluate the utility of analyzing low-volume samples for viral indicators. These samples were submitted to the Wisconsin State Laboratory of Hygiene by the Wisconsin Department of Natural Resources for microbial source-tracking (MST) analysis in response to home-owner water-quality complaints.

Results and Discussion: Using original, "broad-spectrum" PCR primer sets designed to detect an array of known and previously unidentified AdV and PyV, BAdV were detected in 13% of cattle fecal samples, 90% of cattle urine samples, and 100% of cattle manure samples; 44% of BAdV-positive samples contained DNA from two genetically distinct AdV genera, *Atadenoviridae* and *Mastadenoviridae*. BPyV were detected less frequently than BAdV in these samples, at rates of 17% in cattle feces, 14% in cattle urine and 73% in cattle manure. Four previously unknown bovine viruses were detected, three BAdV and

one BPyV. Shedding rates by cattle for two specific bovine viruses, BAdV-10 and BPyV-1, supported targeting these viruses for fecal source attribution.

For private groundwater samples, the recovery of exogenous bacteriophage PRD1 by HFUF varied considerably within and between sites (0-113%). Samples with visible iron solids in HFUF concentrates demonstrated, on average, lower PRD1 recoveries (3 ± 5%, n = 8) compared with samples with no apparent iron in HFUF concentrates (23 ± 33%, n = 13), though the difference was not statistically significant (p = 0.116). The effective groundwater volumes analyzed by PCR (per 5 µL DNA extract) were 1500 mL, 256 mL or 48 mL for samples with PRD1 recoveries of 100%, 16% or 3%, respectively. Of the 24 private groundwater samples collected by HFUF, 17 were positive for coliform bacteria, eight were positive for *E. coli*, six were positive for *R. coprophilus* and three were positive for at least one viral indicator (AdV or PyV) of fecal contamination. A single viral indicator was detected at two of the five sites targeted for repeated sample collection. Of the six homes where groundwater was collected only once, one was positive for two viral markers. The detection of corroborating host-specific microbial markers is required for confident source attribution. Therefore, only for the latter site (Site R, for Rock County) could an actionable contamination source, human, be reliably attributed. Subsequent site investigation by the WI Department of Natural Resources revealed a compromised pipe in the home owner's septic system. An additional site (Site 3) showed "animal/non-human" contamination, supported by the detection in the same HFUF concentrate of an animal AdV of unknown host and *R. coprophilus*. More confident source attribution was possible for several of the low-volume groundwater samples submitted to the WSLH: 8/28 samples were positive for BPyV-1; four of these eight were also positive for BAdV-10. The effective groundwater volume analyzed by PCR for these low-volume groundwater samples was 1 to 10 mL for virus recoveries ranging from 10 to 100%, respectively.

Conclusions and Recommendations: Human AdV and PyV, BAdV-10 and BPyV-1 were detected in groundwater samples collected from private wells, demonstrating their utility as fecal source indicators. Groundwater samples collected using HFUF showed large concentration factors (~10^4) and were often contaminated by coliform bacteria. However, in most cases, even the concentration of large sample volumes did not reveal sources or overcome the intermittent nature of groundwater contamination by fecal materials. On the other hand, despite low volumes, samples analyzed in response to immediate home-owner complaints frequently yielded positive results for bovine viral indicators, resulting in definitive source attribution. Therefore, the concentration and analysis of these types of groundwater samples for AdV and PyV is recommended. Where potentially contaminated groundwater presents an immediate and unacceptable risk to human health (e.g., for vulnerable wells serving daycare facilities, nursing homes, or communities), the application of the simple HFUF method described here is also recommended. For example, at Site R, human viruses were detected in HFUF concentrate with low measured PRD1 recovery, but were absent in a 400-mL sample collected and processed in parallel. Additional optimization of HFUF methods for processing groundwater samples is advised. In particular, assessing the utility of beef extract or peptone solutions for concentrate recovery would streamline sample processing and may improve recoveries.

Related Publications:

Sibley, S.D., T.L. Goldberg and J.A. Pedersen. 2011. Detection of Known and Novel Adenoviruses in Cattle Wastes via Broad-spectrum Primers. *Appl. Environ. Microbiol.*, **77**:5001-5008.

"*Testing Well Water for Microorganisms*." Fall/Winter 2010, Aquatic Sciences Chronicle, University of Wisconsin Sea Grant and Water Resources Institute.

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INTRODUCTION

Many rural homeowners have as their immediate source of water a private well that has a contemporary record of suspected fecal contamination (based on repeated detection of *Escherichia coli* in well water samples). Unfortunately, the simple *detection* of commonly targeted fecal indicators, like fecal coliforms and *E. coli*, provides little information about contamination source(s) (e.g., human vs. livestock), and dedicated resources are typically lacking for more thorough investigations that may elucidate the sources of groundwater contamination. To address these concerns, need exists to (i) investigate microbial indicators, such as host-specific viruses, whose detection in groundwater provides reliable information on contamination sources, and (ii) explore methods to concentrate and detect source-diagnostic microorganisms in groundwater samples from such "problem wells."

Adenoviruses (AdV) and polyomaviruses (PyV) have been advocated as potentially valuable, microbial indicators of fecal contamination (Hundesa et al., 2006). For both groups of viruses, species (or genotypes) infecting specific human or livestock hosts exist; many of these species have been detected in excreta of asymptomatic individuals and/or in aggregated waste samples (Maluquer de Motes et al., 2004; McQiaig et al., 2006, 2009). Because DNA viruses, such as AdV and PyV, are thought to co-evolve with their hosts (Pérez-Losada et al., 2006), PCR assays can be designed to target specific virus gene segments (e.g., capsid protein genes) that differ significantly, depending on host species. When properly designed, these PCRs can be invaluable for attributing source(s) of fecal contamination. However, the majority of research targeting viruses for fecal source attribution has focused on human viruses, in part because more complete information is available on the diversity and ecology of human (vs. livestock or wildlife) viruses that cause mild or asymptomatic infections (i.e., those viruses that are most promising for source tracking). Prior to this study, eleven distinct bovine adenoviruses (BAdV) and one bovine polyomavirus (BPyV) had been identified (Schuurman et al., 1990; Lemkuhl and Hobbs, 2008). The detection of several of these viruses in bovine excreta (Maluquer de Motes et al., 2004, Wong and Rose, 2009) encouraged their consideration as specific indicators of livestock fecal contamination.

Even with a reliable viral fecal source tracking (FST) PCR assay in hand, a significant challenge remains: fecal contamination of groundwater is expected to be intermittent, and the concentrations of fecal microbes (viruses, in particular) in aquifers may be small and variable. Therefore, it is commonly accepted that large sample volumes (e.g., 50- to 500-L) must be concentrated to capture a sufficient number of viruses to be detectable by molecular methods (Lambertini et al. 2008; Smith and Hill, 2009; Knappett et al., 2011). Thus, the optimization of methods for collecting viruses from a large water sample while eliminating (or reducing the influence of) compounds (e.g., inorganic colloids, dissolved organic matter) that interfere with molecular virus detection is a fundamental hurdle in nearly all current FST investigations of surface water and groundwater. Several methods have been advocated for concentrating groundwater samples. However, most of these have been primarily (or exclusively) validated in laboratory settings and usually without explicit consideration for how the methods might be implemented efficiently in a field setting by anyone other than the a highly trained practitioner. One of the most promising, yet challenging, methods in this category is hollow fiber ultrafiltration (HFUF).

Hollow fiber ultrafiltration is a size-exclusion method for concentrating microorganisms. Water under pressure is forced into ("dead-end" format; Smith and Hill, 2009) or cycled through ("tangential flow" format; Hill et al., 2005) a sterile, prepackaged hemodialysis filter containing thousands of hollow, porous plastic fibers. Water exits the filter through pores (30 kDa molecular weight cutoff, ~5 nm) in the fiber sidewalls, while particles, including microorganisms and other natural colloids, are retained. Laboratory investigations of HFUF in dead-end and tangential flow formats have been completed by the Centers for Disease Control and others for the detection of microbial pathogens in water (Hill et al., 2005). Recently, HFUF was employed *in situ* for the concentration of microorganisms in surface water (Leskinen and Lim, 2008) and groundwater (Gibson and Schwab, 2011; Knappett et al., 2011). Yet, few environmental investigations have employed the simpler "dead-end" filtration format, which requires little operator training, is conducive to rapid response implementation for field sampling (Smith and Hill, 2009).

The objectives of this study were: (1) to ascertain the utility of bovine AdV (BAdV) and bovine PyV (BPyV) as fecal contamination indicators by determining their prevalence in cattle wastes; (2) to

quantify the efficiency of hollow fiber ultrafiltration (HFUF) for concentrating viruses from large natural groundwater samples; and (3) to evaluate the likelihood that AdV and PyV will be detected and prove useful for fecal source attribution in private groundwater samples deemed vulnerable to fecal contamination. Human and animal waste samples collected in southern Wisconsin were investigated for human and bovine AdV and PyV using both previously validated and novel PCR assays. Subsequently, selected PCR methods were used to detect AdV and PyV in groundwater samples collected from private wells with demonstrated or suspected fecal contamination.

PROCEDURES AND METHODS

Excreta Sample Collection. Catch samples of dairy and beef cattle (*Bos primigenius taurus*) feces (n = 32) and urine (n = 21) were acquired opportunistically from individual animals between August 2008 and January 2010; eleven dairy cows provided paired fecal and urine samples. Eleven additional manure samples (i.e., mixed wastes, including feces and urine, from multiple cattle) were obtained: one sand-separated, dewatered manure sample, three bedding samples, two liquid manure lagoon samples, and five bedding-percolate samples. Waste samples from several additional animal species were examined for comparison. Five human sewage samples were collected from the 9-Springs wastewater treatment facility (Madison, WI). All excreta samples were collected in sterile containers, transported on ice, and stored for < 1 week at 4 °C or at -80 °C until analysis (except for one liquid manure sample, which was held at 4º C for approximately one year until analysis).

Dead-end Hollow fiber Ultrafiltration System. The HFUF system (Figure 1) employed a REXEED-21S hemodialysis filter (Asahi Kasei America Inc.) housing thousands of polysulfone hollow fibers (26.6 cm length, 185 µm inner fiber radius; 30-kDa molecular weight cutoff; 2.1 m^2 total surface area). The HFUF system was configured to accept flow directly from a common garden hose connection, allowing continuous concentration of an arbitrary sample volume from most private wells. During sample collection, the filter was positioned vertically and operated in the "dead-end" configuration (Leskinen and Lim, 2008; Smith and Hill, 2009) by keeping the filter outlet port capped. Groundwater fed by the home's water pressure into the filter inlet port was driven laterally through pores in the hollow fibers, achieving permeate flow rates of ~2 L min^{-1} at ~13 psi (controlled at the faucet). Platinum-cured silicone tubing was used (VWR International, no. 60985-730, 60985-738), and tubing-tubing connections were completed with interchangeable male/female polyethylene quick-disconnects (Bel-Art Scienceware, no. 197280000, 197290000). The male connectors in this series fit securely into the filter inlet and outlet ports and were used to equip the HFUF with a standard, leak-free barbed fitting for connecting tubing. To minimize microbe aggregation, a filter-sterilized (Corning, 430015) dispersant/chelating solution consisting of either (A) 10% sodium polyphosphate (NaPP, Sigma Aldrich), (B) 10% NaPP/7.5% EDTA or (C) 0.5 M EDTA was added continuously during filtration via syringe pump, operated at 2 mL min^{-1}. Considering a filtration rate of 2 L min^{-1}, the dispersant was diluted 1000-fold into the influent groundwater sample.

Groundwater Sample Collection by Hollow fiber Ultrafiltration. Between March 2010 and February 2011, 28 groundwater samples (~120 L, each) were collected by HFUF from twelve private wells; five of these wells were sampled on three to four separate occasions (Figure 2). Well selection was informed by state officials familiar with site histories. Samples were typically collected from an outdoor garden hose faucet, which was purged until electrical conductivity, temperature, pH and dissolved oxygen concentrations, measured with a YSI 556 multiprobe, stabilized (typically 10-15 min). The HFUF system was connected to the faucet with the filter outlet port open to purge the filter of storage solution. The filter outlet port was then capped, and the syringe pump feeding dispersant was activated. During filtration, system pressure was maintained at 13 ± 3 psi by adjusting the regulator on the home's faucet, and dispersant was refilled as needed. To reduce system pressure fluctuations, home owners were advised to limit water use during sample collection. Where water flow was poorly regulated by the home's existing fixture, a simple flow regulator was mounted to the faucet prior to initiating sample collection.

Following filtration of the first 60 L, 100 µL of PDR1 stock (strain D4; HER 23, Laval University) was suspended in 10 mL of ultrafiltered groundwater (collected from a tubing junction installed downstream of the permeate port) and injected (21G needle) through alcohol-swabbed tubing into the HFUF system upstream of the filter. PRD1 stocks were generated on *Salmonella enterica* serovar

Typhimuadditirium (strain LT2 pLM2 1217; Laval University), and the 100 µL addition was titered to 4 ± 2 × 10^8 genome equivalents (G.E.; see below) or 4 ± 2 × 10^7 colony forming units (cfu; USEPA Method 1602, 2001). To conclude filtration, water flow was stopped, and filter pressure was allowed to relax. Tubing was carefully disconnected (ensuring no loss of concentrate), filter caps were replaced, and the filter was placed in a plastic bag on ice for transport back to the laboratory, where concentrate was recovered. The time required for ultrafiltration of 120 L was consistently 60 minutes. Tubing was sterilized between uses with 10% bleach (≥ 30 min contact time); following disinfection, tubing was thoroughly rinsed (10-15 min) with distilled water or groundwater from the next sampling site. To assess the influence of concentrate recovery method (elution vs. back-flushing) on PRD1 recovery, paired groundwater samples were collected at three sites by splitting the influent water flow to two parallel HFUF systems.

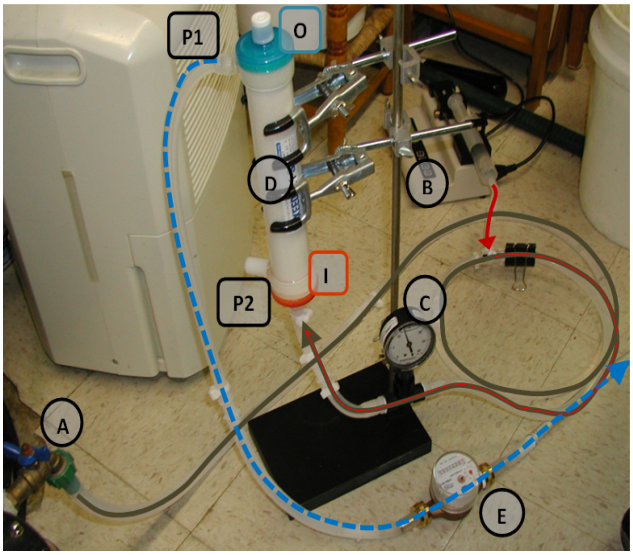

Figure 1. The HFUF was connected directly to a garden hose faucet (A), and sample water (gray, solid line) was fed into the filter inlet port (I) using the home's water pressure. HFUF system pressure was monitored with a liquid-filled pressure gauge (C) and maintained at ~13 psi by adjusting the tap (A). The HFUF system was operated in a dead-end configuration by leaving the filter outlet (O) and lower permeate (P2) ports closed. With port O closed, groundwater under pressure was driven laterally through 2.1 m^2 of hollow fibers contained within the filter housing; filtered water (blue dashed line) exited the open permeate port (P1) at ~2 L min^{-1}, verified using a flow totalizer (E). A dispersant solution (red solid line) was added continuously during filtration upstream of the filter via syringe pump (B), operated at 2 mL min^{-1}. The sampling event pictured occurred near Beloit, WI; the concentrated well sampled was positive for human AdV and PyV.

HFUF Concentrate Recovery and Secondary Concentration. Groundwater concentrate was recovered from the filter by back-flushing or elution 6-16 h after collection. Two concentrate recovery solutions (CRS) were used: (1) 0.01% NaPP + 0.1% Tween 80 and (2) 0.01% NaPP + 0.01% Tween 80. Concentrate recovery by back-flushing (Smith and Hill, 2009; Figure A1) involved reversing the direction of fluid flow through the filter relative to sample collection. To begin, both permeate ports were opened, and the permeate reservoir (i.e., the space within the filter housing *outside* of the hollow fibers) was purged with 500 mL of CRS1 using a peristaltic pump. The lower permeate port was then closed, the inlet port was opened, and 200 mL of CRS1 was pumped through the open permeate port and into the hollow fibers, displacing the sample concentrate into a 250-mL polypropylene centrifuge bottle. After 5-min contact time between the fibers and the CRS, the peristaltic pump was restarted and an additional 200-mL concentrate sample was collected. Filter elution was accomplished by opening the filter inlet and outlet ports and pumping 200 mL of CRS2 directly through the hollow fibers and into a 250-mL polypropylene centrifuge bottle. Following 5-min contact time, the peristaltic pump was restarted and an additional 200-mL concentrate samples was collected. The 400 mL of CRS recovered represents 3.6× the REXEED-21S filter priming volume (i.e., the volume of the full concentrate reservoir) of 112 mL.

Each 200-mL CRS sample was further concentrated by polyethylene glycol (PEG) precipitation. Briefly, CRS was supplemented to 3% beef extract, 0.3 M NaCl and 10% PEG 8000, pH 7.3. Following overnight incubation (4 °C, 120 rpm, ≥ 16 h), microorganisms were collected by centrifugation (60 min, 4550*g*, 4 °C); PEG supernatants were discarded, and PEG pellets from the first 400 mL of CRS were recovered with one 0.75 mL aliquot of Zymo Research Soil Microbe (ZRsm) DNA extraction kit lysis buffer. The third 200 mL was recovered with a separate 0.75 mL aliquot of ZRsm lysis buffer. All PEG pellets were stored at -20 °C until DNA extraction. Iron-rich samples from two households (both sampled repeatedly) were clarified (10 min, 4500*g*) prior to PEG precipitation in an attempt to remove iron solids.

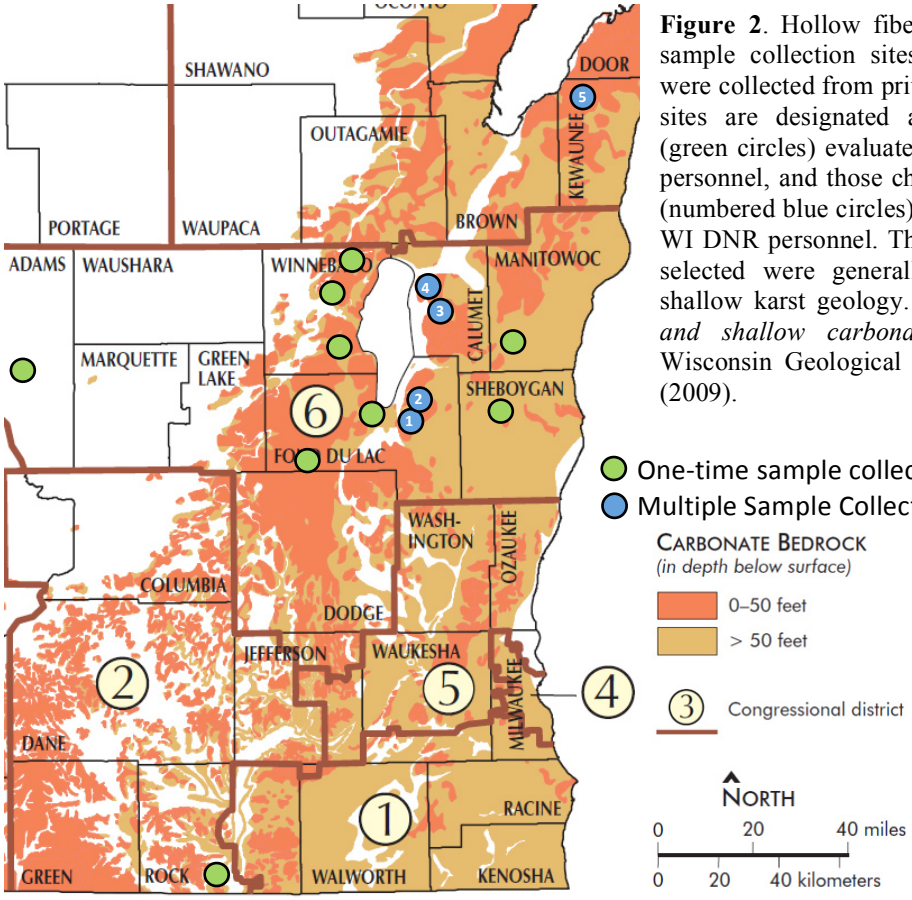

Figure 2. Hollow fiber ultrafiltration groundwater sample collection sites. All groundwater samples were collected from private wells. Sample collection sites are designated as one-time sampling sites (green circles) evaluated at the request of WI DNR personnel, and those chosen for repeated evaluation (numbered blue circles) chosen with the guidance of WI DNR personnel. The groundwater samples sites selected were generally located in regions with shallow karst geology. Figure adapted from, *Karst and shallow carbonate bedrock in Wisconsin*. Wisconsin Geological and Natural History Survey (2009).

Low-volume Groundwater Samples. Twenty-seven groundwater samples (200-600 mL), archived by the Wisconsin State Laboratory of Hygiene (WSLH), were concentrated by PEG precipitation and assayed by PCR for bovine BAdV-10 and BPyV-1. These samples were submitted to WSLH by the Wisconsin Department of Natural Resources for microbial source-tracking (MST) analysis in response to homeowner water quality complaints. The samples were investigated here to evaluate the utility of analyzing low-volume priority samples for viral markers of fecal contamination.

Bacteria and Virus Detection. Groundwater samples concentrated by HFUF were analyzed for coliform bacteria, *E. coli,* livestock and human AdV and PyV, and *Rhodococcus coprophilus*. Culturable *E. coli* and total coliforms in 100 mL of unfiltered groundwater (collected with 20 of 28 HFUF samples) and 1 mL of the first 400 mL of HFUF CRS (prior to PEG precipitation) were detected using the Colilert^{TM} reagent with the Quanti-tray^{TM} 2000 system (IDEXX Laboratories Inc.). Selected PCR methods were used to detect AdV, PyV and *R. coprophilus* and to quantify PRD1 in DNA extracted from PEG-precipitated HFUF groundwater sample concentrates. PRD1 was quantified by Taqman PCR against a $\log_{10}$ dilution series (10^2-10^7) of purified plamid DNA containing the target amplicon (TOPO TA, Invitrogen). Prior to PCR, the purified plasmid DNA concentration was quantified spectrophotometrically (Nanodrop ND 1000, Wilmington, DE). The cloned PRD1 amplicon was verified by sequencing. DNA extracted from PEG pellets of low-volume groundwater samples were assayed for BAdV-10 and BPyV-1. Primer sequences and the conditions employed in PCR assays are provided in Table A1.

DNA Extraction. All DNA extracts were prepared using the ZRsm DNA kit (Zymo Research Corp.). For livestock wastes, DNA was extracted directly from 0.5-g "solid" (feces, sand-separated manure and soil) or 0.5-mL liquid/slurry samples. Human sewage samples (100 mL) were clarified (4500*g*, 20 min, 4 °C) and PEG precipitated. For sewage samples and all groundwater concentrates, PEG pellets were extracted directly with the ZRsm kit, with minor modifications to the manufacturer's

sample-lysis protocol: samples were suspended with ZRsm lysis buffer by vortexing and incubated at 70 °C (10 min) immediately prior to bead beating (2800 oscillations·min^{-1}, 1 min; Mini Beadbeater, BioSpec Products). Subsequently, DNA was extracted from 400 µL of clarified lysate (15,000*g*, 10 min for HFUF concentrates and 1 min for low-volume groundwater samples).

Oligonucleotide Selection and Design. Published primer sets for the specific amplification of BPyV-1 (Wang et al., 2005) and HPyV-BK plus HPyV-JC (McQuaiq et al., 2006) were adopted for this study without modification. Available complete capsid protein gene sequences for AdV (hexon gene) and PyV (VP1 gene) were obtained from GenBank and aligned by viral genus using ClustalW, executed in BioEdit (v.7.0.9.0). Broad-spectrum (BS) primers targeting atadenoviruses (AtAdV), mastadenoviruses (MaAdV) and polyomaviruses were designed to amplify an array of known and novel viruses. Primers were tested empirically for their ability to generate specific amplicons of the expected sizes from (i) DNA extracts of HAdV-41, BAdV-1, BAdV-2; (ii) purified plasmid DNA of BAdV-4, -6, -7 -8, and OdAdV (kindly provided by H. Lehmkuhl), and of HPyV-BK and simian (polyoma)virus type 40 (kindly provided by J. Mertz); and (iii) DNA extracts of AdV- and PyV-positive bovine manure and human wastewater samples. A BAdV-10-specific primer set targeting hexon gene hypervariable region 1 was designed using Primer3. A PRD1-specific Taqman PCR assay was designed by inspection of a multiple genome alignment of bacteriophages L17 (AY848684), PR3 (AY848685), PR4 (AY848686), PR5 (AY848687), PR772 (AY848688) and PRD1 (AY848689). Primer3 was used to verify the compatibility the primers and probe selected. The PDR1 Taqman assay was linear over six orders of magnitude, demonstrated 90% amplification efficiency (slope = -3.59), and exhibited no amplification when bovine (n = 3) and human waste samples (n = 6) were assayed. The expected target range and specify of all oligonucleotides used during this study was assessed *in silico* using the Specificity Check feature of Primer-BLAST (National Center for Biotechnology Information).

PCR. Conventional PCRs (50 µL) were prepared with GoTaq® Green Master Mix (Promega, Inc.) and 5 µL of DNA extract or 1 µL flanking PCR product (for nested and semi-nested reactions). All degenerate primers were included at a concentration of 300 nM × primer degeneracy (D), except MaAdF2 (D = 8), which was employed at 1600 nM total; non-degenerate primers were employed at 500 nM. Identical TD-PCR programs were used for both rounds of semi-nested amplification using BS AdV primer sets: 94 °C for 4 min, followed by 10 cycles of 94 °C for 30 s, 65 °C for 30 s (with a decrement 1 °C per cycle), and 72 °C for 1 min. An additional 30 cycles were completed as follows: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, finishing with a 72 °C (7 min) extension. All other conventional PCRs were completed using the following reaction conditions: 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 or 58 °C for 30 s, and 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. All PCRs were prepared in a cooling block (4 °C) before placement in the preheated (94 °C) Eppendorf Mastercycler® Thermocycler. PCR products were detected under UV light after agarose gel electrophoresis (2%) and ethidium bromide staining. Quantification of PRD1 DNA was completed in 20-µL reactions (5 µl of DNA extract) using LightCycler® TaqMan® Master Mix on the LightCycler® 2.0 Real-Time PCR System (Roche Applied Science, Inc.). qPCR conditions were as follows: DNA polymerase activation at 95 °C for 10 min, followed by 45 cycles of DNA denaturation (95 °C, 15 s), primer annealing (58 °C, 40 s; 72 °C, 1 s), and polymerase extension (60 °C, 60 sec); the temperature ramp rate was 20 °C·s^{-1}. The oligonucleotides employed were synthesized by the University of Wisconsin-Madison (UW) Biotechnology Center or Integrated DNA Technologies (Coralville, IA); their sequences and PCR annealing temperatures are reported in Table A1.

RESULTS AND DISCUSION

Excreta Evaluation for AdV and PyV. We determined the prevalence of BAdV and BPyV in excreta samples from cattle to ascertain the utility of these viruses as fecal contamination indicators (Table A2). Using original, "broad-spectrum" PCR primer sets designed to detect an array of known and previously unidentified AdV and PyV, BAdV were detected in 13% of cattle fecal samples, 90% of cattle urine samples and 100% of cattle manure samples; 44% of BAdV-positive samples contained DNA from two genetically distinct AdV genera, *Atadenoviridae* and *Mastadenoviridae*. BPyV were detected less frequently than BAdV in these samples, at rates of 17% in cattle feces, 14% in cattle urine and 73% in

cattle manure. BAdV excretion in urine was observed commonly during this investigation but had not been previously documented. Additionally, five previously unknown bovine viruses were detected and partially sequenced during this investigation, four BAdV and one BPyV, increasing the number of BAdV genotypes to 15 and BPyV genotypes to two.

Observed detection rates for two specific bovine viruses, BAdV-10 and BPyV-1 (e.g., 50% and 27%, respectively, in manure) supported targeting these viruses for fecal source attribution. BPyV-1 has been detected frequently in other investigations of bovine serum and excreta (Wang et al., 2005; Wong and Rose, 2009). However, no previous study has demonstrated prevalent, asymptomatic shedding of BAdV-10 by cattle. Based on this finding, we designed and validated a PCR assay for the specific detection of BAdV-10. Our assay targets a "hypervariable" region of the AdV hexon protein gene, making the PCR assay highly specific for BAdV-10. This attribute is critical, since knowledge of AdV (and PyV) genetic diversity across potential host species is incomplete. Therefore, PCR assays capable of detecting *multiple* viruses have an increased chance of detecting a previously unidentified virus with an unknown host, obscuring fecal source attribution.

We also evaluated all of our bovine excreta samples, plus five sewage influent samples, using a previously published PCR assay for HPyV (McQuaig et al., 2006). The specificity of the HPyV assay has been verified previously by testing 152 animal waste samples (from 13 species) (McQuaig et al., 2009). However, no information was available to validate its usefulness locally. In our hands, the assay showed no amplification of PyV in bovine samples and 80% HPyV detection in sewage (Table A2). The presence of HPyV at high concentration in sewage has been documented, and HPyV have shown higher prevalence in individual septic tanks than HAdV (Harwood et al., 2009; McQuaig et al., 2009). These factors encouraged our application of the HPyV PCR for fecal source tracking in domestic well water samples.

Hollow fiber ultrafiltration system configuration. Most previous laboratory and environmental investigations using HFUF to concentrate microorganisms employed a tangential-flow configuration. This method requires comprehensive operator training and is not conducive to rapid-response field sampling (Smith and Hill, 2009). We designed our HFUF system to operate in a dead-end configuration (Smith and Hill, 2009), which is permitted by the large surface area (2.1 m^2) of the hemodialysis filter. The streamlined system plumbing and sample collection routine associated with dead-end HFUF allows direct sample collection from a garden hose faucet. This simplification reduces the likelihood of sample contamination, since no intermediate vessel (e.g., a plastic garbage can) is employed during sample collection, and significantly reduces the operator training required for system operation. In addition, the dead-end HFUF is well-suited for rapid-response field implementation. Overall, the virus-concentration routine assembled here meets sample-processing criteria suggested for fecal source tracking investigations (Harwood et al., 2009): the method co-concentrates a variety of microbial targets using an affordable, commercially-available filter and co-purifies DNA using a commercially available extraction kit.

PRD1 recovery by dead-end HFUF. The efficacy of HFUF for concentrating microorganisms, including viruses, in controlled laboratory experiments has been demonstrated repeatedly, with optimized microorganism recoveries of 50 to 100% (Hill et al., 2005; Smith and Hill, 2009). However, as the chemistry of water samples becomes more complex, microorganism recoveries may diminish and/or become more variable (Leskinen and Lim, 2008; Knappett et al., 2011; Gibson and Schwab, 2011b); in these situations, significant correlations between marker recoveries and water chemistry parameters become more difficult to define (Hill et al. 2007). The expected site-to-site variability of groundwater chemistry challenges the utility of further (e.g., beyond Smith and Hill, 2009) laboratory optimization of dead-end HFUF. Therefore, to quantify the cumulative efficiency of our virus detection protocol, we spiked bacteriophage PRD1, an enteric virus surrogate, into the HFUF system during groundwater sample collection from domestic wells.

A large phage spike ($4 \pm 2 \times 10^8$ G.E.) was selected, anticipating that *in situ* microorganism recoveries from groundwater might be low and/or variable (Gibson and Schwab, 2011b), which was in fact, the case (Table 1). Considering 100% recovery of viruses from a groundwater volume of 115 L, our sampling and analysis protocol provides a theoretical concentration factor of $10^{5.5}$ (i.e., the 0.005 mL of concentrated "groundwater" DNA analyzed during PCR corresponds to an extrapolated native groundwater volume of 1500 mL). In practice, we observed an average PRD1 recovery of 16 ± 29% (n =

20), corresponding to an effective concentration factor of $10^{4.7} \pm 10^{4.9}$ and an average analysis of 250 ± 420 mL of groundwater by PCR.

Method recoveries varied widely for PRD1 among sample collection sites (Figure 3). The factor(s) promoting reproducibly strong PRD1 recoveries at Site 3 (31% ± 6%), but not the other sites, are unclear. Conversely, consistently low PRD1 recoveries observed at Sites 2 and 4 may be associated with the significant (> 5 mL) volumes of colloidal iron collected by HFUF at these locations. This iron appeared to have little impact on bacteria recovery (90% and 83% for Site 5), but was the most striking (though not statistically significant, $p = 0.12$) factor predictably associated with low PRD1 recovery. Of note, groundwater at these sites had detectable dissolved oxygen (0.7-5.6 mg L^{-1}) and low dissolved iron concentrations (<0.006-0.044 mg L^{-1}) (Walt Kelley, Illinois State Water Survey, personal communication). These observations (a) suggest that iron precipitation had occurred prior to sampling and exposure to atmospheric oxygen; and (b) appear responsible for negating the effectiveness of EDTA addition during HFUF sample collection as a method for reducing iron accumulation (Knappett et al., 2011).

In contrast with measured PRD1 recoveries, for sites where coliform bacteria were present in unfiltered groundwater and HFUF concentrates, the recovery of *in situ* coliform bacteria was impressive (46 ± 34%, $n = 9$). The higher recoveries estimated for bacteria are consonant with literature reports of stronger recovery of bacteria (than viruses) from environmental HFUF concentrates (Gibson and Schwab, 2011b). Unlike for PRD1, coliform recoveries by HFUF were determined without need for a secondary concentration or DNA extraction steps. Therefore, optimization of these methods could hold the key to improving virus detection in groundwater samples concentrated by ultrafiltration.

Paired samples were collected at three unique sites to determine if HFUF concentrate recovery methods could be adjusted to improve PRD1 recovery. For the three sites, groundwater concentrate was displaced from the HFUF by either elution or back-flushing. Consonant with published laboratory experiments (Hill et al., 2005) and our initial strategy, PRD1 recoveries for all three sites demonstrated stronger (though not statistically significant, $p = 0.16$) virus recovery by back-flushing compared with elution.

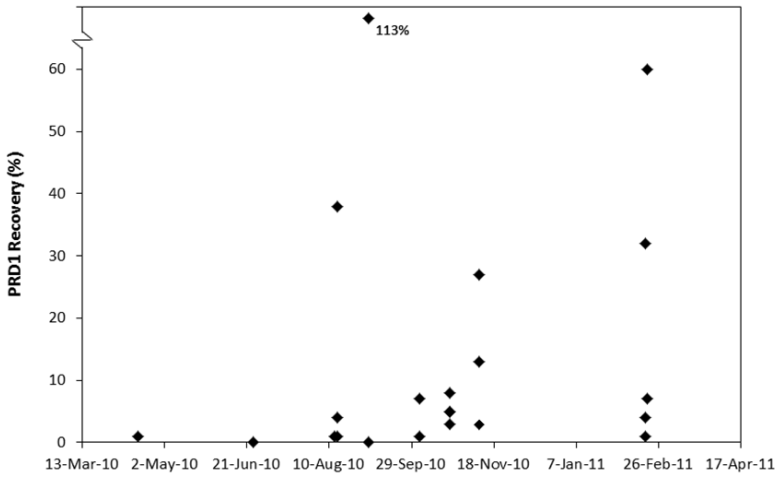

Figure 3. The recovery of exogenous bacteriophage PRD1 by HFUF from groundwater (115 ± 15 L) samples was highly variable. Average PRD1 recoveries (%) showed no statistical dependence on the factors (*a*) sampling site ($p = 0.43$), (*b*) dispersant (NaPP vs. EDTA solutions added during HFUF; $p = 0.75$), (c) colloidal iron (presence/ absence in HFUF concentrates; $p = 0.12$), or (*d*) collection month ($p = 0.50$). These results indicated a lack of predictable influence of changes in groundwater chemistry or variations in sample processing strategy on PRD1 recovery.

Fecal Source Attribution in Groundwater Samples: The primary motivation for this study was to evaluate the likelihood that AdV and PyV would be detected and prove useful for fecal source attribution in private groundwater samples deemed vulnerable to fecal contamination. Of the 24 groundwater samples collected by HFUF, 17 were positive for coliform bacteria, eight were positive for *E. coli*, six were positive for *R. coprophilus* and three were positive for at least one viral marker (AdV or PyV) of fecal contamination. A single viral marker was detected at two of the five sites targeted for repeated sample collection; of the six homes where groundwater was collected only once, one was positive for two viral markers (Site R, for Rock County). The detection of corroborating host-specific microbial markers is required for confident source attribution. Therefore, only for the latter site could an

actionable contamination source, human, be reliably attributed. Subsequent site investigation by the WI Department of Natural Resources revealed a compromised pipe in the homeowner's septic system.

Table 1. PRD1 recovery and fecal indicator detection in domestic well water.

		PRD1 Recovery			Microbial Indicator Detection					Putative Source
	n	*n*[a]	Ave. (%)	SD (%)	Coliform Bacteria	*E. coli*	*R. coprophilus*	AdV or PyV		
Site 1	3	3	41	62	0/3	0/3	0/3	0/3	NA	Unknown
Site 2	5	4	18	28	3/5	0/5	1/5	0/4	NA	Unknown
Site 3	3	3	31	6	3/3	1/3	1/4	1/3	AtAdV[b]	Animal
Site 4	4	4	3	2	1/4	1/4	3/4	1/4	HPyV	Ambiguous
Site 5	3	3	5	8	3/3	2/3	1/3	0/3	NA	Unknown
Others	7	4	2	3	6/7	4/7	0/7	1/7	HAdV HPyV	Human[c]

[a] Number of HFUF samples collected that were spiked with PRD1.
[b] AtAdV detected showed 89% identity with BAdV-6.
[c] Source for Site R.

At Site 3, an AdV was detected with our AtAdV PCR assay. The expected target range for this assay comprises BAdV-4 through -8, plus ovine AdV-7 and goat AdV-1. During our investigation of bovine excreta, no AtAdV with less than 96% identity to prototype AtAdV genotypes were amplified using this assay. However, in this groundwater sample an AtAdV with only 89% identity with the closest known genotype (BAdV-6) was discovered. This novel AtAdV is predicted to have a livestock host, based on comparative phylogenetic analysis (Figure 4), and a livestock source of contamination is supported by the coincident detection in this sample of *R. coprophilus,* a bacterium that grows in the dung of herbivores and provides useful support for other ruminant fecal markers (Oragui and Mara, 1981; Savill et al., 2001; Gilpin et al., 2008). However, a wildlife host cannot be excluded. This scenario exemplifies one of the main problems with viral genetic markers of livestock contamination: potential host-ambiguity due to preponderance of unidentified genotypes. For that reason, PCRs that can amplify multiple livestock viruses require extensive validation prior to use in source tracking without confirmatory DNA sequencing. The detections of an HPyV and *R. coprophilus* at Site 4 were unsupported by corroborating host markers. Contamination at this site could be resulting from humans, grazing herbivores, or both.

In most cases, even the concentration of large sample volumes did not reveal sources or overcome the intermittent nature of groundwater contamination. For example, Sites 4 and 5 were sampled for bacteria by a research group from the Illinois State Water Survey one day prior to our February 2010 sampling trips. Using the same detection method (Colilert/Quantitray, IDEX), their results differed dramatically from ours: at Site 4, their 100 mL sample exceeded the upper detection limit of the Quantitray method (>2419 cfu) for coliform bacteria and was *E. coli* positive; conversely, our sample, collected 29 h later, showed *no* bacterial contamination. Nearly the opposite result was obtained for Site 5, where the IL group observed 5 cfu/100 mL coliform bacteria (*E. coli* absent) whereas *our* sample exceeded the Quantitray upper detection limit for coliforms and was *E. coli* positive (6 cfu/100 mL). These simple data highlight the significant challenge in "correctly" timing sample collection for fecal source tracking.

Large sample volumes are generally thought to be necessary when targeting viruses in groundwater. However, confident fecal source attribution was possible for several low-volume groundwater samples submitted to WSLH for microbial source tracking in response to homeowner water quality complaints. These samples were analyzed for BPyV-1 and BAdV-10, and frequently yielded positive results for these bovine viral indicators: eight of 28 samples analyzed were positive for BPyV-1, while four of these eight were also positive for BAdV-10. The effective groundwater volume analyzed by PCR for these low-volume groundwater samples was 1 to 10 mL for method recoveries ranging from 10 to 100%, respectively. Knappett et al. (2011), working in Bangladesh, recently detected human viruses in low-volume (250-mL) groundwater samples collected from shallow wells, supporting our findings.

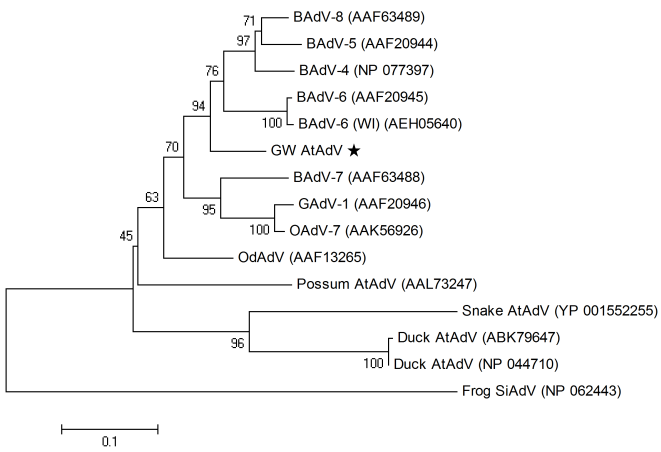

Figure 4. Neighbor-joining phylogenetic analysis suggested a livestock host for the AtAdV ("GW AtAdV") detected at Site 3. Prior to the analysis of this sample, the PCR assay employed had not amplified an AtAdV with less than 96% identity to known BAdV. This scenario is exemplary of one of the main problem with genetic markers for FST livestock contamination (and animal sources, more broadly): ambiguity when the sequence is identified due to preponderance of unidentified markers. For that reason, PCRs that can amplify multiple viruses (in particular livestock viruses) require extensive validation before they should be used for source tracking without DNA sequencing to identify the presumptive amplicon.

CONCLUSIONS AND RECOMMENDATIONS

Our detection of HAdV, HPyV, BAdV-10 and BPyV-1 in groundwater samples collected from private wells supports the utility of these viruses as fecal source indicators. In particular, our discovery of prevalent shedding of BAdV-10 by cattle is significant, and our implementation of a BAdV-10 PCR assay adds a highly cattle-specific marker to the toolbox of methods available to the source tracking community. Groundwater samples collected using HFUF showed large concentration factors (~10^4) and were often contaminated by coliform bacteria. However, in most cases, even the concentration of large sample volumes did not reveal contamination sources or overcome the intermittent nature of groundwater contamination by fecal material. On the other hand, low-volumes samples analyzed in response to immediate home-owner complaints frequently (and unexpectedly) yielded positive results for bovine viral indicators, resulting in definitive source attribution. Therefore, the analysis of these types of groundwater samples for AdV and PyV is recommended. Where potentially contaminated groundwater presents an immediate and unacceptable risk to human health (e.g., for vulnerable wells serving daycare facilities, nursing homes, or communities), the application of the dead-end HFUF method described here is also recommended. For example, at Site 4 and Site R, human viruses were detected in HFUF concentrate with low measured PRD1 recovery (0% and 1%, respectively) but were absent in a 400-mL sample collected and processed in parallel.

Strong *in situ* recovery of bacterial indicators (versus PRD1) suggests that improved virus recovery may be gained by further optimization of sample secondary concentration and DNA purification methods. For example, targeted treatment of CRS (e.g., incubation with EDTA for sample iron removal) or a reduction in the total volume of CRS collected may reduce the impact of method inhibitors. The use of beef extract as a CRS, and perhaps as a sample amendment immediately following filtration, would streamline sample processing and might result in improved virus recovery if virus attachment to the filter matrix is an issue.

Our results suggest that mobilizing a sampling effort to a particular site at the "right" time – which from a practical standpoint may be unknowable – is challenging. Alternatively, our successful detection of host-specific viral markers in low-volume groundwater samples suggests an alternative approach for site-dedicated source tracking: the homeowner could be enlisted in the collection of ~500-mL samples over a several day period; focusing on periods following large rain events or snow melts might improve the chances of fecal source attribution. Following the collection of a series of low-volume samples, the investigator could retrieve samples and perform source tracking following the methods described here.

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APPENDIX A: Awards, Publications, Reports, Patents and Presentations

Sibley, S.D. and J.A. Pedersen. 2011. Detection of Known and Novel Adenoviruses in Cattle Wastes via Broad-spectrum Primers. Appl. Environ. Microbiol., **77**:5001-5008.

"*Testing Well Water for Microorganisms*." Fall/Winter 2010, Aquatic Sciences Chronicle, University of Wisconsin Sea Grant and Water Resources Institute.

APPENDIX B:

TABLE A1. Oligonucleotides used for PCR. All assays, except for BPyV-1 and HPyV, were designed and validated during this investigation.

Assay	Direction	Oligo ID	Oligo Conc. (nM)	Annealing Temp (°C)	Sequences (5' - 3') [a]	Product size (bp)
MaAdV	Forward	MaAdF1		55	CAGTGGTCHTACATGCACATC	
	Reverse	MaAdR1		55	GCATAAGACCCGTAGCAWGG	599 – 725[b]
	Forward	MaAdF2		55	CATGCACATCGCSGGNCAGGA	588 – 714[b]
AtAdV	Forward	AtAdF1	500	55	CACATTGCGGGTAGAAATGC	
	Reverse	AtAdR1	600		TAAGCWGTTCCTCCATAAGG	323
	Forward	AtAdF2	500	55	GCGGGTAGAAATGCGAGG	
	Reverse	AtAdR2			TGTTGGAGCTACAAAAGGATCTC	114
BAdV-10	Forward	B10F	500	58	TTACGCCCAACTTCCTTTTG	
	Reverse	B10R			CCACGCGTCTACTCCGTATT	127
BPyV-1[c]	Forward	VP1F	500	58	GGTATTCGCCCTCTGCTGGTCAAG	
	Reverse	VP1R			GCTGGCAATGGGGTATGGGTTCT	527
	Forward	VP2F	500	58	ATT TCAAAGCCCCCTATCATC	
	Reverse	VP2R			GCCTACGCCATTCTCATCAAG	263
HPyV[d]	Forward	SM2	500	58	AGTCTTTAGGGTCTTCTACCTTT	
	Reverse	P6			GGTGCCAACCTATGGAACAG	176
R. c.[e]	Forward	RcF	200	58	GGGTCTAATACCGGATATGACCAT	
	Reverse	RcR			GCAGTTGAGCTGCGGGATTTCACAC	443
PRD1	Forward	PRD1F	500		AGCTTAATGACTACGCCAGT	
	Reverse	PRD1R		58	GGAAGATTCCGTTTGAACA	161
	Taqman	PRD1q	100		TAATGATTATTTGGCTTCACAAGCGGG	
PyV	Forward	PyV-F2900		46, 58	AATGAIAACACIAGRTAYTWTGG	
	Reverse	PyV-R4160	1000	46	GGTTGTITTTGARGATGTIAARGG	~1260
	Reverse	PyV-R0		58	CAIAGIGGICCMACNCCATTYTCAT	120

[a] H = C+A+T; W = A+T; B = C+T+G; S = G+C; degenerate positions are underlined.
[b] Amplification of hypervariable region, V1 (6) by MaAdV primers results in variably-sized products.
[c] Wang et al. (2005)
[d] McQuaig et al. (2009)
[e] *Rhodococus coprophilus*; Savill et al. (2001)

TABLE A2. Summary of excreta and low-volume groundwater sample evaluations by broad-spectrum and virus-specific PCRs.

		Detection Rate					
Sample	Sample Info./Animal Age [a]	HPyV	MaAdV	AtAdV	PyV	BAdV-10	BPyV-1
Bovine Feces							
Dairy Calf	<14 weeks; AARS-DCU	0/9	0/9	0/9	1/8	0/9	2/8
Adult	1-10 years; UW-Dairy, AARS-BNC	0/21	4/23	0/23	3/22	0/9	0/22
Bovine Urine							
Beef Cow	AARS-BNC; ~ 15 months	0/3	2/3	3/3	2/3	2/3	0/3
Dairy Cow	UW-Dairy (*n* = 17); Farm A (*n* = 1); 2.5 to 9 years	0/18	13/18	11/18	1/18	13/18	0/18
Bovine Manure							
Dairy Lagoon Slurry	Two private dairies	0/2	2/2	2/2	2/2	1/2	1/2
Dairy Manure	Sand-separated	0/1	1/1	1/1	1/1	1/1	1/1
Bedding Percolate	Dairy Exposition	NA	5/5	5/5	4/5	NA	1/5
Beef Cattle Bedding	AARS-BNC; ~15 month cattle; moist, soiled hay	0/3	3/3	3/3	0/3	1/3	0/3
Environmental							
Field Mud [b]	Water station, Farm E	0/2	0/2	2/2	1/2	0/2	0/2
Field Runoff [c]	Drainage ditch, Farm E	0/1	1/1	1/1	1/1	1/1	0/1
Groundwater	≤ 600 mL; Archived by WSLH	NA	NA	NA	NA	4/28	8/28
Other							
Human sewage [d]	NSWTF, 24-h composites	4/5	5/5	0/5	5/5	0/5	0/5
Pig feces	AARS-P; 1 sow, 1 piglet	0/2	0/2	0/2	0/2	0/2	0/2
Pig wash water [e]	AARS-P; newborn to finished	0/1	1/1	0/1	0/1	0/1	0/1
Deer feces		0/4	0/4	0/4	0/4	0/4	0/4
Rabbit feces	One fecal pellet	NA	1/1	0/1	0/1	0/1	NA
Dog feces	3 to 4 year	0/2	0/2	0/2	0/2	0/2	0/2
Goose feces	10 scat composite	0/1	0/1	0/1	0/1	0/1	0/1

[a] Abbreviations: AARS, Arlington (WI) Agricultural Research Station; -DCU, Dairy Calf Unit; -BNC, Beef Nutrition Center; -P, Porcine Research Center; UW-Dairy, University of Wisconsin-Madison Dairy; NSWTF, Nine Springs Wastewater Treatment Facility (Madison, WI); NA, Not Analyzed.

[b] Collected approximately 3 and 6 meters from a water station.

[c] Collected from a muddy pool adjacent to a large cattle lot.

[d] Collected May, Jun and Oct 2009; Jan and Feb 2010. HAdV-31 detected once; HAdV-41-WI detected in all sample.

[e] Pen wash-water recirculated throughout the facility, sampling newborn to finished swine.

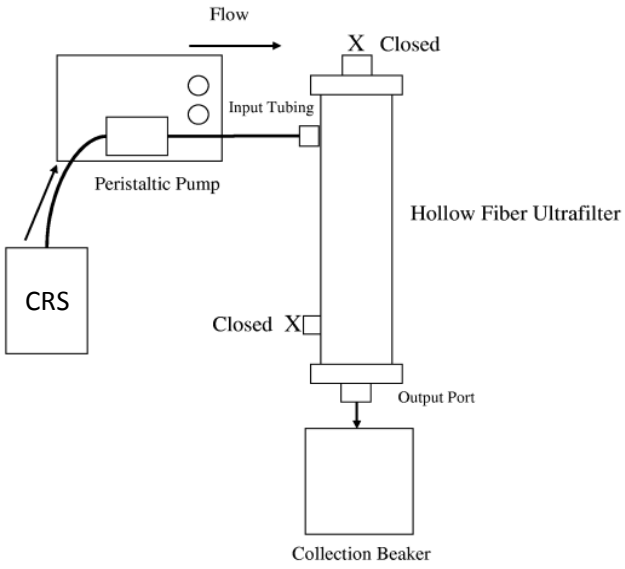

Figure A1. Configuration used for back-flushing the hollow fiber ultrafilter (from Smith and Hill, 2009). Four-hundred milliliters of a 0.01% sodium polyphosphate and 0.1% Tween 80 concentrate recovery solution (CRS) was used to displace and carry microorganisms collected within the filter's hollow fibers into two 250-mL centrifuge bottles for subsequent secondary concentration by polyethylene glycol precipitation.