Use of human and bovine adenovirus for fecal source tracking. [DNR-195] 2008

Kluender, S.; McMahon, K. D.; Pedersen, Joel A.
Madison, Wisconsin: Wisconsin Department of Natural Resources, 2008

195

FINAL PROJECT REPORT

for

Use of Human and Bovine Adenovirus for Fecal Source Tracking

Submitted to
Wisconsin Department of Natural Resources

by

PIs: S. Kluender, K.D. McMahon and Joel A. Pedersen
Wisconsin State Laboratory of Hygiene and Departments of Environmental Engineering, and Soil Science
University of Wisconsin-Madison

10/04/2008

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

PROJECT SUMMARY

Title: Use of Human and Bovine Adenovirus for Fecal Source Tracking.

Investigators: **S. Kluender**, Microbiologist Supervisor, WI State Laboratory of Hygiene, Madison
K.D. McMahon, Associate Professor, Departments of Civil & Environmental Engineering and Bacteriology, University of Wisconsin (UW), Madison
Joel A. Pedersen, Associate Professor, Departments of Soil Science and Civil & Environmental Engineering, UW-Madison
Research Assistant: **Samuel D. Sibley**, Graduate Research Assistant, Environmental Chemistry & Technology, UW-Madison

Period of Contract: July 1, 2006 to June 30, 2008

Background/Need:

Confirming the presence and determining the source(s) of fecal contamination to water is critical for the protection of human health and environmental quality. This is especially true in large portions of Wisconsin, where fractured and/or karst bedrock is located within a few feet of the ground surface, facilitating the rapid transport of fecal pathogens to aquifers. The detection of commonly targeted fecal indicators, such as coliform bacteria and caffeine, suggests that water quality has been compromised. However, these indicators do not adequately track or confirm the presence of several enteric pathogens of concern, including viruses. In addition, current indicators do not discriminate between important sources of fecal contamination (e.g., human vs. livestock) without labor-intensive and time-consuming investigation. Therefore, the causes of elevated indicator concentrations may be misjudged or remain unidentified.

Adenoviruses (AdV) have been advanced in recent literature as fecal indicators that have the potential to both distinguish contamination sources and track the environmental transport of enteric viruses. Despite these promising features, a number of challenges have prevented the efficient and confident utilization of AdV as source-specific fecal indicators. These challenges include (1) current polymerase chain reaction (PCR) methods designed for AdV detection were not developed to simply or definitively discriminate between AdV of human vs. livestock origin; (2) filters commonly used to collect/concentrate viruses in 10- to 100-L water samples often exhibit poor virus recovery and are costly; and (3) compounds present in concentrated samples (e.g., chemicals added to elute viruses from filters, natural substances in water co-concentrated with viruses) often interfere with DNA amplification by PCR, an exquisitely sensitive biochemical reaction.

Objectives:

The objectives of this study were (1) to develop a quantitative PCR (qPCR) method capable of simply and confidently distinguishing between human and bovine AdV; and (2) to optimize virus recovery from environmental samples by two newly-advocated, competitively priced filters while minimizing concomitant concentration or introduction of PCR-inhibiting compounds. To achieve these goals we addressed the following questions:

1: Considering the similarity between group I bovine adenoviruses (BAdV) and all human adenoviruses (HAdV), can a simple and specific qPCR assay be designed capable of detecting multiple AdV of one group while excluding representatives from the other?

2a: To what extent can adsorbed viruses (bacteriophage, then adenoviruses) be recovered from NanoCeram® filters, a highly electropositive medium allowing efficient removal of viruses from water?

2b: If virus recovery is achievable, will successful eluate(s) inhibit down-stream PCR

applications?

2c: Will Fresenius Hollow Fiber Ultrafilters, which exhibit strong virus recovery, allow the co-concentration of other microorganisms/pathogens of public health and fecal source tracking interest (facilitating the broad deployment of the HFUF system for public health monitoring/assessment)?

2d: If so, will adenovirus, for which recovery data are absent, be recovered to a similar extent as surrogate bacteriophages (and other viruses previously examined)?

These questions must be addressed before adenoviruses can be confidently employed to assess the prevalence and concentrations of AdV of human and livestock origin in impacted versus nonimpacted waters. Although additional work is required to fully validate AdV-based fecal source tracking (most notably, the prevalence of non-human *Mastadenovirus* in fecal samples must be determined and the genomes of any detected viruses must sequenced), the focused effort of this study allows the confident concentration, detection and discrimination of human vs. group I bovine adenoviruses for virus monitoring and incipient AdV-based fecal source tracking.

Methods:

Oligonucleotides for the detection of HAdV and of BAdV were designed based on alignments of all available non-redundant sequences of the hexon gene (or complete AdV genome) present in the NCBI GenBank database. Primers were evaluated first by conventional PCR and agarose gel electrophoresis (with ethidium bromide staining) and subsequently by quantitative PCR using SYBR green dye or specific TaqMan probes. Cationic Nanoceram® filters were evaluated for the retention and recovery of two bacteriophages (MS2 and PRD1) and two AdV (bovine 1 and human 41) by virus adsorption-elution (VIRADEL). A variety of eluent solutions were investigated based on their previously demonstrated or hypothesized ability to disrupt virus-filter interactions. Fresenius hollow-fiber hemodialysis ultrafilters were evaluated for the recovery of bacteria (*Escherichia coli* and *E. faecalis*), bacteriophages (MS2 and PRD1), BAdV 1 and HAdV 41. AdV were assayed by qPCR, while bacteriophage were enumerated via plaque-forming-unit (pfu) assay on host bacteria cultures. *E. coli* and *E. faecalis* were enumerated using the quantitray application of the Colilert and Enterolert assays (IDEXX, Inc.). All experiments were conducted at the Agriculture Drive branch of the Wisconsin State Laboratory of Hygiene (WSLH), where the molecular biology and biosafety level 2 facilities required for this research were available. Daily interaction with WSLH personnel was of mutual benefit, as their input helped craft the techniques they will eventually deploy.

Results and Discussion:

Published PCR methods were unavailable (BAdV) or deemed inadequate (HAdV) for purposes proposed here. While designing a PCR assay capable of distinguishing between (i.e., amplifying specifically) human vs. group I (GI) bovine AdV, both belonging to the genus *Mastadenovirus*, represented a significant challenge, we successfully designed and evaluated a set of primers capable of specific HAdV vs. group I BAdV amplification. These primers take advantageous of three sets of previously undescribed consecutive mismatches between HAdV vs. BAdV hexon gene sequences that are conserved within the sequences of each of these groups. The specificity of the technique is derived from the placement of consecutive dual nucleotide mismatches (DNM) at the 3' end of both the forward and reverse primers: our data show that extension (duplication) of DNA from the 3' end of a primer/template complex by Taq Polymerase, the enzyme responsible for PCR, is completely inhibited when otherwise homologous primers demonstrate these consecutive 3' mismatches with the target sequence. To our knowledge, ours represents the first promulgated qPCR assay for BAdV.

NanoCeram® filters demonstrated quantitative removal and 0 – 90% recovery of phages from feed water, depending on eluent composition and bacteriophage identity. Optimized eluents showed no

PCR inhibition but were surprisingly ineffective at dislodging AdV (HAdV 41 and BAdV 1) from the filters. We observed higher than 99% retention of AdV on the NanoCeram® filters, but less than 5% recovery of these AdV with eluents optimized for bacteriophage. No peer reviewed reports on these filters are currently published, so low recoveries could not be verified by comparison. Favorable microorganism recoveries (≥ 58%), including for HAdV 41 (72%), were observed for the Fresenius hollow fiber ultrafilter. The hollow-fiber ultrafiltration system (HFUF) designed here is therefore preferred for future virus and pathogen collection and is ready for deployment by WSLH personnel.

Conclusions/Implications/Recommendations:

As a result of its empirically demonstrated specificity, the novel PCR assay described here represents a significant advance towards the implementation of AdV-based fecal source tracking. In addition, the specificity of the assay allows for the adoption of SYBR green-based qPCR. Though more straight-forward in application, SYBR green binds to double stranded DNA non-specifically and, accordingly, is often avoided in assays requiring the extra specificity potentially derived from TaqMan probes (which bind to specific sequences within a target). However, the use of SYBR green may be preferred in instances when (a) resources for TaqMan probes may be unavailable, (b) assay simplification is desired, and (c) investigators require an additional homogeneous check of assay results (i.e., because amplicons generated for BAdV vs. HAdV in our assay differ in size, they can be independently confirmed/distinguished at the conclusion of a PCR by melting curve analysis that is possible with SYBR green but not TaqMan chemistry). We note that AdV belonging to the genus *Mastadenovirus* that have yet to be isolated and sequenced could share one or both of the 3'DNP around which our assay derives its specificity. This uncertainty is not unique to the present study; the design of PCR primers and probes (and their resulting specificity) is always limited by the availability of sequence data upon which primers are based. That said, the primer designed here (1) are based on two sets of 3' dual nucleotide mismatches, when one 3'DNM alone would likely provide sufficient discrimination, and (2) are not predicted to amplify any known non-target AdV (based on an evaluation of our oligonucleotides with the BLAST algorithm of the NCBI database).

While poor recovery of AdV discouraged further evaluation of NanoCeram® filters, the HFUF unit is ready to be deployed by the WSLH for simultaneous concentration of multiple pathogens and indicators (including AdV) of interest for fecal source tracking. Future research into the spatial and seasonal distribution of livestock and wildlife AdV is recommended, as the information acquired during such surveys will make AdV-based fecal source tracking assays more robust. Any work completed in this regard should be accompanied by the acquisition of genetic data through cloning/sequencing of AdV-positive PCR products. In this way, the database of available animal AdV sequences will be enhanced, allowing for continuing evaluation/validation of the specificity of the primers/probes designed here. In addition, considering the improved HFUF configuration, which allows for sample concentration without user supervision, a logical next step is the modification/evaluation of this HFUF system for the collection of very large (500- to 1000-L) water samples. Specifically, modifications of the current system facilitating (1) continuous filtration from a water source, (2) injection of the NaPP dispersing agent during filtration (as opposed to one-step addition at when commencing to acquire 50- to 100-L samples), and (3) real-time sample preservation (e.g., installation of a cooling jacket around the sample concentrate bottle) would significantly expand an investigator's ability to efficiently concentrate viral (and other) pathogens from potentially contaminated groundwater.

Related Publications:

Sibley, S.; McMahon, K.D.; Pedersen, J.A. Development and evaluation of a novel fecal source tracking TaqMan PCR assay for discriminating bovine and human adenoviruses based on specific polymerase extension from 3' dual nucleotide mismatches. (*in prep.*)

Sibley, S.; McMahon, K.D.; Pedersen, J.A. Capture and recovery of bacteriophage MS2 and PRD1, bovine adenovirus Type 1 and human adenovirus type 41 from novel nanoaluminum oxide fiber filters. (*in prep.*)

Key Words: Adenovirus, bovine, bacteriophage, nanoaluminum, NanoCeram©, hollow fiber ultrafiltration, fecal source tracking, indicator.

Funding: UW-Water Resources Institute.

INTRODUCTION

Groundwater contamination by human and livestock feces introduces nutrients, pharmaceuticals, hormones and pathogenic microorganisms that may pose risk to human and animal health and degrade water quality, restricting its use. Rapid identification of fecal contamination and its source(s) are critical for preventing human exposure to pathogens and addressing contamination at its source. Current fecal source tracking (FST) methods can be grouped in four categories (Fong and Lipp, 2005): (1) genotypic library-based methods (e.g., ribotyping); (2) phenotypic library-based methods (e.g., multiple antibiotic resistance; MAR); (3) library-independent bacterial host marker methods; (4) direct measurement of host-specific viral pathogens and bacteriophages. Many of these techniques have been used successfully for source tracking, but none of these methods have been agreed upon for regulatory purposes, and all have advantages and disadvantages that must be weighed. For example, traditional bacterial indicators, such as fecal coliform or *Escherichia coli* counts, can distinguish between human and animal sources only by employing labor- and time-intensive library-based methods such as MAR profiling and ribotyping (Scott et al., 2002; Rangdale et al. 2003; Fong and Lipp, 2005). The detection of commonly targeted fecal indicators, such as coliform bacteria and caffeine, suggests that water quality has been jeopardized. However, these indicators do not adequately track or confirm the presence of several enteric pathogens of concern, including viruses. In addition, current indicators do not distinguish between important sources of fecal contamination (e.g., human vs. livestock) without labor-intensive and time-consuming investigation. Therefore, the causes of elevated indicator concentrations may be misjudged or remain unidentified.

The detection of human and livestock enteric viruses (e.g., enterovirus, adenovirus) is a promising alternative for identifying the sources of environmental fecal contamination (Rangdale et al., 2003; Fong and Lipp, 2005). In particular, adenoviruses (AdV) have been promoted in several recent publications as potentially valuable fecal source tracking agents (e.g., Noble et al., 2003; Maluquer de Motes et al., 2004; Pond et al., 2004; Fong et al. 2005). AdV are non-enveloped, icosahedral viruses and contain a linear, double-stranded DNA genome (Madigan et al., 2000). AdV capsids are composed of 240 hexon proteins and 12 vertices (called the penton), which consist of a complex of two proteins, a base and an outward-extending fiber protein. This protein mediates binding to host cells and initiates AdV infection. Several genetically distinct AdV serotypes (i.e., viruses with unique surface proteins, or antigens, targeted by species-specific antibodies) infect humans and livestock species and have been detected in the environment (Heim et al. 2003; Maluquer de Motes et al., 2004; Fong and Lipp, 2005).

Group I bovine AdV (BAdV serotypes 1, 2, 3 and 10) and all 52 identified human AdV (HAdV), which are currently broken into six species, A through F, belong to the genus *Mastadenoovirus*, while group II BAdV (4, 5, 6, 7, and 8) belong to the genus *Atadenovirus* (Büchen-Osmond, 2006; Lehmkuhl and Hobbs, 2008). Despite their genetic similarity (especially among *Mastadenovirus*), these viruses are putatively host-specific, making their use for fecal source tracking library-independent, and are released in large numbers in feces (10^4 – 10^8 particles•g^{-1}) from infected individuals (Maluquer de Motes et al., 2004; Fong and Lipp, 2005; Carter, 2005; Lehmkuhl et al., 1999). BAdV spanning both *Atadenovirus* and *Mastadenovirus* have been isolated from bovine manure. American Type Culture Collection (ATCC, Manassas, VA) BAdV 1 and BAdV 2 isolates were derived from the "feces of normal calf" (Klein et al., 1959, 1960); Lehmkuhl et al. (1999) isolated BAdV 10 from calf feces; and three of the four bovine fecal samples investigated by Maluquer de Motes et al. (2004) were positive for the presence of BAdV. This latter study, though limited in scope, found half of the

young animal samples and all of the old animal samples positive for BAdV. Sequenced amplicons were similar to strains 2, 4 and 7. As DNA viruses, AdV persist in the environment to a larger extent than do enteric RNA viruses (e.g., enteroviruses) and current microbial indicators (Gerba et al., 2002; Meng and Gerba, 1996; Fong et al., 2005). Thus, use of RNA viruses or bacteria as indicators of fecal contamination may not be sufficiently protective when enteric DNA viruses contaminate groundwater. In principle, PCR analysis for DNA viruses (e.g., AdV) is more streamlined than for RNA viruses in that a reverse transcriptase step is not required. Unlike bacterial fecal indicator organisms that may multiply in the environment, AdVs can not replicate outside host organisms. These factors support the potential of AdV as conservative tracers of fecal contamination and enteric viruses and are among the reasons why AdV were designated as a U.S. Environmental Protection Agency Candidate Contaminant.

Groundwater contamination by human enteric viruses has been documented, though relatively little data exist on the types and concentrations of viruses contaminating groundwater (Fout et al., 2003). In particular, very few investigations have targeted AdV. Abbaszadegan et al. (1999) found that 30.1% of 150 wells sampled (spanning different geographical locations in a variety of physical, chemical, and geological settings) contained human enterovirus RNA; when primers for specific viruses were employed, 8.6% of samples were considered positive for the presence of hepatitis A virus (HAV) RNA, while 13.8% were positive for rotavirus RNA. In a similar study, 31.5% and 20.7% of groundwater samples (448 total, from 35 states) were positive for viral nucleic acid and bacteriophages (Abbaszadegan et al., 2003). Analysis of water from 48 municipal wells in La Crosse, Wisconsin, revealed a 50% detection rate for enteric viruses, including echovirus, coxsackievirus, rotavirus, HAV, and norovirus (Borchardt et al., 2004). In a study by Fout et al. (2003), 16% of the 321 samples collected and 72% of the 29 sites (sampled monthly) examined were positive for RNA from enteroviruses (5%), reoviruses (10%), HAV (1%), and Norwalk virus (3%). Investigating a groundwater-associated outbreak that affected approximately 1,450 people on South Bass Island, Ohio (July to September 2004), Fong et al. (2007) detected AdV DNA in 2 of the 16 wells sampled. When assessing the influence of cess-pool latrines on groundwater quality in rural communities in the State of São Paulo (Brazil), 8 of the 15 wells sampled were adenovirus-positive by PCR (Piranha et al., 2006).

Virus concentration. Because viruses are present in low quantities in natural waters, in all of the groundwater investigations cited above, virus detection became possible only through the concentration of large sample volumes (10 to >1000 L). The concentration of water samples for virus detection has historically been accomplished by *vi*rus *ad*sorption-*el*ution ("VIRADEL"), where contaminated water is passed through a filter [e.g., electropositive Zeta Plus™ 1MDS (1MDS) cartridge filter, CUNO, Inc.) from which viruses are eluted, typically with a glycine-buffered 1.5 to 3% beef extract solution. Subsequent to initial virus filtration, concentrated water samples often require "secondary" concentration. In beef extract-containing samples, this is typically accomplished by flocculation/sorption (e.g., polyethylene glycol precipitation, aluminum hydroxide or celite addition) followed by centrifugation, techniques that typically co-concentrate viruses and beef extract in the final sample volume to be assayed (Schwab et al., 1995; Fout et al., 2003). More recently, primary and secondary virus collection by ultrafiltration (i.e., techniques based on virus capture by size exclusion) has gained popularity (Winona et al., 2001; Jiang et al., 2001; Hill et al., 2005; Fong and Lipp, 2005). With the viruses/microorganisms of interest, ultrafiltration will co-concentrate dissolved/colloidal organic matter to a degree inversely related to the filter molecular weight cut-off (MWCO). After sample concentration by these techniques, viruses are most commonly detected today by PCR.

Polymerase chain reaction. The principle method currently employed to detect viruses in environmental samples is PCR. During a conventional PCR (cPCR) cycle, a forward (or left) and a reverse (or right) user-designed oligonucleotide "primer" that is complementary to the DNA of a specific target (or group of targets) binds ("anneals") with that target, if it is present in the reaction mixture. The enzyme, *Thermus aquaticus* (Taq) DNA polymerase, then recognizes each primer/target duplex and initiates DNA replication from the 3' end of the primers. DNA replication is controlled by rapidly cycling the reaction mixtures through a series of temperatures that (a) denature (separate) double stranded DNA (94° C); (b) promote primer annealing (typically, 50 to 60° C; depends on primer length and nucleotide composition); (c) optimize Taq Polymerase activity (72° C); and (d) denature newly generated double stranded DNA molecules. During cPCR, amplified DNA is typically visualized after 30 cycles by gel electrophoresis with ethidium bromide staining under a UV light source. However, this technique is qualitative, since DNA "band" intensity is not linearly related to starting DNA concentration for this end-point measurement.

Quantitative (real time) PCR (qPCR), on the other hand, is used to detect and quantify PCR products (amplicons) by monitoring DNA amplification in real-time (i.e., as the reaction cycles progress rather than at the conclusion of a PCR routine as in cPCR). Several variations of this technique exist, each employing a fluorescent molecule that binds to the amplicon. TaqMan chemistry detects amplicons during thermal cycling through the inclusion of a double fluorescence labeled oligonucleotide ("probe") specific for a certain (or set of certain) DNA targets. This probe is designed to anneal with the target proximal to one of the primer binding sites and is cleaved during each amplification cycle by the 5'-3' exonuclease activity of Taq polymerase (Fairchild et al., 2006; Heim et al., 2003). Thus, the fluorescent molecule on the 5' end of the probe is separated from *both* the target sequence and the fluorescence-quencher on the opposite (3') end of the oligonucleotide. As a result, a fluorescent signal builds and is measured with each amplification cycle. SYBR green chemistry involves the non-specific binding (intercalation) of a fluorescent dye to the double-stranded DNA replicated with each PCR cycle. The dye is active when bound to double-stranded DNA (when real-time measurement occurs). During the standard increase in temperature to near boiling (94° C) at the conclusion of each PCR cycle, the SYBR green dye disengages as the double-stranded DNA separates, permitting further DNA replication (and repeated SYBR-green binding) during subsequent cycles. Because of its generic nature, SYBR green can be used with any optimized set of PCR primers for qPCR but introduces no additional specificity to the qPCR assay. Quantification in either of these cases is possible because of the linear relationship between the initial concentration of viral nucleic acid and the number of amplification cycles required to increase fluorescence over a threshold level (referred to as the threshold cycle, C_t). This relationship is usually linear over ≥ 5 orders of magnitude (Jiang et al., 2005; Jothikumar et al., 2005). Since PCR detects nucleic acid, viruses need not be intact or infectious for impacted groundwater to be diagnosed. While this may call into question whether detected viruses pose risk, this feature is desirable for fecal source tracking because it should provide conservative results.

Several recent studies reported qPCR methods for detecting and quantifying HAdV in a variety of biological and environmental fluids. For example, Heim et al. (2003) and Jothikumar et al. (2005) used consensus, nondegenerate, primers (i.e., primers designed to return HAdV despite the presence of an optimized minimum number of mismatches with all target sequences) to detect and quantify all 51 HAdV serotypes in human blood and water, respectively; van Heerden et al. (2005) quantified HAdV in treated drinking water and river water samples; and He

and Jiang (2005) quantified HAdV in sewage using degenerate primers (*vide infra*) predicted to detect approximately 13 HAdV types. Heim et al. (2005) and Jaing et al. (2005) found qPCR more sensitive than one-step PCR and nested PCR for human blood and seeded environmental samples, respectively. On the other hand, no qPCR assays have been published for livestock AdV, preventing the use of AdV to discriminate human from livestock sources of fecal contamination on a quantitative basis.

Consensus and degenerate primers in PCR. To detect multiple AdV species with a single set of primers, "degenerate" (He and Jiang, 2005) or non-degenerate "consensus" primers (Heim et al., 2003; Jothikumar et al. 2005) have been employed. Degenerate or consensus primers are employed in situations where a gene to be detected (e.g., the AdV hexon protein gene) is similar (but not identical) among targets. Nucleic acid sequences coding for the same (or very similar) series of amino acids can demonstrate considerable variability owing to genetic mutations and codon degeneracy (i.e., nucleotide triplets that differ yet code for the same amino acid). To detect these variable sequences by PCR, several individual primers homologous for nearly all possible targets could be synthesized and used during PCR. Two additional, alternative strategies can be employed: with consensus primers, two oligonucleotides (a forward and a reverse) are designed to optimize the number of bases that match the majority of target sequences, while allowing primer/target mismatches; and by employing a permissive T_A during PCR, target sequences having multiple (usually between one and four) mismatches with the primers can still be amplified. With a degenerate primer, a set of oligonucleotides is manufactured for PCR use in a single synthesis reaction with the introduction of multiple nucleotides at variable positions in target sequences. The degenerate (forward or reverse) primer that results represents a mixture of similar primers that can be expected to be homologous (or nearly so) to the majority of target organisms (in some case, even targets with nucleic acid sequences that have yet to be isolated/identified). For clarification, consider an aligned set of nucleic acid sequences that are identical except for three variable nucleotide positions. If the first, second and third variable positions can be occupied by C or T, G or T, and A or C or G, then a primer designed to target all possible permutations of these sequences would have a total degeneracy of $2 \times 2 \times 3 = 12$. Every possible combination of the bases filling these three positions would be present in the degenerate primer whether a potential target exists or not. This can be a drawback when, for example, one variable base is always accompanied by another variable base for a certain set of sequences and, as a result, a number of the primers in the mixture have no exact target. An additional drawback to the use of degenerate primers is the low relative concentration of any particular primer in solution; low primer concentration reduces sensitivity and requires the use of low PCR annealing temperatures (T_A) and large bulk primer concentrations, conditions that are typically required for consensus primers, as well, and that directly contribute in both cases to reduced PCR specificity.

Challenges in enumerating and discriminating among AdV species in environmental samples by PCR. Despite the promise of PCR, use of this method to distinguish HAdV from livestock AdV in fecal source tracking and to enumerate AdV in environmental samples has been limited by a number of issues: (1) current PCR methods designed for AdV detection were not developed to simply or definitively discriminate between AdV of human vs. livestock origin, and no qPCR assays have been published for BAdV; (2) filters commonly used to collect/concentrate viruses in 10- to 100-L water samples often demonstrate poor virus recovery and are costly, limiting their use; and (3) compounds present in concentrated samples (e.g., chemicals added to

elute viruses from filters, natural substances in water co-concentrated with viruses) often interfere with DNA amplification by PCR, increasing the potential for false-negative results.

Before qPCR-based fecal source tracking methods can be confidently applied, target specificity of primers must be ensured. Fundamental considerations related to the design of PCR primers that can specifically amplify more than a single previously identified AdV pose a challenge to attaining this goal. PCR primers must strike a balance between amplification/detection of many viruses (accomplished by making primers less specific or more degenerate) and ensuring adequate amplification specificity (accomplished by increasing primer specificity). Nearly all previously described PCR procedures for AdV detection targeted some portion of the well-conserved genomic sequence that codes for the AdV hexon protein, which forms the majority of the virus capsid (i.e., outer protein packaging). More sequence data are available for the hexon gene than for any other potential AdV genomic target. However, the conserved regions of these sequences (which must be targeted by primers if amplification of multiple viruses is the goal) are well conserved among many *Mastadenoviruses* that infect different species. Therefore, the majority of previously described primers/probes have enough similarity to other mammalian AdVs to potentially amplify non-target viruses. Except for the large similarity between species C HAdV (particularly HAdV type 1) and a single putative feline AdV (GenBank accession # AY512566, detected in Hungary), precluding its exclusion as a target for hexon-based HAdV primers (Jothikumar et al., 2005), this nuance has received little attention in the literature to date. Design of PCR primers and probes that will amplify specifically all (or most) of the HAdV and exclude all BAdV (and vice versa) represents a daunting task. The vast majority of published AdV oligonucleotides were originally derived for human clinical settings (e.g., Heim et al., 2003). Accordingly, little (or insufficient) care was taken to definitively preclude the possibility that non-human AdV belonging to the *Mastadenovirus* genus (e.g., porcine AdV and group I BAdV) may be returned by these primers. For BAdV, a single nested PCR protocol published for the detection of livestock AdV (Maluquer de Motes et al., 2004) showed discriminating potential; however, this assay was not suitable for an efficient qPCR assay, since (a) the published assay was nested (i.e., multiple primer sets and amplifications were required to effect specificity), (b) the primers were highly degenerate, and (c) three of the seven primers designed for groups I and II BAdV were of questionable quality: these primers each exhibited a terminal 3' adenosine, a base from which Taq Polymerase extends very inefficiently (Ayyadevara et al., 2000). Considered together, these factors will reduce primer efficiency and limit their suitability for qPCR.

Despite continued interest in the environmental significance of AdV, few studies have examined their quantitative recovery from water; studies that have been completed using standard filters have generally demonstrated poor to modest AdV recovery and have employed beef extract-based eluents. Currently, 1MDS cartridges are the most commonly used filters for virus sample collection (USEPA, 2001b). Sobsey and Glass (1984) determined the recoveries of simian AdV SV-11 spiked in raw and finished drinking water samples (1.3 L; lake source-water) from two layers of 1MDS membrane filters (47 mm diameter). Depending on feedwater chemistry, 18% to 46% of the spiked AdV passed through the membranes. Elution from filters with two successive 7.5-mL volumes of 0.3% beef extract (0.05 M glycine, pH 9.5) resulted in AdV recoveries (based on the initial virus addition) between 13 and 22%. In a similar study, 19% to 33% (n = 4) recoveries were observed for HAdV 40 spiked into 113-L tap water samples, filtered with 1MDS cartridge filters and eluted using 900 ml of 1.5% beef extract (0.05 M glycine, pH 9.5) (Enriquez and Gerba, 1995). In this study, AdV adhered to the cationic 1MDS

media to a greater extent than the two enteroviruses, poliovirus (recovery: 36–57%) and echovirus (recovery: 14–83%), examined. Most recently, a user-assembled sodocalcic glass wool column filter (a 1MDS alternative) was evaluated for virus recovery (VIRADEL) by Marshfield Center (Marshfield, WI) researchers (Lambertini et al., 2008). Recovery of HAdV 41 from 10- to 1500-L water samples (pH 6.5) varied widely from 4% to 58% (n = 32), depending most strongly on source water type (groundwater vs. tap water). Separate trials to determine the influence of sample pH found approximate HAdV 41 recoveries from 20-L tap water samples of 12%, 12%, 9% and 5% at pH 6.0, 6.5, 7.0 and 7.5. In each trial, filters were eluted with 3% beef extract (0.5 M glycine, pH 9.5). These filters are very inexpensive, but sample acidification is occasionally necessary to promote optimal virus adsorption, and AdV recoveries were generally low and variable. A number of studies have shown strong (> 60%) recovery of a variety of viruses from water using ultrafiltration systems (hollow-fiber and tangential-flow). While no ultrafiltration data are available for AdV recovery from water samples, laboratory-scale (the exact volume processed was not provided) concentration of HAdV 5 vector preparations by hollow fiber ultrafiltration (500 kDa, polysulfone; Amersham, Inc.) resulted in 70% recovery (Peixoto et al., 2006). A significant benefit of ultrafiltration over other filtration methods is this technique's suitability for concentrating/recovering multiple microorganisms at once. This is advantageous for fecal source tracking, public health monitoring and bioterrorism surveillance activities where multiple target organisms are potentially of interest or concern.

Several investigators have noted inhibition of PCR by substances that are either introduced to (e.g., beef extract) or variably co-concentrated with (presumably humic acids and/or multivalent metals) samples (Schwab et al., 1995; Fout et al., 2003; Fong and Lipp, 2005; Jiang et al., 2005). In light of the rapidly expanding use of PCR for virus detection in natural waters, methods that improve virus recovery by (i) avoiding the use of beef extract, and/or (ii) minimizing the co-concentration of environmental PCR-inhibiting compounds are desired (USEPA, 2003; WHO, 2004). The elution of viruses with beef extract alternatives, though few have been optimized, is one promising approach. Alternative virus concentration techniques, such as hollow fiber ultrafiltration (Hill et al., 2005), which can recover viruses without the addition of PCR-inhibiting compounds, also deserve consideration in this regard. Furthermore, improvements in the quality of qPCR results require development and consistent use of recovery and internal standards (Lebuhn et al., 2004). Most published qPCR studies have included positive and negative PCR controls; but the additional incorporation of recovery standards (i.e., surrogate virus spiked into the sample before and enumerated after concentration) and PCR amplification controls (e.g., salmon sperm DNA spiked into PCR reactions with concentrated sample and amplified to elucidate the presence of PCR inhibitors) will facilitate assessment of procedural losses and/or PCR inhibition (Lebuhn et al., 2004). This study addressed most of the major shortcomings of virus quantification described above that are required for the practical implementation of human and bovine AdVs in fecal source tracking.

Objectives and significance. To advance the use of AdV as fecal source tracking agents, the predominant goals of this study were (1) to develop a quantitative PCR (qPCR) method capable of simply and confidently distinguishing between human and bovine AdV; and (2) to optimize virus recovery from water samples by two recently-advocated and competitively priced filters, NanoCeram® electropositive nanoaluminum and Fresenius hollow fiber hemodialysis filters, while minimizing concomitant concentration or introduction of PCR-inhibiting compounds. To achieve these goals we addressed the following questions:

1: Considering the similarity between group I bovine adenoviruses (BAdV) and all human

adenoviruses (HAdV), can a simple and specific qPCR assay be designed capable of detecting multiple AdV of one group while excluding representatives from the other?

2a: To what extent can adsorbed viruses (bacteriophage, then adenoviruses) be recovered from NanoCeram® filters, a highly electropositive medium allowing efficient removal of viruses from water?

2b: If virus recovery is achievable, will successful eluate(s) inhibit down-stream PCR applications?

2c: Will Fresenius Hollow Fiber Ultrafilters, which exhibit strong virus recovery, allow the co-concentration of other microorganisms/pathogens of public health and fecal source tracking interest (facilitating the broad deployment of the HFUF system for public health monitoring/assessment)?

2d: If so, will adenovirus, for which recovery data are absent, be recovered to a similar extent as surrogate bacteriophage (and other viruses previously examined)?

Fecal contamination of groundwater is a significant concern for both the Wisconsin Department of Natural Resources (DNR) and the Wisconsin State Laboratory of Hygiene (WSLH). Both agencies are faced with questions such as the following. Has a neighboring farm contaminated a landowner's drinking water well? Is a septic system impacting public health through contamination of a community well or gaining stream? For legal and/or remedial action to be taken, the source of fecal contamination must be identified unambiguously and efficiently. To date, WSLH has not validated a method to achieve this goal. Therefore, the validation of a method capable of distinguishing the source(s) of fecal contamination is a key step towards improvement of groundwater quality and the protection of public health in Wisconsin. Since other enteric viruses have been documented in Wisconsin groundwater (Borchardt et al., 2004, 2007) and show extended periods of infectivity at colder temperatures characteristic of groundwater, AdV quantification is important and their application for fecal source tracking is promising. The results of this study will directly benefit local state agencies (e.g., DNR, WSLH) mandated to monitor water quality and to address the fundamental causes of water quality degradation.

MATERIALS AND METHODS

Virus sources. Enteric HAdV 41 (ATCC VR-930), BAdV 1 (ATCC VR-313), BAdV 2 (ATCC VR-314) and murine adenovirus 1 (MAdV; ATCC VR-550) were purchased from the American Type Culture Collection (ATCC). HAdV 40 and 41 have been detected in the environment (e.g., Jiang et al., 2001; van Heerden et al., 2005) and are the second leading cause of gastroenteritis in young children; BAdV 1 and 2 stocks from ATCC were isolated from manure. These viruses were cultured in appropriate cell lines [human embryonic kidney 293A (HEK293A, ATCC CRL-1573) for HAdV; Madin-Darby bovine kidney cells for BAdV (MDBK, ATCC CCL-22); and BALB-3T3 cells (ATCC CCL-163) for MAdV], which were used to generate in-house virus stocks: once >75% cytopathic effect was reached, viruses were released from remaining intact cells by three freeze-thaw cycles, and cellular debris was removed by ultracentrifugation. Clarified cell culture supernatant (CCS) was aliquoted (0.5 ml) and stored at –80° C. The infectivity of these stocks was determined as $TCID_{50}$, or the dilution level at which 50% of dosed cell cultures are virus positive (Karber Method), for BAdV and MAdV and by qPCR for BAdV 1 and HAdV 41. MAdV was obtained for use as a surrogate standard for

assessing virus loss during sample processing. The molecular assay designed for MAdV was specific, and MAdV was not amplified by the molecular assays designed for BAdV and HAdV (below).

Bacteriophages MS2 (American Type Culture Collection, ATCC, 15597-B1) and PRD1 (strain D4; HER 23, Laval University) were enumerated using a modified single agar layer procedure (USEPA, 2001a) on appropriate host cultures [F-Amp *E. coli* (ATCC 700891) and *Salmonella typhimurium* (strain LT2 (pLM2) 1217; HER 1023, Laval University), respectively]. PRD1 was obtained to provide variability in the physicochemical properties of microorganisms used to challenge filtration systems, has been suggested as an adenovirus surrogate for fate and transport studies (Harvey and Ryan, 2004; Davies et al., 2005), and is believed to share a common evolutionary ancestor with AdV (Huiskonen et al., 2007). Selected physicochemical properties of the viruses studies are provided in TABLE 1.

TABLE 1: Physicochemical properties of viruses evaluated during this study.

		Virus Properties		
Study Virus	**Size (nm)**	**p*I***	**Lipid Content (%)**	**Fibers (nm)**
Adenoviruses	80 – 110	~5[b], 4.5[i]	0	16.0 – 37.3[c] (20.0[c,d], 34.0 [c,e])
MS2[a]	24	3.9	0	–
PRD1	70 [f]	4.2 [a]	16 [a]	27.0 [f, g]

[a] Dowd et al. (1998)

[b] Mann et al. (2000); Herzer et al. (2003); both approximations based on HAdV 5. p*I* values of other AdV may differ.

[c] Favier et al. (2002)

[d] Short HAdV 41 fiber; [e] Long HAdV 41 fiber; Enteric HAdV 40 and 41 have two fibers of different length (short and long at ~1:1 abundance).

[f] Huiskonen et al. (2007)

[g] Referred to by PRD1 researchers as "spikes," putatively, PRD1 has two fiber-like projections (or a single branching spike) from its penton protein, P31 (which shows analogy with AdV; Huiskonen et al., 2007)

[i] Trilisky et al. (2007); estimate for recombinant HAdV 5

Molecular assay design. Use of AdV in fecal source tracking requires improvement in molecular methods for the detection/discrimination of human vs. bovine AdV. To this end, we focused our degenerate oligonucleotide-design efforts first on *in silico* (computer-based) methods. We evaluated the ability of the following software packages to design sufficiently selective and specific primers: GeneFisher (Giegerich et al., 1996), SCPrimer (Jabado et al., 2006), and AlleleID (Premierbiosoft, Inc.). For straightforward design considerations (e.g., the detection of a single target organism through cPCR), PCR software is typically the first resource exploited because the vast number of considerations inherent in PCR primer design are automatically taken into account for the user. After finding degenerate primer design using available software inadequate, we transitioned to the labor-intensive process of designing fecal source tracking oligonucleotides manually by inspection of sequence alignments and evaluation of candidate sequences with a variety of programs providing specific information on critical oligonucleotide properties (e.g., primer-target annealing temperature, potential primer-primer interactions). All non-degenerate primers (e.g., those simple sets used for cloning or general

amplification of a single target) were designed with the aid of the online program, Primer3 (Rozen and Skaletsky, 2000).

AdV nucleotide sequences considered. For human AdV belonging to the AdV genus *Mastadenovirus* 65 hexon gene or complete genome sequences previously considered for HAdV primer/probe design (Xagoraraki et al., 2007) were retrieved from GenBank (Benson et al., 2008), aligned using the ClustalW algorithm in the program, BioEdit (v. 7.0.9.0, Hall, 1999), and re-evaluated during this study. For BAdV, sequences were aggregated and aligned according to genus: *Mastadenovirus* BAdV (i.e., group I BAdV) 1, 2, 3 and 10, plus ovine adenoviruses (OAdV) 2 through 5 and porcine AdV (PAdV) 3 and 5, were evaluated together; *Atadenovirus* BAdV (i.e., group II BAdV) 4, 5, 6, 7 and 8, plus *Odocoileus hemionus* (black-tailed deer) AdV (OdAdV) and duck AdV 1, were evaluated together. Compared to HAdV, far fewer unique BAdV (or livestock, in general) hexon gene or complete viral genome sequences have been described and deposited in GenBank; all those providing non-redundant information were aligned and considered. The inclusion of non-bovine AdV in these sequence alignments was sometimes unavoidable due to the similarity existing between the hexon gene sequences of closely related animals (e.g., porcine adenoviruses 3 & 5 and ovine adenoviruses 2 – 5 with group I BAdV; Maluquer de Motes et al., 2004; Büchen-Osmond, 2006). Evaluating these other bird/animal viruses facilitated the design of primers that confidently discriminated BAdV from HAdV and most livestock AdV (and/or informed of which non-BAdV targets may be amplified). All of the primers/probes described here were compared with the recently isolated/sequenced HAdV 52 (Jones et al., 2007), which is proposed to represent an entirely new AdV species (HAdV G).

Oligonucleotide design criteria. To the extent possible, the stringent rules listed below were adhered to during manual selection and design of degenerate PCR primers and probes based on AdV sequence alignments. In general, these criteria focus on factors expected (a) to maximize the binding strength between primers (with emphasis on the nucleotides near the 3' end, from which Taq Polymerase initiates DNA replication) and target sequences, and (b) to facilitate (or inhibit) primer extension by Taq Polymerase, promoting amplification efficiency while minimizing primer annealing with closely-related non-target sequences. The rules followed included (1) identify regions ($\geq$ 18 nucleotides) in alignments of BAdV or HAdV hexon protein gene sequences that are sufficiently conserved so that primers or TaqMan probes with low degeneracy ($\leq$ 8) could be designed to return all, or a large number of, the considered sequences; (2) among candidate oligonucleotides, reject or adjust the length of primers exhibiting 3' terminal adenosines (from which Taq polymerase extends poorly; Ayyadevara et al., 2000) or probes possessing 5' guanines (which quench adjacent real-time fluorescent molecules and reduce the resulting qPCR signal); (3) evaluate candidate oligonucleotides against the non-target alignments (i.e., compare BAdV candidates with HAdV alignments and vice versa) manually and *in silico* (described below) for differences that could promote species-specific amplification; (4) adjust primer and probe lengths to achieve melting temperatures (T_M values) around 60° C and > 70° C, respectively; (5) reject oligonucleotides exhibiting significant hairpin or dimer formation; (6) position TaqMan probes as close as possible (within adequately conserved nucleotide stretches) to the 3' end of the eventual upstream primer; (7) reduce mismatches between oligonucleotides and target sequences to no more than two by selective incorporation of degenerate positions (i.e., positions within an otherwise identical primer/probe group that exhibit different nucleotides) and inosine (a nucleotide capable of pairing to varying extents to each of the four DNA bases) substitutions; (8) avoid primer/target mismatches,

degenerate positions or inosine substitutions within the first eight base pairs of the 3' end of primers, especially for important targets (e.g., the enteric HAdV 40/41) (i.e., allowed mismatches or substitutions were typically 5' of the 8th base pair from 3' end); (9) design oligonucleotides that do not co-amplify mouse MAdV, a *Mastadenovirus* chosen as the AdV method recovery control; and (10) if possible, align the terminal 3' nucleotides of primers with nucleotides that are conserved among either HAdV *or* BAdV hexon alignments but that *differ* between AdV infecting these different hosts. The final criterion represents advancement in pathogen detection and was motivated by literature describing PCR assays designed to detect single nucleotide polymorphisms (SNPs, or single nucleotide substitutions that can be prognostic of disease in human DNA);

In silico oligonucleotide evaluation. After manual comparison of potential primers and probes against non-target AdV alignments, target-specificity was evaluated using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) according to the parameters described in Table B1 (in Appendix B). Oligonucleotides were screened for potentially disruptive hairpin or dimer formations using AutoDimer (Vallone and Butler, 2004). A sodium ion concentration of 100 mM and a total strand concentration of 10 μM were assumed; these concentrations were purposefully high to enhance potential primer/probe interactions and to introduce a margin of error in the program's predictions. Melting temperatures for oligonucleotides were predicted using the BioMath web utility (http://www.promega.com/biomath/calc11.htm; Promega, Inc., Madison, WI). BioMath is considered one of the most accurate freely-available algorithms for predicting oligonucleotide T_M values (Chavali et al., 2005). Melting temperatures for degenerate primers (match and mismatch degenerate primer configuration) with target and non-target sequences were predicted using the MeltCalc spreadsheet (Schutz and von Ahsen, 1999); these predictions aided the design of PCR thermocycling profiles and the evaluation of PCR results. The use of thermodynamic parameters for the calculation of oligonucleotide duplex stability provides the best estimates of oligonucleotide T_M values (Schutz and von Ahsen, 1999). Because the MeltCalc spreadsheet and the Promega BioMath website both employ thermodynamic considerations, these program predict melting temperatures for the same oligonucleotide that are typically within 0.5° C under comparable input conditions (e.g., salt and oligonucleotide concentrations).

During Autodimer analysis and T_M prediction with MeltCalc, inosines (I), which are not accommodated by these programs, were replaced with guanines (G); these nucleotides share cytosine (C) as their "Watson-Crick" complementary nucleotide, based on hydrogen bonding interactions (Martin et al., 1985). Since I behaves most-similarly to G, replacing I for G logically highlights potentially problematic dimer or hairpin formation that might occur for inosine-containing primers and likely leads to the most accurate T_M predictions.

Experimental primer evaluation. Finalized candidate primers and probes were synthesized at the University of Wisconsin – Madison Biotechnology Center DNA Synthesis Laboratory. Primers were initially tested by cPCR (Eppendorf Inc., Thermocycler; GoTaq Green master mix, Promega, Inc.) followed by 1.5% agarose gel electrophoresis with ethidium bromide staining for their ability to amplify the target sequences of interest. Subsequent to successful target amplification, PCR reactions involving the primers were optimized with regard to primer concentration, target specificity and annealing temperature (T_A). Once conditions were optimized by cPCR, selected assays were transitioned to a 20 μL reaction volume qPCR platform (Lightcycle 2.0, Roche, Inc.). Purified (Wizard SV Gel and PCR Clean-Up Kit, Promega, Inc.)

cPCR products generated using primers that annealed outside of primers of interest were used as template standards for qPCR. DNA concentrations of these purified PCR products were determined by fluorescence using the Quant-iT Broad Range assay kit for the Qubit Quantitation Platform (Invitrogen, Inc.). Concentrated templates were aliquoted, stored at −20° C, and diluted before use with GENEMate nuclease-free water (ISCBioExpress, Inc). Diluent was augmented with tRNA (Ambion, Inc.) to 20 ng•μL^{-1} to prevent/minimize template loss to dilution- and PCR-tubes. For qPCR reactions spiked with 5 μL sample, the final reaction tRNA concentration was 5 ng•μL^{-1}. Probes were evaluated at an initial reaction concentration of 100 nM and a range of template concentrations; probes that generated signals were evaluated at two addition final reaction concentrations, 50 and 200 nM. Cycling conditions (e.g., annealing time, extension temperature and time) recommended by Roche were employed for qPCR without additional optimization.

Specific evaluation of 3' dual nucleotide mismatch (3'DNM) PCR assay. Inhibition of DNA replication due to terminal 3' primer/template mismatches is well recognized, but the extent to which Taq polymerase extension is inhibited varies considerably among investigations and appears to depend on a number of factors that have yet to be fully characterized (Ayyadevara et al., 2000, Latorra et al., 2003). To validate the hypothesized specificity of the 3'DNM fecal source tracking assay described here, we generated three sets of PCR primers and two purified DNA templates for both HAdV 41 and BAdV 1 (Appendix C). Degenerate primers targeting several HAdV or BAdV were not employed during this evaluation to minimize factors that could contribute to negative outcomes (e.g., observed primer non-specificity or non-amplification). Refer to Appendix C for a primer map indicating the locations and sequences of the primers describes here; all PCR products generated for qPCR templates were verified by gel electrophoresis and subsequently purified using the Wizard SV Gel and PCR Clean-Up Kit (Promega, Inc.).

For both HAdV 41 and BAdV 1, a "true" match virus template was generated by amplifying with primers labeled C1 and C2; a "mismatch" template, where the 3'DNM characteristic of BAdV was introduced into a HAdV 41 template (and vice versa), was generated using on-purpose mismatch primers (OPMM) 3'OPMM-T1 and 3'OPMM-T2 (see Appendix D). Both cPCR reactions were conducted using GoTaq Green master mix (Promega, Inc.) at T_A = 58° C. Predicted T_M values for the 3'OPMM-T1 and -T2 primers were ≥ 75.8° C. The permissiveness of PCR conducted at 58° C allowed for primer annealing and PCR amplification, despite the internal mismatches with the target sequences. As a result, the desired mismatches were introduced into the PCR products. For clarification, the BAdV 1 "mismatch" PCR product (i.e., the template standard for evaluating the specificity of BAdV primers) effectively represents a hypothetical HAdV that is *identical* to BAdV 1, except for the bases aligned with each of the (forward and reverse) primers' two terminal 3' nucleotides; these were adjusted by design to be characteristic of HAdV. The converse is true for the HAdV 41 "mismatch" template.

With "true" and "mismatch" templates in hand, two sets of qPCR experiments were conducted for each virus: the "true" PCR primers (3'DNM1 and 3'DNM2; 500 nM final concentration for each) were challenged with five concentrations (~3×10^1 through ~3×10^5 template copies per 20 μL qPCR reaction) of each template. Likewise, the "mismatch" primers (3'OPMM-A1 and 3'OPMM-A2; 500 nM final concentration for each) were challenged with the same concentrations of "true" and "mismatch" templates. Assuming the hypothesized 3' DNM technique was valid, amplification should be observed only for "true" primer/"true" template and "mismatch" primer/"mismatch" template combinations. Challenging the 3'DNM primers in this

manner allows verification of the technique's specificity against the worst-case scenario (i.e., a non-target virus that is completely homologous with the target virus, except for the 3'DNMs). Quantitative PCR was accomplished for HAdV using the "Sense FST Probe," (Appendix D) and for BAdV using SYBR green chemistry.

Filter Evaluation. Two filters, operating by fundamentally different mechanisms (adsorption/anion-exchange vs. size exclusion) were evaluated for their ability to concentrate viruses without introduction or co-concentration of PCR-inhibiting compounds. The filters evaluated were recently advocated as potentially more efficient, lower cost alternatives to those that currently dominate environmental virus sampling (e.g., 1MDS cartridge filters). In particular, the Fresenius Hemodialysis hollow-fiber ultrafilter is suitable for concentrating multiple classes of microorganisms (Hill et al., 2005; Polaczyk et al., 2008), an attractive feature for agencies seeking broad microbiological data from a single sample during public health investigations.

NanoCeram® filters. We explored the laboratory-scale adsorption/elution of bacteriophage MS2 and PRD1 (enteric virus surrogates), BAdV 1 and HAdV 41 from moderately hard water (MHW; EPA 821/R-02-012; $[NaHCO_3]$ = 96.0 mg·L^{-1}, $[CaSO_4{\cdot}2H_2O]$ = 60.0 mg·L^{-1}, $[MgSO_4]$ = 60.0 mg·L^{-1}, [KCl] = 4.0 mg·L^{-1}, pH = 7.4-7.8, hardness = 80-100 mg $CaCO_3{\cdot}L^{-1}$, alkalinity = 57-64 mg $CaCO_3{\cdot}L^{-1}$) using a recently developed and promising electropositive, nanoaluminum oxide filter (NanoCeram®, Argonide Corporation; http://www.argonide.com/). These filters consist of 2-nm diameter boehmite (γ-AlO(OH)) fibrils end-bonded to a microglass fiber and are fused into a membrane using conventional paper-making technology (Tepper and Kaledin., 2006). A variety of solutions, chosen for their demonstrated or hypothesized (i) ability to disrupt electrostatic interactions between negatively-charged viruses and cationic surfaces and (ii) compatibility with PCR, were evaluated as possible eluents. To optimize capture and recovery of viruses in general, an array of eluents were first evaluated using the bacteriophage MS2 and PRD1. These bacteriophage differ in fundamental biophysicochemical properties (e.g., size, lipid content; TABLE 1). We hypothesized that conditions optimized for the recovery of both bacteriophages would be transferable to many viruses, including AdV. Subsequent to bacteriophage evaluation, filters were challenged with AdV, which were eluted with the four most promising eluents.

Bacteriophages were inoculated into MHW to a final concentration of 500-1000 plaque forming units•mL^{-1} (pfu•mL^{-1}). Final BAdV 1 and HAdV 41 concentrations in MHW feedwater were 2.6×10^5 and 3.1×10^5 genomic copies per mL (G.C.•ml^{-1}), respectively. Duplicate 25-mm NanoCeram® syringe filters were then rinsed with 25 mL of filter-sterilized, double deionized water and challenged with 25 mL of the virus feedwater solution, filtered at ~0.5 mL•s^{-1}. Within 15 min, the viruses were eluted from the filters (typically with 5 mL of eluent). The eluents used are listed in TABLE 2. Approximately 1 mL of eluent was filtered initially, to purge the syringe filter of air. The filter in contact with eluent was then set aside for 10-min incubation at room temperature. Following this contact time, the remaining ~4 mL of eluent was filtered at ~0.25 mL•s^{-1}. MS2 and PRD1 concentrations were determined via plaque-forming assay in triplicate for the virus stock and the eluent solutions; AdV concentrations in virus stock, filtrate and eluate samples were determined by SYBR green qPCR after viral DNA extraction using the Qiagen DNeasy Blood and tissue kit (Qiagen, Inc.).

Virus samples typically require a secondary concentration step, during which the concentration of PCR-inhibiting compounds can be increased dramatically. We therefore evaluated NanoCeram® filters as possible secondary concentration devices for samples

containing 0.01% sodium polyphosphate (NaPP) + 0.01% Tween 80 (polyoxyethylene (20) sorbitan monooleate, a nonionic surfactant) or 0.05 M sodium carbonate buffer + 0.01% Tween 80 (pH 8.0). The former solution corresponds to the primary concentrate from Fresenius Hollow-Fiber Ultrafilters (HFUF); the latter represents a pH-adjusted (from 9.8 to 8.0) candidate eluate for NanoCeram® filters (*vide infra*) and addresses whether NanoCeram filters, which are available in a variety sizes/configurations, could be used sequentially to achieve higher overall virus concentration. Bacteriophage were spiked (as before) into 25 mL of each of the mock primary concentrates, and these solutions were passed through the NanoCeram® media as described above. 1.0% NaPP + 0.1% Tween 80 (which demonstrated strong recovery for both phages, but inhibited PCR; *vide infra*) and 0.1% NaPP + 0.01% Tween 80 (which demonstrated moderate to strong virus recovery with no PCR inhibition; *vide infra*) solutions were used to recover *phages* from the HFUF and NanoCeram mock concentrate trials, respectively. A separate trial was conducted to determine the potential for sequential concentration of AdV by NanoCeram filters using the carbonate eluent: BAdV 1 (5×10^6 G.C. per 25 mL) was spiked into 0.05 M Na_2CO_3 0.01% Tween 80 (pH 8.0); this solution was filtered and eluted (as above) with 0.05 M Na_2CO_3 0.01% Tween 80 at pH 9.8. Feedwater, filtrate and eluate samples were enumerated for bacteriophage and BAdV 1, and the passage/recovery of these viruses was calculated.

TABLE 2. Solutions used to elute bacteriophages from cationic NanoCeram® filters. All eluents were dissolved in a background buffer of 10 mM Tris-HCl, 10 mM NaCl. A pH of 7.5 was employed, except where noted.

1.0 M KCl
0.33 M $MgCl_2$ (1.0 M ionic strength)
0.1 M EDTA
10 mM SDS
3% beef extract
0.01 to 1.0% sodium polyphosphate (NaPP) + 0.001 to 0.1% Tween 80
0.5 M DL-threonine (DL-T; pH 7.5 and 9.1)
0.05 M sodium carbonate buffer, pH 9.8 + 0.01% Tween 80

Fresenius hollow-fiber ultrafilter. Based on research conducted at the Centers for Disease Control and Prevention (Atlanta, GA; Hill et al., 2005; Polaczyk et al., 2008) demonstrating the successful concentration/recovery of a variety of microorganisms, including viruses, from 10- to 50-L water samples, we evaluated the Fresenius Optiflux F200NR polysulfone hollow fiber ultrafilter (HFUF) for virus and bacteria concentration. This high-flux HFUF was advanced for its high active surface area (2.0 m^2), narrower molecular weight cutoff compared to other HFUF units (nominally 30 kDa), low relative cost, and its ability to recover multiple microorganisms from large-volume water samples (Hill et al., 2005, 2008). Bacterial pathogens/indicators were

included selectively in these trials as a result of the Wisconsin State Lab of Hygiene's interest in stream-lining the collection of a variety of fecal indicators and/or pathogenic microorganisms.

Chemically blocking non-specific binding sites on nylon and nitrocellulose membranes is a critical step toward ensuring specific binding of probes used during molecular biology blotting experiments. Membrane surfaces are treated with high-concentration cocktails of, for example, high molecular weight polymers and/or proteins, including bovine serum albumin and nonfat powdered milk (Sambrook and Russell, 2001). An analogous technique has been advanced for reducing the non-specific binding (i.e., loss) of pathogens to HFUF membranes (Winona et al., 2001; Hill et al., 2005). However, few data were available regarding the effectiveness of various blocking agents in pathogen recovery. With this in mind, we compared three blocking agents: fetal bovine serum (FBS), horse serum (HS; to avoid products of bovine origin), and non-fat instant milk (NFIM; Carnation), an inexpensive and readily available alternative to serum. Fresenius ultrafilters were blocked by recirculating 5% FBS, 5% HS or 5% NFIM (with 0.01% antifoam A and 0.02% NaN_3, pH 6.8) for 5 min followed by overnight incubation at room temperature.

For FBS- and NFIM-blocked filters, ultrafilter recovery experiments were initiated by rinsing blocking agent from the filters with 1 L of double-deionized water. Next, a 10-L phosphate buffered saline (PBS) solution with 0.01% NaPP (a dispersant) was spiked with 2.4×10^7 colony forming units (cfu) of *E. coli* and 4.64×10^6 cfu or pfu of both *Enterococcus faecalis* and male-specific coliphage MS2. For the HS-blocked filter, a 50-L test volume was spiked with MS2, PRD1, BAdV 1, HAdV 41 and *E. coli* to approximate final concentrations of 10 pfu•mL^{-1}, 100 pfu•mL^{-1}, 7×10^3 G.C.•mL^{-1}, 8×10^4 G.C.•mL^{-1} and 1 cell•mL^{-1}, respectively. For each trial, solutions were stirred for 10 min after microbe addition, and initial samples were collected for PFU enumeration using appropriate bacteria for phages, qPCR (following viral DNA extraction using the Qiagen DNeasy Blood & Tissue Kit) for AdV, and by the Colilert and Enterolert Quanti-Tray systems (IDEXX, Inc.) for *E. coli* and *E. faecalis*, respectively. Each filter was then eluted by (1) closing the ultrafilter permeate port, adding eluent (a 500 mL 0.01% Tween 80, 0.01% NaPP, and 0.001% antifoam A solution) to the sample reservoir, and, after swirling the eluent around the sample carboy, recirculating the combined concentrate/eluent for 5 min; (2) concentrating the elutriate solution by opening the permeate port; and (3) purging the ultrafiltration system (tubing and filter) of solution with compressed air (FBS- and NFIM-blocked) or by reversing the direction of fluid flow in the system (HS-blocked).

Potential PCR inhibition. To evaluate the potential for eluents employed during virus concentration protocols (described above) to inhibit downstream PCR applications, we characterized the pre- and post-DNA extraction tendencies of these solutions to interfere with qPCR. Performing a pre-DNA extraction test provides information on the inherent PCR-inhibiting properties of each of the compound investigated. The post-DNA extraction test indicates (1) the extent to which potential DNA inhibitors are removed from samples by the DNA extraction kits evaluated, and (2) the tendency for an eluent to interfere with the viral DNA extraction process.

Prior to DNA extraction - eluent inhibition. The efficiency of PCR can be evaluated based on the C_t (threshold cycle) value determined during a quantitative PCR run. The C_t represents the amplification cycle number at which the fluorescence of the indicator dye is significantly brighter than background fluorescence. As initial sample DNA concentration increases, C_t values decrease; the presence of inhibitors significantly increases C_t values. Therefore, we evaluated the amplification efficiency of salmon sperm DNA (a common, and

EPA-endorsed, internal amplification standard) spiked into aliquots of the most promising eluents dissolved in a 10 mM Tris buffer + 10 mM NaCl background buffer by SYBR green qPCR. A 10 mM Tris buffer + 10 mM NaCl solution, a dilute (~5×) version of the background composition of most PCR master-mix buffers, alone was employed as a no-inhibition control.

Post DNA extraction – eluent inhibition. Using primers 3'DNM1 and 3'DNM2 for HAdV 41 described in Appendix C, we assessed the influence of the most promising filter eluents on virus extraction and subsequent DNA amplification using two competing commercial DNA extraction kits. Briefly, 100 µL of HAdV 41-infected cell culture supernatant was diluted 10-fold in each eluent. A 200-µL aliquot of each dilution was then extracted with the Qiagen DNeasy Blood and Tissue DNA kit and with the Epicentre Water kit. These kits were chosen for (1) their mutual inclusion of a protease K incubation step, a factor shown to improve DNA recovery (Read, 2001), and (2) their competing methods/mechanisms of final DNA concentration [silica adsorption/desorption (Qiagen) vs. alcohol precipitation (Epicentre)]. Manufacturer instructions were followed with two exceptions. First, for the Epicentre kit, we replaced the membrane capture/1× lysis buffer wash step (used for collecting bacteria from water samples by size exclusion) with incubating 200 µL of sample with 200 µL of 2× lysis buffer. This procedure was formulated by consultation with the manufacturer. Second, we modified the final step in each protocol (either DNA elution from a silica support [Qiagen] or dissolution of precipitated DNA [Epicentre]) so that the final volumes of extracted DNA solutions were equal (100 µL), facilitating kit comparison. The combined success of DNA extraction and subsequent amplification was screened by cPCR followed by agarose gel electrophoresis and then by SYBR green qPCR.

RESULTS AND DISCUSSION

Primer Design.

Published primer and degenerate primer software evaluation. Successful PCR primer design was critical for the ultimate success of this project. Considering the number of published sets of primer/probes (oligonucleotides) for the detection of human adenoviruses, we initially intended to adopt primers/probes for our HAdV assays from those previously designed and evaluated; for BAdV, adenoviruses with far fewer previously published oligonucleotides and PCR protocols, we planned to design PCR primers/probes using available software packages.

As described above (and visualized in the alignments in Figure B2), the similarity between group I BAdV and HAdV hexon gene sequences in regions sufficiently conserved to position degenerate primers is remarkable. This fact alone presented a challenge to the adoption/development of broadly-reactive primers for group I BAdV and HAdV that amplify many viruses from one group while excluding those in the other. The potentially permissive nature of PCR posed an additional challenge. For example, Jothikumar et al. (2005) demonstrated amplification of 56 HAdV isolates (spanning species A through F) with primers that were nearly non-degenerate (the reverse primer contained a single inosine) despite the presence of as many as four primer/template mismatches. In addition, consensus primers designed by Heim et al. (2003) to amplify all 51 known HAdV types demonstrated as few as one mismatch with each of the group I BAdV - some of the HAdV amplified empirically with these primers possessed more primer/template mismatches than BAdV. The permissiveness of these primers and the degenerate nature of many of the broadly reactive HAdV primers previously published (both factors expected to reduce primer specificity) provided little assurance that available primers for HAdV would be sufficiently discriminating.

After rejecting published primers, we evaluated two free and one commercial degenerate primer design utilities: GeneFisher (Giegerich et al., 1996), SCPrimer (Jabado et al., 2006), and AlleleID (Premierbiosoft, Inc.). However, these programs, which are intended for degenerate primer design for multiple aligned sequences, returned either non-discriminating, highly degenerate primers or multiple individual primers. Repeated consultation with Premierbiosoft support failed to improve the outcome. Thus, the programs evaluated were unable to return broadly reactive degenerate primers that excluded closely related sequences.

Manual Primer Design: Manual primer design was pursued following the rejection of degenerate primer design software. While the design of *non-discriminating* degenerate primers based on AdV hexon gene sequences was fairly straight-forward [we designed so-called "anchor primers" for interrogating potential source samples for previously unidentified AdV – in fact, published primers by Heim et al. (2003) for HAdV are also potentially suitable anchor primers], initial attempts at manual design of potentially discriminating primers were met with unsatisfactory options. Our evaluation included an intermediate analysis of the few available group I BAdV fiber protein sequences, which are not well conserved and, thus, were not viable primer targets. Utilization of non-specific anchor primers with possible TaqMan probes capable of discriminating HAdV from BAdV was considered and rejected; no suitably conserved and discriminating TaqMan probes could be designed. Subsequently, we shifted our focus to PCR techniques for distinguishing highly similar targets. Almost entirely, this literature exists outside of environmental and microbiological fields. Of the techniques considered, those aimed at discriminating single nucleotide polymorphisms (SNPs), or single base mutations in genomes often associated with disease (e.g., Latorra et al., 2003) seemed the best to pursue. While internal single-base primer/template mismatches provide little enhanced primer specificity, mismatches at or near the 3' end of primers significantly impact the efficiency of Taq polymerase extension/DNA replication (Maerten et al., 2006). This is especially true when mismatches result in purine-purine (A:A or G:G) or pyrimidine-pyrimide (C:C or T:T) pairing. These "transversion" mismatches present unfavorable hydrogen bond configurations between paired nucleotides and show the greatest potential to discriminate SNPs (Latorra et al., 2003).

Mastadenovirus. We investigated the HAdV and BAdV alignments for bases conserved within one group but differing from the other. While SNPs were rare, side-by-side comparison of HAdV and group I BAdV alignments revealed three locations where *consecutive* nucleotides were completely (in the case of BAdV) or nearly completely (for HAdV) conserved within a group but different between the groups (e.g., Figure B2). Subsequent primer design focused on aligning these "dual nucleotide mismatches" (DNM) with the 3' terminal and penultimate nucleotides of potential forward or reverse primers (henceforth, 3'DNM). Based on the extent the sequences 5' of the DNM were conserved, each 3'DNM site was evaluated as a potential forward or reverse primer binding site. Forward *and* reverse primers containing a 3'DNM were designed (Appendix D) meeting the majority of the aforementioned criteria and evaluated for HAdV and group I BAdV. HAdV and group I BAdV forward primers overlap (exercise the same 3'DNM site) while the reverse primers for these groups are positioned on either of the two remaining 3'DNMs. The HAdV sequences aligned with the BAdV 3'DNM reverse primer were not sufficiently conserved for FST primer placement; on the other hand, the BAdV sequences aligned with the HAdV 3'DNM reverse primer were sufficiently conserved for use as a reverse FST primer. Employing non-aligned reverse primers (i.e., by exercising different conserved 3'DNMs for HAdV vs. BAdV) results in PCR amplicons of *different* sizes, facilitating fecal source screening by cPCR and gel electrophoresis alone.

To our knowledge, this is the first description of PCR assay for microorganism detection/discrimination based on 3'DNMs. Since a single base mismatch on a forward or reverse primer can reduce DNA amplification by 40- to 100-fold (Ayyadevara et al., 2000), we hypothesize that the existence of 3'DNM primer/template mismatches on *both* forward and reverse primers might (1) completely inhibit Taq polymerase extension against non-target sequences possessing both opposing 3'DNMs, and (2) largely (or completely) inhibit the amplification of non-target viruses that only display a single opposing 3'DNM. Fortuitously, both mismatches in all 3'DNMs described here are transversions, increasing their specificity (Ayyadevara et al., 2000). An initial test of this hypothesis was conducted using 3'DNP-containing primers designed for BAdV 1 and HAdV 41 (Appendix C). These primers were tested against DNA extracted from MAdV type 1, BAdV type 1 and HAdV type 41. The primers showed specific amplification of their intended target and no amplification of the other two challenge viruses. We therefore designed a more sophisticated evaluation of the specificity of this technique: HAdV 41- and BAdV 1-specific 3'DNM (non-degenerate) test primers were challenged with several concentrations of two templates, one homologous with the primers (the "true" template) and one containing the 3'DNP characteristic of the opposing group (the "mismatch" template; Appendix C). In these evaluations (FIG. 1), strong specific amplification of "true" templates was demonstrated while non-target amplification was absent. Furthermore, amplification profiles for the five non-target template concentrations were indistinguishable from no-template controls (horizontal lines in FIG. 1a and 1b).

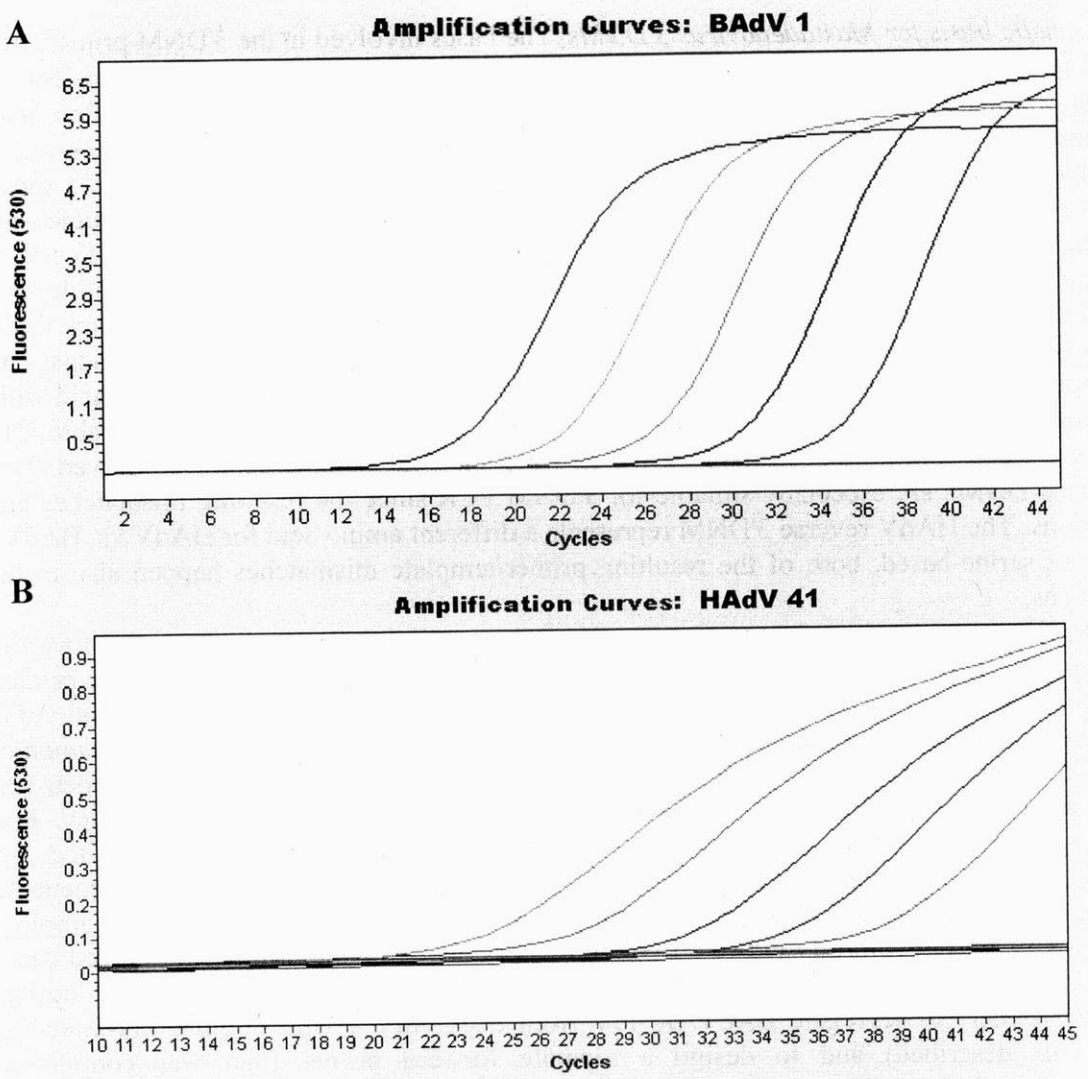


FIG. 1. Quantitative PCR (qPCR) amplification curves using "true" match PCR primers for five "true" and five "mismatch" (purified PCR-product) template concentrations from 3.0×10^1 to 3.0×10^5 template copies per reaction (copies) for (A) HAdV 41 and (B) 2.4×10^1 to 2.4×10^5 copies for BAdV 1. "True" templates are exact matches with the BAdV 1 or HAdV 41 hexon gene in the region targeted by our FST primers. The "mismatch" templates were designed to introduce the BAdV 3'DNMs into the HAdV 41 target sequence (and vice versa). Therefore, in the case of HAdV 41, the mismatch template represents a hypothetical BAdV that is identical to HAdV 41 except for the two 3' terminal nucleotides (which are those possessed by group I BAdV). In other words, these templates represent the most similar hypothetical BAdV hexon gene sequence to HAdV 41 that could be expected (and vice versa). Therefore, the amplification of "true" templates and the absence of "mismatch" template amplification for both BAdV and HAdV 3'DNM test primers strongly supports the hypothesized specificity of the novel application described here. In addition, the specificity of this assay supports the applicability (if desired) of SYBR green dyes for FST assays using the degenerate versions of the 3'DNM fecal source tracking primers. Primers used during this experiment are diagramed in Appendix C.

Genetic basis for Mastadenovirus 3'DNMs. The bases involved in the 3'DNM primers are bold and underlined in the sequence translations in Appendix C (the entire codon, tri-nucleotide units that code specific amino acids, is bold while the DNM is underlined). The amino acid corresponding to the bases at the 3' end of the group I BAdV and HAdV forward and the group I BAdV reverse 3'DNM primers is serine. Nearly all codons for a particular amino acid show degeneracy in the third position (i.e., the third position in the codon triplet can be occupied by more than one specified nucleic acid base without changing the amino acid that the triplet codes). Accordingly, for 17 of the 20 naturally occurring amino acids, the first two codon nucleotides are invariable, with the third nucleotide demonstrating degeneracy. Two of the remaining three amino acids, arginine and leucine, show degeneracy in the first and third codon positions, but always possess a stable second nucleotide. Serine, on the other hand, is the *only* amino acid with codons that exhibit degeneracy in all three positions of the nucleotide triplet (see Table C1, Appendix C), allowing for a potential DNM even when the coded amino acid is conserved. The serine-based DNMs are especially suitable for 3'DNM PCR since the resulting mismatches are transversions. The HAdV reverse 3'DNM represents a different amino acid for HAdV vs. BAdV. Though not serine-based, both of the resulting primer/template mismatches happen also to be transversions.

Atadenovirus: Design of degenerate primers for group II BAdV was more straightforward than for group I BAdV. However, the low numbers of aligned sequences upon which the primer designs are based and the similarity of these group II BAdV sequences to other animal AdV hexon gene sequences that have been deposited in GenBank [specifically *Odocoileus hemionus* (OdAdV, black-tailed deer) and avian egg drop syndrome (Duck 1) AdV], leaves open the potential that as of yet unidentified animal *Atadenovirus* could potentially be amplified. For example, one of two OdAdV alignments (Accession Number AF198354, deposited by Lehmkuhl, H.D.) present in GenBank is quite similar to BAdV group II hexon sequences; therefore, is not reasonable at this time to assume that degenerate primers designed to amplify group II BAdV will not amplify OdAdV (including, possibly, AdV that infect white-tailed deer; Woods et al., 2001). Thus, the approach taken for the design of group II BAdV was to design degenerate primers to segregate BAdV/OdAdV from duck AdV 1 (the single avian-infecting *Atadenovirus* described) and to design a separate forward primer (that was completely homologous) and a separate TaqMan probe specific to the most-similar OdAdV sequence available in GenBank. In combination with other group II primers designed here, the OdAdV forward primer and probe could be used to exclude the possibility that positive results from the BAdV group II primer/probe set resulted from amplification of AdV from wild deer. On the other hand, the *Atadenovirus* hexon sequences are quite distinct from HAdV sequences, lending confidence that BAdV group II fecal source tracking primers/probes will not return HAdV (and vice versa). *Atadenovirus* primers have been designed and synthesized but not yet evaluated. We have contacted Howard Lehmkuhl (USDA, Aims, IA) who has agreed to provide us with positive controls against which our BAdV group II and OdAdV primer sets can be challenged.

Fecal Source Tracking Primer and Probe Optimization. The final series of degenerate primers and probes designed for HAdV, group I BAdV and group II BAdV are provided in Appendix D. When incorporating a degenerate position into a primer increased the overall degeneracy to > 8, a separate primer was instead designed to maintain low degeneracy while maximizing the AdV detected by any given primer. This resulted in the design of separate forward fecal source tracking primers for group I BAdV and separate reverse fecal source tracking primers for HAdV (dividing HAdV by species). Optimized conditions for the majority

of degenerate FST primers included a T_A of 55° C and forward and reverse primer concentrations near 1000 nM each. Degenerate anchor primers, those designed to non-specifically amplify as many *Mastadenovirus* as possible, required a more permissive T_A of 50° C before significant amplification of test viruses (HAdV 41, BAdV 1 and BAdV 2) was observed.

The HAdV sense probe demonstrated optimal amplification of a range of template concentrations at 100 nM. Amplification with the group I BAdV probe was poor, potentially resulting from primer and probe T_M values that are insufficiently different. However, because of the proven assay specificity, SYBR green chemistry is a well suited option for future use with these primers. SYBR green PCR reactions require far fewer optimization steps and, thus, have the potential for rapid implementation in fecal source tracking. No HEX labeled probes (including our group I BAdV and HAdV 40/41 specific) generated an amplification signal even though target amplification was verified by gel electrophoresis. The reason for the probes' failure is unclear at the time of writing, but seems to result from a defect in manufacturing associated with the fluorescent molecule (HEX vs. FAM) responsible for generating the real-time signal. Further evidence for probe defect is derived from the identical FAM-labeled group I BAdV TaqMan probe we ordered which did demonstrate a real-time amplification signal. We intend to have the defective probes remade to evaluate their utility.

Summary of degenerate primer specificity. The assays designed here represent a significant advance in the field of AdV-based fecal source tracking. The specificity of the primers generated, particularly those for discriminating between HAdV and group I BAdV, should allow for the confident PCR-based source identification of AdV-contaminated samples. Still, the limited numbers of aligned sequences upon which the BAdV primer designs were based and the over-all similarity of hexon genes among all of *Mastadenovirus* sequences that have been deposited in GenBank, leaves open the potential that as of yet unidentified animal AdV could potentially be amplified by any of the primer sets. This uncertainty is not unique to this study; the design of PCR primers and probes (and their resulting specificity) is always limited by the availability of sequence data upon which primers are based. That said, since all HAdV sequenced to date contain at least two 3' mismatches with the BAdV group I primers, the available data supports the specificity of the HAdV primers designed here to discriminate against livestock and most other animal AdV. One possible exception is feline adenovirus (FeAdV). One putative FeAdV sequence (deposited without an accompanying publication; GenBank accession # AY512566) has been deposited in the NCBI nucleotide database. This sequence is highly homologous with species C HAdV (particularly HAdV 1) and, unlike all other livestock/animal AdV (except for Simian AdV, which are actually classified as HAdV), shares both 3'DNM with HAdV. Based on a comparison with this previously deposited FeAdV hexon sequence, a possible FeAdV was recently isolated from a one year old child in Japan (Phan et al., 2006). However, no known contact between the child and a cat could be established. Because of the large similarity of the possible FeAdV with known HAdV, until addition work is completed elucidating the possible prevalence and genetic character of more FeAdV, it is reasonable to question whether the single FeAdV hexon sequence deposited was actually a HAdV. For group I BAdV, primers designed will also target ovine adenoviruses 2 through 5 and porcine adenoviruses 3 and 5, since these viruses are highly similar the BAdV and share both 3'DNMs. Based on BLAST evaluation of these primers against all sequences deposited in the NCBI database, no additional non-target AdV amplification is predicted. While the isolation and sequencing of additional livestock and wildlife AdV will certainly improve confidence in these

assays, the work presented represents the first qPCR assay designed for BAdV and a significant improvement in techniques available for AdV-based fecal source tracking.

Comparing our HAdV FST primers with HAdV 52, all three 3'DNMs described here are maintained in this newly described HAdV that tentatively represents a new species (Jones et al., 2008), supporting the integrity of the 3'DNM assay. In fact, only two discrepancies exist between the FST primers/sense probe and this new HAdV: (1) the HAdV sense probe has a single mismatch with HAdV 52 (three bases from the primers 5' end, or 5' n-3); and (2) the codon overlapping the reverse HAdV 3'DNM primer is ACC vs. ACG (both code the amino acid, threonine). As a result, HAdV 52 has a 3' n-3 mismatch with the HAdV RVS primer. While this mismatch *may* reduce amplification efficiency, it does not jeopardize the specificity of the 3'DNM assay. We note that the HAdV reverse 3'DNM differs among HAdV 3, 7, 11, 14, 16, 21, 34, 35 and 50 (subgroup B). The amino acid coded for here is ATG (methionine, start codon) vs. ACG (threonine). This inconsistency presents no difficulties in the 3'DNM method, since consecutive 3'DNM between group B HAdV and group I BAdV are maintained.

Design of recovery and internal standards. Primers specific for MAdV (Appendix D), a *Mastadenovirus* similar to HAdV and group I BAdV, were designed using Primer3 to allow this virus to be used as logical surrogate recovery standard during field filtration activities. Two TaqMan probe options were designed manually. Primers and probes were evaluated as MAdV-specific by BLAST searching. While primers demonstrated strong target amplification during cPCR trials, (as described above) the HEX-labeled probes (as expected) failed to generate a real-time fluorescence signal and will be re-synthesized and retested.

Filter evaluation.

NanoCeram® filters. NanoCeram® filters have received accolades for their ability to remove viruses from water (http://www.argonide.com). Little attention, however, has been paid to the recovery of sequestered viruses from this medium. Based on literature describing the elution of a variety of viruses from many substrates (e.g., cationic membrane filters, sewage, manure, glass wool), we chose several promising eluents (TABLE 2) for evaluation with NanoCeram® filters. *A priori*, electrostatic interactions between the viruses examined (which possess a net negative surface charge at pH $\geq$ 4.5 to 5) and the nanoaluminum filters (which possess a net positive charge below pH ~10) were expected to be the dominant binding mechanism requiring consideration. Thus, the eluents chosen were those previously shown or hypothesized (1) to disrupt electrostatic interactions between viruses and various media and (2) to be (or likely be) compatible with PCR (Farrah et al., 1991; Lytle and Routson, 1995; Fujito and Lytle, 1996; Shieh et al., 1997; Lukasic et al., 2000; Hill et al., 2005). PCR compatibility was a key aspect in eluent choice: 1.5–5% beef extract solutions, which are capable of disrupting both electrostatic and hydrophobic interactions between viruses and a variety of media, are the standard filter eluents used in environmental virology. However, elution with beef extract introduces PCR inhibitors to samples, the influence of which is likely enhanced when beef-extract containing eluate is subjected to necessary secondary concentration techniques (e.g., polyethylene glycol precipitation, centrifugal ultrafiltration) that co-concentrate PCR-inhibiting compounds with viruses (Schwab et al., 1995; Fout et al., 2003). We determined the efficacy of each eluent to disrupt the binding between two bacteriophages, MS2 and PRD1, and NanoCeram® filters. Optimized eluents were evaluated for the recovery of bound HAdV 41 and BAdV 1. In addition, considering the need for secondary concentration of most environmental virus samples (Fong and Lipp, 2005), we evaluated the capture/recovery of phages by

NanoCeram filters from two mock primary concentrates (representing optimized eluate from HFUF and NanoCeram filters).

Initial tests verified, with two exceptions, that a constant number of phages spiked into each eluent and enumerated generated comparable pfus (i.e., to determine if the phage was inactivated or otherwise influenced by eluent chemistry). Two of the eluents (0.1 M EDTA and 10 mM SDS) negatively influenced the plaque-forming ability of PRD1, precluding recovery estimation for these eluents. Similar disruption of PRD1 by SDS has been reported previously (Lytle and Routson, 1995; Fujito and Lytle, 1996). The strong retention of viruses by these filters indicated by the manufacturer (Tepper and Kaledin, 2006) was confirmed: filtrate samples evaluated for MS2 and PRD1 demonstrated ≤ 1 pfu (usually 0 pfu), which translated to a virus retention by the filter of ≥ 99.6 %. On the other hand, the recovery of phage by the different eluents varied considerably (TABLES 3 and 4).

A wide range of eluent chemistries were evaluated initially (TABLE 3). The presence of multivalent cationic salt (> 0.01 M $MgCl_2$) in feedwater was shown to significantly interfere with the adsorption of viruses to cationic 1MDS filters (Lukasic et al., 2000). In the present study, phage were not recovered from NanoCeram® filters by eluent containing 0.33 M $MgCl_2$ or 1.0 M NaCl. This finding corroborates claims made by the filter's manufacturer regarding efficient virus capture in solutions with elevated ionic strengths (Tepper et al., 2006). At concentrations $\geq$ 10 mM (neutral pH), the anionic surfactant SDS had been shown an effective eluent ($\geq$ 85% recovery) for several bacteriophages (MS2, ΦX174 and T7) from positively charged BioTrace HP membranes (Lytle and Routson, 1995; Fujito and Lytle, 1996). This observation was confirmed here, as 10 mM SDS recovered > 70% of MS2 bound to NanoCeram filters. Considering previously observed interference of virus adsorption to metal-oxide-modified diatomaceous earth by 0.1 M Na-citrate (Farrah et al., 1991), we evaluated the chelating agent, 0.1 M EDTA for its ability to displace sorbed phages from NanoCeram filters. We found low recovery (10-15%) of MS2 with this eluent, which was eliminated from consideration. Shieh et al. (1997) found 0.5 M threonine (pH 7.5), a uncharged polar amino acid, as effective as 3% beef extract (pH 7.5) at eluting viruses from separated sewage solids without inhibiting PCR. In our hands, we obtained modest recovery (33-44%) of MS2 with this eluent, but 0% recovery of PRD1. Attempting to increase the chemical complexity of the amino acid preparation and improve phage recovery, we mixed five amino acids differing in polarity and charge. This amino acid cocktail performed poorly as an eluent and was not considered further. Tepper and Kaledin (2005), reported 70% recovery of MS2 with 3 mL of a 3% beef extract/0.37% glycine (pH 7.4) solution. This represents the only disseminated estimate of virus recovery from NanoCeram filters. For reference, we found comparable results with 3% beef extract (pH 7.5) as an eluent.

The NaPP and NaPP+Tween 80 solutions evaluated as eluents of bacteriophages PRD1 and MS2 from NanoCeram® syringe filters were first advocated as eluents for hollow-fiber ultrafilters (Hill et al., 2005); in the range of 0.01 to 0.1%, these compounds are compatible with qPCR (Hill et al., 2005; *vide infra*). Tween 80 is a non-ionic detergent that disrupts hydrophobic interactions between microorganisms and surfaces. The anionic dispersant NaPP is employed to disrupt electrostatic interactions, which were expected to dominate during bacteriophage adsorption to NanoCeram® filters. For eluents containing NaPP alone, bacteriophage recovery increased and then decreased with increasing NaPP concentration (Table 3). This effect was most pronounced for PRD1: approximate recovery values of 2%, 25% and 2% were observed for the 0.01%, 0.1% and 1% NaPP concentration trials, respectively. The 1% NaPP trial was repeated to verify the unexpected decrease in recovery observed at this concentration, while preliminary

experiments ruled out toxic (or otherwise negative) influences of these eluents on phage plaque forming ability. Next, we examined the influence of Tween 80 on bacteriophage elution. A 0.1%, solution of Tween 80 alone was unable to disrupt phage/filter binding. However, addition of Tween 80 to NaPP eluents improved slightly the recoveries of MS2 and significantly recoveries of PRD1. Previous investigations into virus/surface interactions have revealed similar trends (e.g., Farrah et al., 1982; Lytle and Routson, 1995; Lukasik et al., 2000). Overall, solutions containing as low as 0.1% NaPP + 0.01% Tween 80 demonstrated equivalent or improved phage recovery from these filters compared with standard eluent formulations with beef extract.

TABLE 3. Efficiency of candidate eluents in recovering bacteriophage MS2 and PRD1 from NanoCeram® filters.

	Recovery (mean ± 1 standard deviation, %)[a]			
Eluent	**MS2**		**PRD1**	
1.0 M KCl	0 (0)	0 (0)	0 (0)	0 (0)
0.33 M $MgCl_2$	0 (0)	0 (0)	0 (0)	0 (0)
10 mM SDS	73 (21)	72 (13)	ND	ND
0.1 M EDTA	15 (5)	10 (2)	ND	ND
3% Beef Extract (for ref.)	75 (12)	79 (11)	60 (18)	61 (14)
0.5 M DL-Threonine (pH 7.6)	44 (11)	33 (7)	0 (0)	0 (0)
0.5 M DL-Threonine (pH 9.1)	4 (1)	8 (2)	0 (0)	0 (0)
0.43 M Amino Acid Cocktail[b]	2 (1)	2 (1)	0 (0)	0 (0)
0.01% NaPP	13 (5)	6 (2)	3 (1)	2 (2)
0.1% NaPP	60 (2)	56 (10)	24 (7)	27 (8)
1% NaPP[c]	55 (6)	65 (7)	1 (1)	2 (1)
1% NaPP[c]	47 (8)	38 (8)	3 (3)	2 (1)
0.1% Tween 80 (10 mL eluent)	0 (0)	0 (0)	0 (0)	0 (0)
0.01% NaPP + 0.01% Tween 80	20 (6)	20 (7)	12 (5)	18 (3)
0.1% NaPP + 0.01% Tween 80	81 (18)	85 (17)	59 (9)	64 (12)
0.1% NaPP + 0.1% Tween 80	86 (18)	71 (18)	68 (8)	75 (10)
0.1% NaPP + 0.1% Tween 80 [d]	56 (12)	56 (17)	60 (5)	56 (7)
1% NaPP + 0.01% Tween 80	70 (6)	65 (4)	54 (9)	52 (11)
1% NaPP + 0.1% Tween 80	87 (9)	80 (17)	91 (14)	86 (16)

[a] Recovery for duplicate filtration experiments are reported in separate columns; numbers in parentheses are one standard deviation from triplicate phage enumerations of initial (feedwater) and final (eluate) samples. All eluents were made in solution of 10 mM Tris-HCl, 10 mM NaCl, pH 7.5. A 5-mL volume of eluent was used for all experiments except where noted otherwise.

[b] Solution of the following amino acids: threonine (0.1M), leucine (0.1M), lysine (0.1 M), glycine (0.1 M); saturated aspartic acid (0.03 M).

[c] The 1% NaPP trial was repeated to verify the low recoveries observed for PRD1 compared with MS2.

[d] Filtrations completed with two layers of NanoCeram® disc filters enclosed in a Pall, Inc. user-assembled filter housing. These 0.1% NaPP + 0.1% Tween 80 trials were repeated to provided standards for comparison between elution experiments using pre-manufactured 25-mm syringe filter vs. user-assembled 25-mm syringe filter housings (TABLE 4).

Further evaluations were completed using a series of Na_2CO_3 and Na_2CO_3 + Tween 80 solutions as eluents of MS2 and PRD1 from NanoCeram® filters (TABLE 3). These chemicals were advocated without supporting data as eluents by Argonide, Inc. (http://www.argonide.com). Here, virus recovery experiments were conducted using two 25-mm NanoCeram® cationic filter membranes housed within a Pall, Inc. syringe filter housing (in contrast to previous experiments, which used pre-manufactured syringe filters containing two layers of NanoCeram® media). This change in procedure resulted from a shift in product availability from the manufacturer. To establish a reference for comparing data acquired with the two different syringe-filter configurations, we duplicated the adsorption/elution experiment for MS2 and PRD1 using the 0.1% NaPP + 0.01% Tween 80 eluent (bullet d, TABLE 3). Recoveries for this eluent were always lower for user-assembled vs. pre-manufactured syringe filters. Repeated trials with these housings (and with a syringe filter housing from a different manufacture, Millipore, Inc.) verified the lower recoveries.

TABLE 4. Recovery of bacteriophage MS2 and PRD1 from NanoCeram® filters using carbonate-based eluents.

	Recovery (%)[a,b]			
Eluent	**MS2**		**PRD1**	
0.05 M Na_2CO_3, pH 9.8	59 (16)	69 (19)	1 (0)	1 (0)
0.05 M Na_2CO_3, pH 9.8 + 0.01% Tween 80	69 (19)	69 (17)	70 (3)	68 (6)
0.05 M Na_2CO_3, pH 8.0 + 0.01% Tween 80	0 (0)	0 (0)	0 (0)	0 (0)

[a] Recovery for duplicate filtration experiments are reported separately; numbers in parentheses are one standard deviation involving triplicate phage enumerations of initial (feedwater) and final (eluate) samples. All eluents were dissolved in a background buffer of 10 mM Tris-HCl, 10 mM NaCl, pH 9.8.

[b] Experiments conducted with 25 mm user-assembled Pall, Inc. filter housing containing two layers of NanoCeram® disc filters. A 10-mL volume of eluent was used for all experiments to ensure maximal elution from the alternative filter housings.

Similar trends were observed for carbonate eluents (pH 9.8) as for NaPP eluents. For eluents containing Na_2CO_3 alone, MS2 recovery was high (~65%) while PRD recovery was minimal (~1%) (TABLE 4). The addition of Tween 80 to Na_2CO_3 eluents slightly improved MS2 recovery. On the other hand, we observed a dramatic (~70%) increase in PRD1 recovery resulting from the addition of 0.01% Tween 80 to the eluent. When the same eluent containing Tween 80 was employed at pH 8.0, no phages were recovered from these filters. At pH 9.8, <50% of carbonate ions in solution are present as CO_3^{2-}. The two negative charges of this anion are dispersed over the three electronegative oxygen atoms so that each has an effective charge of -2/3. The tridentate carbonate anion may displace negatively-charged virus from the filter by complexation with the nanoaluminum cationic media. Conversely, at pH 8.0 more than 90% of carbonate species in solution are present as HCO_3^-, an anion with potentially weaker interaction with the positively-charged nanoaluminum filter media. The results of these trials demonstrate (1) the comparable recoveries of phage achieved by NaPP and carbonate based eluents; (2) the large pH dependence of virus elution for Na_2CO_3 eluents; and (3) the dramatic influence of Tween 80 addition for PRD1 recovery from these filters.

We evaluated the recovery of HAdV 41 and BAdV 1 from these NanoCeram® filters using selected eluents optimized during bacteriophage recovery experiments (TABLE 5). Despite the demonstrated recovery of phage by these solutions, in all cases, AdV recovery was poor (or absent). The reason(s) for low recovery of AdV from NanoCeram filters is unclear but cannot be attributed to AdV passage through the filter in feedwater: eight filtrate samples were investigated for BAdV 1 or HAdV 41; three of eight filtrate samples for AdVs demonstrated DNA amplification below the level of quantification (as defined automatically during software sample analysis); extrapolating, these filtrate samples contained ≤ 1.4% of the original virus spike. The remaining filtrate samples were AdV-negative. Precedent for poor AdV recovery from electropositive filters exists: Sobsey and Glass (1984) observed AdV recoveries from 1MDS disc filters between 13 and 22%. In that study, AdV adhered to the cationic 1MDS media to a larger extent than did the two enteroviruses, poliovirus (recovery: 36–57%) and echovirus (recovery: 14–83%), examined.

TABLE 5: Recovery of HAdV 41 and BAdV 1 from NanoCeram® filters using selected eluents optimized for bacteriophage recovery.

	Recovery (%)[a,b]			
Eluent[c]	**BAdV 1**		**HAdV 41[d]**	
3% Beef Extract (for ref.)	4	3	0.2	0.2
0.1% NaPP + 0.01% Tween 80 (pH 9.8)	2	1	1	1
0.05 M Na_2CO_3, pH 9.8 + 0.01% Tween 80	Neg.	Neg.	ND	ND
10 mM SDS	Neg.	Neg.	ND	ND

[a] Recoveries for duplicate filtration experiments reported separately; standard deviations were not determined. All eluents were prepared in 10 mM Tris-HCl, 10 mM NaCl. A pH of 7.5 was employed, except for the carbonate-based eluent (pH 9.8). Eluent volume = 5-mL.

[b] Three of eight filtrate samples for AdVs (not shown) demonstrated DNA amplification below the level of quantification (as defined automatically during software sample analysis); extrapolating, these filtrate samples contained ≤ 1.4% of the original virus spike. The remaining filtrate samples were AdV-negative.

[c] 1% NaPP + 0.1% Tween 80 was not a viable eluent for AdV due to strong PCR inhibition (*vide infra*).

[d] Samples for the Na_2CO_3 and SDS eluents were not enumerated for HAdV 41 after poor recoveries were observed first for BAdV 1.

Abbreviations: Neg., Negative; ND, Not Determined.

NanoCeram® filters were evaluated as possible secondary concentration devices for 0.01% NaPP + 0.01% Tween 80 and pH-adjusted Na_2CO_3 + 0.01% Tween 80 eluents. Phages were spiked into 25 mL solutions of 0.05 % Na_2CO_3 + 0.01% Tween 80 (at pH 8.0) and these solutions were passed through the NanoCeram® media as described previously. A 0.1% NaPP + 0.1% Tween 80 solution was used to recover phages from the filters for these experiments. Filtrate and eluate samples were enumerated. The passage/recovery of bacteriophage is shown in TABLE 6. When present in the 25 mL feedwater, 0.05% Na_2CO_3 + 0.01% Tween 80 (pH 8.0) showed little interference with bacteriophage capture by the NanoCeram® media, while the 0.01% NaPP + 0.01% Tween 80 feedwater, an eluent that was only moderately effective, made the NanoCeram media nearly transparent towards the viruses investigated. Considering these results, only the 0.05% Na_2CO_3 + 0.01% Tween 80 feedwater was evaluated for BAdV 1 (TABLE 6). In contrast with phage, low concentrations of BAdV 1 were determined in both filtrate and eluate. This result was in agreement with the poor recovery of AdV observed for all

of the eluents evaluated. Overall, our results indicate that NanoCeram® filters would be ineffective secondary concentration devices for HFUF concentrates (as formulated) and effective secondary concentration devices for pH-adjusted Na_2CO_3 eluents for some (the phages surveyed) but not all (e.g., AdV) viruses; again, the reason for this discrepancy is unclear at this time.

TABLE 6. Evaluation of NanoCeram® filters as secondary concentration devises for two mock primary concentrates: (**A**) 0.01% NaPP and 0.01% Tween 80 and (**B**) 0.05 M Na_2CO_3 + 0.01% Tween 80 (adjusted to pH 8.0); recovery (%) of MS2, PRD1 and BAdV 1 in the filtrate and eluate (± 1 standard deviation) from 25 mm NanoCeram® syringe filters for duplicate adsorption/elution experiments.

Recovery (mean ± standard deviation, %)[a,c]					
MS2		**PRD1**		**BAdV 1[d]**	
Filtrate	Eluate	Filtrate	Eluate	Filtrate	Eluate
		A			
97 (17)	2 (2)	107 (19)	3 (1)	ND	ND
93 (20)	2 (2)	99 (19)	3 (1)	ND	ND
		B[b]			
0 (0)	43 (6)	1 (1)	77 (8)	≤ 1.4 [e]	Neg.
0 (0)	50 (8)	2 (1)	72 (9)	≤ 1.4 [e]	≤ 1.4 [e]

[a] The feed solutions employed simulated (A) hollow fiber ultrafilter concentrate (Hill et al., 2005; vide infra), or (B) NanoCeram® concentrate from a candidate 0.05 M Na_2CO_3+0.01% Tween 80 eluent.
[b] This trial simulates the secondary filtration (after pH adjustment from 9.8 to 8.0) of a primary eluent from a larger-scale NanoCeram® cartridge filter to determine if NanoCeram filters may be used in series to achieve greater virus concentration.
[c] Bacteriophage were eluted using 1% NaPP/0.1% Tween 80 solutions (trial A) and 0.1% NaPP/0.1% Tween 80 solutions (trial B); elution was attempted for BAdV 1 in both trials with 0.05 M Na_2CO_3+0.01% Tween 80 (pH 9.8).
[d] ND, Not determined; Neg., negative (no DNA amplification observed);
[e] DNA amplification observed, but below quantification limit (determined automatically during sample analysis by Roche Lightcycler 2.0 qPCR software).

Prior to the present study, MS2 was the only virus used to challenge NanoCeram filter for virus recovery. Considering the high filtration rates, strong virus retention, limited, but strong, MS2 recovery and replacement potential for 1MDS filters advertised for these NanoCeram® units, their evaluation was warranted. However, our results do not support the use of these filters for virus collection at this time. We demonstrated that (a) MS2 was not a good surrogate for PRD1, and (b) that neither phage, using multiple optimized eluents, accurately predicted the poor recovery of AdV. This is despite the fact that AdV and PRD1 share similar morphologies, and PRD1 has been suggested as a surrogate for enteric viruses, including AdV, for environmental fate and transport studies (Harvey and Ryan, 2004). Given the successful recovery of two bacteriophage with fundamentally different properties (e.g., size, lipid content; *cf.* TABLE 1) from these filters, the poor recovery of HAdV 41 and BAdV 1 was unexpected. Overall, while NanoCeram® filters may provide an effective means for primary or secondary concentration of some viruses in water samples, the poor AdV recoveries observed here discouraged further evaluation for AdV-based fecal source tracking. Furthermore, just as observed recoveries varied slightly between the two syringe filter formats employed in this study, changes in virus recovery

are expected when transitioning from syringe to cartridge filters capable of concentrating large water samples. For example, Polaczyk et al. (2007) evaluated the recovery of a variety of microorganisms, including viruses, from 1MDS filters in disc and cartridge formats. The optimized beef extract eluent (1.5% beef extract + 0.05 M glycine + 0.1% NaPP + 0.01% Tween 80, pH 8) recovered 89% and 32% of MS2 from disc vs. cartridge filter configurations. Additional research on the physicochemical factors responsible for the poor release of AdV from these filters is critical, especially considering the filter's burgeoning use for the collection of viral water quality samples: a number of research groups and private companies (e.g., Scientific Methods, Inc., Granger, Indiana) are exploring this medium for virus recovery. Until the mechanism(s) responsible for strong AdV binding with these filtered is determined, and can be overcome, it is fair to question the utility of NanoCeram filters for AdV (and possibly other virus) recovery.

Hollow fiber ultrafiltration system evaluation. Hollow-fiber hemodialysis filters have been advocated for the collection/concentration of pathogens in drinking and environmental water samples (e.g., Morales-Morales et al., 2003; Hill et al., 2005; Polaczyk et al., 2008). These filter demonstrate high (typically > 50%) recovery of a variety of microorganisms (including viruses: e.g., Winona et al., 2001; Morales-Morales et al., 2003; Polaczyk et al., 2008) of public health concern and are considerably less expensive than available tangential-flow ultrafiltration units dedicated for water-quality sampling. Therefore, as an alternative to Nanoceram® filters, which collect viruses from water through electrostatic mechanisms, we evaluated the recovery of viruses from water samples using Fresenius Optiflux F200NR hollow-fiber hemodialysis filters, which operate by size exclusion. Considering the WI State Laboratory of Hygiene's overlapping objective of concentrating a variety (in addition to viruses) of fecal indicator organisms and pathogens, we included the bacteria *E. coli* and *E. faecalis* in our evaluation. We assessed the influences of microorganism type and filter blocking agent on recovery by these units.

Filters were incubated with different "blocking" solutions to minimize non-specific binding of test organisms to the filter (Winona et al., 2001; Hill et al., 2005). At a nominal system pressure of 7.5 psi, microorganism-spiked 10-L test volumes of PBS were concentrated after 30 and 60 min for FBS-blocked and NFIM-blocked filters, respectively. At a nominal system pressure of 12 psi, a 50-L test volume of dechlorinated tap water was concentrated within 1 hours (permeate flow was ~ 0.9 $L \cdot min^{-1}$) for the horse-serum-blocked filter. Recoveries for each organism from the three variously-blocked HFUFs are provided in TABLE 7. These experiments support the use of serum blocking agents (for high recoveries and filtration speed). Virus recovery in all cases was $\geq$ 58 %, with a recovery of 72% observed for HAdV 41 for the horse-serum-block HFUF. The HFUF recoveries for phage and bacteria are in agreement with those reported previously for a variety of microorganisms/sample types (Morales-Morales et al., 2003; Hill et al., 2005; Polaczyk et al., 2008); to the best of our knowledge, this study presents the first AdV HFUF recovery estimate. In addition, unlike NanoCeram filters, for which syringe and cartridge filter may show different virus recoveries, no additional scaling issues must be considered with these HFUF units. Overall, these results support the use HFUF systems for collecting AdV-based fecal source tracking samples as well as for future virus- and pathogen-collection activities. In fact, to the extent that current regulations allow, WSLH has already adopted the HFUF system designed here.

TABLE 7: Recovery (%) for test microorganisms from the Fresenius hollow fiber ultrafiltration system..[a]

	Blocking Agent		
	FBS[b]	NFIM[b]	HS[c]
MS2	91	79	95 (45)[e]
E. coli	112	19	83 (8)[d]
E. faecalis	80	80	ND
HAdV 41	ND	ND	72 (19)[e]
BAdV 1	ND	ND	58 (28)[e]
PRD1	ND	ND	63 (7)[e]

[a] Abbreviations: FBS, fetal bovine serum; MS2, male-specific coliphage MS2; NFIM, non-fat instant milk; HS, horse serum; PRD1, bacteriophage PRD1 (strain D4); HAdV 41 (human adenovirus 41); ND, not determined. [b] recoveries were determined from 10-L of phosphate buffered saline without replication; [c] recoveries were determined from 50-L of dechlorinated tap water; [d] Mean and standard deviation of duplicate enumerations of initial and final (concentrate) samples.
[e] Mean and standard deviation of triplicate enumerations of initial and final (concentrate) samples.

Hollow fiber ultrafiltration system configuration. Considerable effort was dedicated to establishing an efficient and reasonably intuitive flow system for the hollow fiber ultrafiltration unit. Several tubing configurations were evaluated before finalizing the configuration illustrated and pictured in Figure B1. We originally situated the sample reservoir so that gravity induced flow directly into the concentrate bottle. This configuration required constant monitoring (to avoid overflowing the system) and for the 50-L carboy to be (a) filled and lifted above the HFUF system (an aspect that is not ideal considering the weight of a filled 50-L carboy), or (b) filled in an elevated location. The system was reconfigured by placing a 0.2-µm-filtered air vent on the concentrate bottle where the gravity-flow sample inlet formerly entered, and inserting a 3-way valve between the concentrate reservoir and the pump to accept sample flow from the carboy. Under this new configuration, a stop-cock below the 0.2-µm filter can be opened initially to allow sample flow from the 50-L sample-filled carboy into the concentrate bottle (this value must be opened initially to allow sample fluid to displace the air in the system); once the concentrate bottle is nearly full, this valve is closed (a) to prevent fluid from overflowing the concentrate bottle and contacting the filter membrane, and (b) to reestablish a "closed system." Under this closed-system configuration, sample is drawn from the carboy at the exact rate that permeate flows from the ultrafilter. Thus, the system can be left unmonitored during the bulk of the filtration process, as opposed to requiring constant adjustment of the fluid flow from the carboy into the concentrate bottle (as before). This represents a significant advance in design, especially if very large water samples are to be collected.

DNA extraction and PCR inhibition.

DNA extraction is required prior to PCR analysis to separate and purify viral nucleic acid from the surrounding viral material (e.g., protein capsid) and, importantly, PCR-inhibiting compounds (Read, 2001). To evaluate the potential for eluents employed during virus concentration protocols (described above) to inhibit downstream PCR applications, we characterized the pre- and post-DNA extraction tendencies of these solutions to interfere with qPCR. Performing a pre-DNA extraction test provides information on the inherent PCR-inhibiting properties of each of the compounds investigated. The post-DNA extraction test

indicates (1) the extent to which potential DNA inhibitors are removed from samples by the DNA extraction kits evaluated, and (2) the tendency for an eluent to interfere with the viral DNA extraction process.

Pre-DNA extraction – eluent inhibition. Comparing C_t values (TABLE 8), the 0.01% NaPP + 0.01% Tween 80, 0.1% NaPP + 0.01% Tween 80, and 3% beef extract solutions showed no inhibition, mild inhibition and strong inhibition of PCR, respectively. The 1.0% NaPP + 0.1% Tween 80 and 10 mM SDS solutions completely inhibited PCR, and no amplification of the salmon sperm DNA was observed.

TABLE 8: Mean C_t values for 5 µL salmon sperm DNA spiked at 0.2 µg/mL into selected candidate solutions (in a 10 mM Tris buffer + 10 mM NaCl background buffer) used for the elution of viruses from NanoCeram® media and hollow-fiber ultrafiltration devices ($n = 3$). A 10 mM Tris buffer + 10 mM NaCl solution alone was employed as a no-inhibition control.

Eluent	Mean (Standard Deviation) C_t
10 mM Tris buffer + 10 mM NaCl	30.5 (0.2)
3% Beef Extract	34.6 (0.6)
10 mM SDS	no amplification observed
0.01% NaPP + 0.01% Tween 80	30.8 (0.4)
0.1% NaPP + 0.01% Tween 80	32.8 (0.2)
1.0% NaPP + 0.1% Tween 80	no amplification observed

Post DNA extraction – eluent inhibition. DNA extraction/purification is accomplished using a variety of procedures/chemistries that can be broken into two general steps: disrupting/denaturing the virus capsid and purifying/concentrating extracted DNA. Capsid (or microorganism cell wall) disruption occurs by chemical/detergent treatment, which can include a proteinase digestion step. Concentration of from digested samples is achieved by DNA-specific chaotrope-induced binding to a silica support or through selective protein precipitation and removal followed by alcohol DNA precipitation (Read, 2001; Sambrook and Russell, 2001). Read (2001) compared the relative efficiencies of hepatitis B virus (DNA) and hepatitis C virus (RNA) nucleic acid purification and recovery by five commercial kits. Large differences in hepatitis B virus DNA recovery were observed among kits, with those employing a protease digestion in addition to chaotropic solutions performing better than those using chemical disruption alone. Therefore, we choose DNA extraction kits that included a proteinase K digestion for our evaluations.

During our initial conventional PCR evaluation of potential eluent inhibition, HAdV 41 DNA was extracted, using Epicenter Water and Qiagen Blood and Tissue kits, and amplified successfully for all eluents except 1.0% NaPP + 0.1% Tween 80; for this eluent, DNA extraction/amplification was unsuccessful. No differences (determined by DNA visualization by ethidium bromide staining agarose gel electrophoresis; not shown) in DNA extraction were apparent either between eluents for a given kit or for a given eluent between kits. Subsequently, extracted HAdV 41 DNA was subjected to amplification by real time PCR using SYBR green detection chemistry. Crossing point values (FIG. 2, TABLE 9) for HAdV 41 DNA amplification were remarkably similar considering the varied chemistries of the eluents surveyed and the fact that two different kits (that concentrate DNA by fundamentally different methods) were compared. These result indicated that PCR would not be differentially influenced by the

chemistry of most of the promising eluents examined and eliminated concerns that eluent inhibition could (1) be responsible for the low recoveries observed for AdV from NanoCeram® filters, or (2) play a role in qPCR downstream of HFUF.

After demonstrating nearly identical average C_t values for each candidate eluent, the Qiagen Blood and Tissue kit was selected for future use because of its simplicity and speed. In comparison with the "Pre-DNA Extraction" cases discussed above, is it clear that the kits evaluated removed inhibitory properties of 3% beef extract and 0.1% NaPP + 0.01% Tween 80, but not of 1.0% NaPP + 0.1% Tween 80 eluent. The lack of inhibition exhibited by 3% beef extract after the incorporation of a DNA extraction step presents an interesting contradiction with many statements in environmental virology literature. This discrepancy may be explained by the following: beef extract has been employed historically for two purposes, to disrupt virus-substrate binding, and to promote co-precipitation of viruses during polyethylene glycol (PEG) (or other flocculation-based) precipitation procedures. Flocculated beef extract samples contain a dramatically larger effective concentration of potential PCR inhibitors than a native 3% beef extract solution. Therefore, the ability of DNA extraction/purification procedures to either concentrate or isolate DNA from the complicated beef extract matrix is likely affected. In fact, it is probable that any attempt at the secondary concentration of beef-extract-containing virus samples will result in increased PCR inhibition (Schwab et al., 1995; Fout et al., 2003).

TABLE 9: Mean C_t values (determined by SYBR green qPCR) for extracted HAdV 41 DNA generated by two competing DNA extraction kits ($n = 3$). HAdV 41 cell culture supernatant was diluted 1/10 into candidate filter (NanoCeram® and Fresenius Hemodialysis) eluents and viral DNA was extracted. Based on C_t value (with lower values representing higher extraction efficiency/lower PCR inhibition), the kits demonstrated extremely similar DNA extraction efficiencies; all C_t values were with 1 cycle of each other.

	Mean (± standard deviation) C_t[c]	
Eluent[a, b]	Qiagen B&T	Epicentre H_2O
10 mM Tris buffer + 10 mM NaCl	16.5 (0.2)	16.5 (0.2)
3% Beef Extract	16.9 (0.3)	16.2 (0.1)
10 mM SDS	16.3 (0.1)	16.1 (0.1)
0.1% NaPP + 0.01% Tween 80	16.8 (0.1)	16.9 (0.1)
0.05 M Na_2CO_3	16.5 (0.1)	16.3 (0.1)

[a] Constituted in 10 mM Tris buffer + 10 mM NaCl background buffer

[b] HAdV 41 extracted from a 10 mM Tris buffer + 10 mM NaCl solution served as the no-inhibition control.

[c] 1 µL of extracted DNA was used in each PCR reaction.

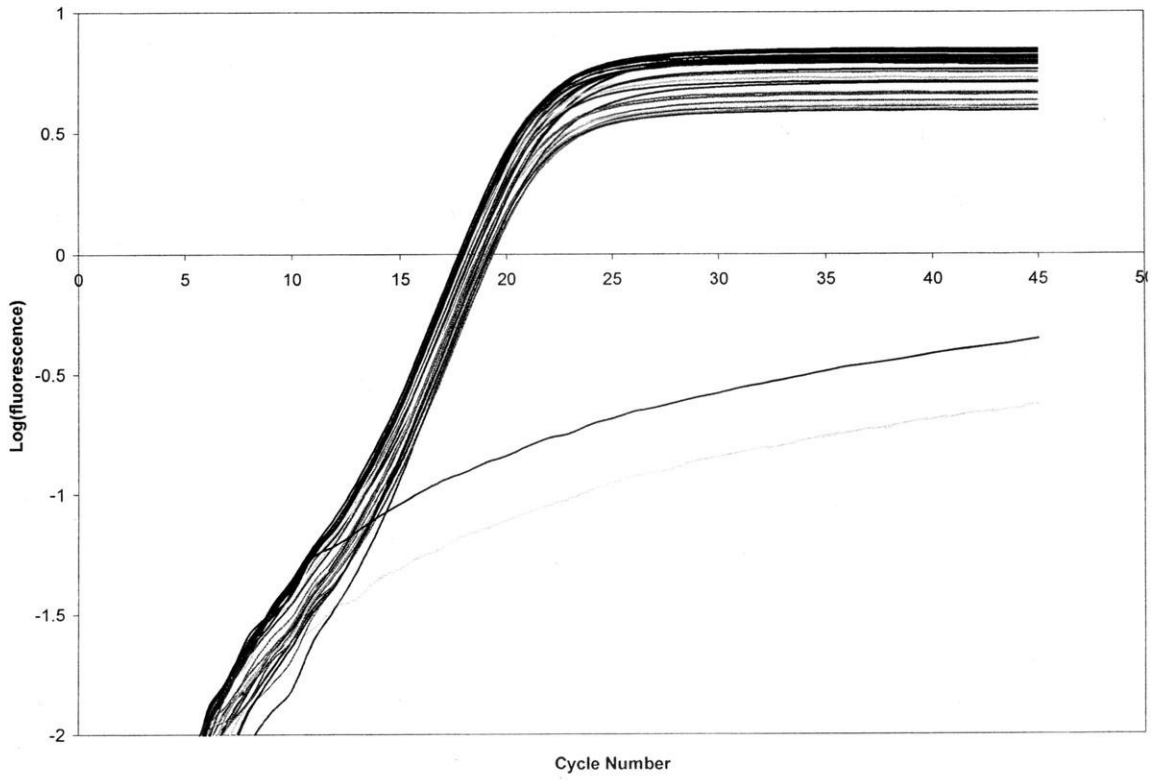


FIG. 2: Real time PCR profiles of HAdV 41 DNA amplification. The virus was spiked in equal quantities into a variety of different eluents and subsequently extracted using both the Epicentre water and Qiagen DNeasy Blood and Tissue kits. The tight grouping of amplification curves (and the resulting C_t values, TABLE 9) indicates that the eluents surveyed exerted little influence on virus extraction efficiency from samples. Curves A and B show no-template control samples. DNA extracted from HAdV 41 spiked into 1.0% NaPP + 0.1% Tween 80 solutions, a promising eluent, showed no amplification during initial conventional PCR experiments. Because of this indication of strong PCR inhibition, these DNA samples were excluded from this qPCR evaluation.

CONCLUSIONS AND RECOMMENDATIONS

As a result of its empirically demonstrated specificity, the novel PCR assay described here represents a significant advance towards the implementation of AdV-based fecal source tracking. In addition, the specificity of the assay allows for the adoption of SYBR green-based qPCR. Though more straight-forward in application, SYBR green binds to double stranded DNA non-specifically and, accordingly, is often avoided in assays requiring the extra specificity potentially derived from TaqMan probes (which bind to specific sequences within a target). However, the use of SYBR green may be preferred in instances when (a) resources for TaqMan probes may be unavailable, (b) assay simplification is desired, and (c) investigators require an additional homogeneous check of assay results (i.e., because amplicons generated for BAdV vs. HAdV in our assay differ in size, they can be independently confirmed/distinguished at the conclusion of a PCR by melting curve analysis that is possible with SYBR green but not TaqMan

chemistry). We note that AdV belonging to the genus *Mastadenovirus* that have yet to be isolated and sequenced could share one or both of the 3'DNP around which our assay derives its specificity. This uncertainty is not unique to the present study; the design of PCR primers and probes (and their resulting specificity) is always limited by the availability of sequence data upon which primers are based. That said, the primer designed here (1) are based on two sets of 3' dual nucleotide mismatches, when one 3'DNM alone would likely provide sufficient discrimination, and (2) are not predicted to amplify any known non-target AdV (based on an evaluation of our oligonucleotides with the BLAST algorithm of the NCBI database).

While poor recovery of AdV discouraged further evaluation of NanoCeram® filters, the HFUF unit is ready to be deployed by the WSLH for simultaneous concentration of multiple pathogens and indicators (including AdV) of interest for fecal source tracking. Future research into the spatial and seasonal distribution of livestock and wildlife AdV is recommended, as the information acquired during such surveys will make AdV-based fecal source tracking assays more robust. Any work completed in this regard should be accompanied by the acquisition of genetic data through cloning/sequencing of AdV-positive PCR products. In this way, the database of available animal AdV sequences will be enhanced, allowing for continuing evaluation/validation of the specificity of the primers/probes designed here. In addition, considering the improved HFUF configuration, which allows for sample concentration without user supervision, a logical next step is the modification/evaluation of this HFUF system for the collection of very large (500- to 1000-L) water samples. Specifically, modifications of the current system facilitating (1) continuous filtration from a water source, (2) injection of the NaPP dispersing agent during filtration (as opposed to one-step addition at when commencing to acquire 50- to 100-L samples), and (3) real-time sample preservation (e.g., installation of a cooling jacket around the sample concentrate bottle) would significantly expand an investigator's ability to efficiently concentrate viral (and other) pathogens from potentially contaminated groundwater.

REFERENCES

Abbaszadegan, M., P. Stewart, M. LeChevallier. 1999. A Strategy for Detection of Viruses in Groundwater by PCR. Appl. Environ. Microbiol. **65**:444-449.

Abbaszadegan, M., M. LeChevallier, C. Gerba. 2003. Occurrence of viruses in US groundwaters. JAWWA, **95**:107-120.

Ayyadevara, S., J.J. Thaden, R.J. Shmookler Reis. 2000. Discrimination of Primer 3′-Nucleotide Mismatch by Taq DNA Polymerase during Polymerase Chain Reaction. Analyt. Biochem. **284**:11-18

Benson, D.A., I. Karsch-Mizrachi, D.J., Lipman, J. Ostell, D.L.Wheeler. 2008. GenBank. Nucleic Acids Res. **36**(Database issue):D25-30 http://www.ncbi.nlm.nih.gov/Genbank/index.html.

Borchardt, M.A., N.L., Haas, R.J., Hunt. 2004. Vulnerability of drinking-water wells in La Crosse, Wisconsin, to enteric-virus contamination from surface water contributions. Appl. Environ. Microbiol. **70**:5937-46.

Borchardt, M.A., K.R. Bradbury, M.B. Gotkowitz, J.A. Cherry, B.L. Parker. 2007. Human Enteric Viruses in Groundwater from a Confined bedrock aquifer. Environ. Sci. Technol. **41**:6606-6612.

Büchen-Osmond, C. (Ed). 2006. Index of Viruses - Adenoviridae. In: ICTVdB - The Universal Virus Database, version 4., Columbia University, New York, USA. http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_index.htm

Carter, M.J. 2005. Enterically infecting viruses: pathogenicity, transmission and significance for food and waterborne infection. Appl. Microbiol. **98**:1354-1380.

Chavali, S., A. Mahajan, R. Tabassum, S. Maiti, D. Bharadwaj. 2005. Oligonucleotide properties determination and primer designing: a critical examination of predictions. Bioinformatics **21**:3918–3925.

Davies, C.M., M.R. Logan, V.J. Rothwell, M. Krogh, C.M. Ferguson, K. Charles, D.A. Deere, N.J. Ashbolt. 2005. Soil inactivation of DNA viruses in septic seepage. J.Appl. Microbiol. **100**:365 - 374

Enriquez, C.E., C.P. Gerba. 1995. Concentration of enteric adenovirus 40 from tap, sea and waste water. Wat. Res. **29**:2554-2560.

Fairchild, A.; M.D., Lee, J.J. Maurer. 2006. PCR Basics, p. 1-26. In J.J. Maurer (ed.), PCR Methods in Foods. Springer Science + Business Media, Inc., New York, NY.

Farrah, S.R.; Shah, D.O.; Ingram, L.O. 1982. Effects of chaotropic and antichaotropic agents on elution of poliovirus adsorbed on membrane filters. Proc. Natl. Acad. Sci. USA. **78**:1229-1232.

Farrah, S.R., D.R. Preston, G.A. Toranzos, M. Girard, G.A. Erdos, V. Vasuhdivan. 1991. Use of Modified Diatomaceous Earth for Removal and Recovery of Viruses in Water. Appl. Environ. Microbiol. **57**:2502-2506.

Fong, T., L.S. Mansfield, D.L. Wilson, D.J. Schwab, S.L. Molloy, J.B. Rose. 2007. Massive Microbiological Groundwater Contamination Associated with a Waterborne Outbreak in Lake Erie, South Bass Island, Ohio. Environmental Health Perspectives. **115**:856-864.

Fong, T., E.K. Lipp. 2005. Enteric Viruses of Humans and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools. Microbiol. Molec. Biol. Rev. **69**:357-371.

Fong, T., D.W. Griffin, E.K. Lipp. 2005. Molecular Assays for Targeting Human and Bovine Enteric Viruses in Coastal Waters and Their Application for Library-Independent Source Tracking. Appl. Environ. Microbiol., **71**, 2070-2078.

Fout, G.S., B.C. Martinson, M.W. N. Moyer, D.R. Dahling. 2003. A Multiplex Reverse Transcription-PCR Method for Detection of Human Enteric Viruses in Groundwater. Appl. Environ. Microbiol. **69**:3158–3164

Fujito, B.T., C.D. Lytle. 1996. Elution of Viruses by Ionic and Nonionic Surfactants. Appl. Environ. Microbiol. **62**: 3470–3473.

Gerba, C.P., D.M. Gramos, N. Nwachuku. 2002. Comparative inactivation of enteroviruses and adenovirus 2 by UV light. Appl. Environ. Microbiol. **68**:5167–5169.

Giegerich R., F. Meyer, C. Schleiermacher. 1996. GeneFisher--software support for the detection of postulated genes. Proc Int Conf Intell Syst Mol Biol. **4**:68-77.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41**:95-98.

Harvey, R.W., J.N. Ryan. 2004. Use of PRD1 bacteriophage in groundwater viral transport, inactivation, and attachment studies. FEMS Microbiol. Ecol. **49**:3–16.

Heim, A., C. Ebnet, G. Harste, P. Pring-Åkerblom. 2003. Rapid and Quantitative Detection of Human Adenovirus DNA by Real-Time PCR. J. Med. Virol. **70**:228-239.

Hill, V.R., A.L. Polaczyk, D. Hahn, J. Narayanan, T.L. Cromeans, J.M. Roberts, J.E. Amburgey. 2005. Development of a Rapid Method for Simultaneous Recovery of Diverse Microbes in Drinking Water by Ultrafiltration with Sodium Polyphosphate and Surfactants. Appl. Environ. Microbiol. **71**:6878–6884.

Herzer, S., P. Beckett, T. Wegman, P. Moore. 2003. Isoelectric titration curves of viral particles as an evaluation tool for ion exchange chromatography. Amersham Biosciences Life Science News. **13**:16-18.

Huiskonen, J.T., V. Manole, S.J. Butcher. 2007. Tale of two spikes in bacteriophage PRD1. Proc. Natl. Acad. Sci. USA. **104**:6666-6671.

Jabado O.J., G. Palacios, V. Kapoor, J. Hui, N. Renwick, J. Zhai, T. Briese, W.I. Lipkin. 2006. Greene SCPrimer: a rapid comprehensive tool for designing degenerate primers from multiple sequence alignments. Nucleic Acids Res. **34**:6605-11.

Jiang, S., R. Noble, W. Chu. 2001. Human Adenovirus and coliphage in urban runoff-impacted coastal water of Southern California. Appl. Environ. Microbiol. **67**:179 - 184.

Jiang, S., H. Dezfulian, W. Chu. 2005. Real-time quantitative PCR for enteric adenovirus serotype 40 in environmental waters. Can. J. Microbiol. **51**:393 – 398.

Jones II, M.S., B. Harrach, R.D. Ganac, M.M.A. Gozum, W.P. dela Cruz, B. Riedel, C. Pan, E.L. Delwart, D.P. Schnurr. 2007. New Adenovirus Species Found in a Patient Presenting with Gastroenteritis. J. Virol. **81**:5978–5984.

Jothikumar, N., T.L. Cromeans, V.R. Hill, X. Lu, M.D. Sobsey, D.D. Erdman. 2005. Quantitative Real-Time PCR Assays for Detection of Human Adenoviruses and Identification of Serotypes 40 and 41. Appl. Environ. Microbiol. **71**:3131-3136.

Klein, M., E. Earley, J. Zellat. 1959. Isolation from cattle of a virus related to human adenovirus. Proc. Soc. Exp. Biol. Med. **102**:1-4.

Klein, M. J. Zellat, T.C. Michaelson. 1960. A new bovine adenovirus related to human adenovirus. Proc. Soc. Exp. Biol. Med. **105**:340-342.

Lambertini, E., S.K. Spencer, P.D. Bertz, F.J. Loge, B.A. Kieke, M.A. Borchardt. 2008. Concentration of Enteroviruses, Adenoviruses, and Noroviruses from drinking water by use of glass wool filters. Appl. Environ. Microbiol. **74**: 2990–2996.

Latorra, D., K. Campbell, A. Wolter, J.M. Hurley. 2003. Enhanced allele-specific PCR discrimination in SNP genotyping using 3 locked nucleic acid (LNA) primers. Human Mutation **22**:79 – 85.

Lebuhn, M., M. Effenberger, G. Garcés, A. Gronauer, P. A. Wilderer. 2004. Evaluating real-time PCR for the quantification of distinct pathogens and indicator organisms in environmental samples. Water Science and Technology. 2004, **50**:263-270.

Lemkuhl, H.D., L.A. Hobbs. 2008. Serologic and hexon phylogenetic analysis of ruminant Adenoviruses. Arch. Virol. **153**:891–897.

Lenaerts, L., E. Verbeken, E. De Clercq, L. Naesens. 2005 Mouse Adenovirus Type 1 Infection in SCID Mice: an Experimental Model for Antiviral Therapy of Systemic Adenovirus Infections. Antimicrobial Agents and Chemotherapy **49**:4689–4699.

Lukasik, J., T.M. Scott, D. Andryshak, S.R. Farrah. 2000. Influence of Salts on Virus Adsorption to Microporous Filters. Appl. Environ. Microbiol. **66**:2914–2920.

Lytle, C.D., L.B. Routson. 1995. Minimized Virus Binding for Tests of Barrier Materials. Appl. Environ. Microbiol. **61:** 643–649.

Maluquer de Motes, C., P. Clemente-Casares, A. Hundesa, M. Martin, R. Girones. 2004. Detection of bovine and porcine adenoviruses for tracing the source of fecal contamination. Appl. Environ. Microbiol. **70**:1448–1454.

Mann, B., J.A. Traina, C. Soderblom, P.K. Murakami, E. Lehmberg , D. Lee , J. Irving, E. Nestaas , E. Pungor Jr. 2000. Capillary zone electrophoresis on a recombinant adenovirus. J. Chromatog. A. **895**:329 – 337.

Martin, F.H., M.M.Castro, F. Aboul-ela, I. Tinoco, Jr. 1985. Base pairing involving deoxyinosine: implications for probe design. Nucleic Acids Research **13**:8927-8938.

Madigan, M., J. Martinko. 2000. Brock's Biology of Microorganisms, 9th ed. Prentice-Hall, Inc. Upper Saddle River, NJ.

Meng, Q.S., C.P. Gerba. 1996. Comparative inactivation of enteric adenoviruses, poliovirus and coliphages by ultraviolet irradiation. Water Resources. **30**, 2665 – 2668.

Morales-Morales, H.A., G. Vidal, J. Olszewski, C.M. Rock, D. Dasgupta, K.H. Oshima, G.B. Smith. 2003. Optimization of a Reusable Hollow-Fiber Ultrafilter for Simultaneous Concentration of Enteric Bacteria, Protozoa, and Viruses from Water. Appl. Environ. Microbiol. **69**:4098–4102.

Noble, R.T., S.M. Allen, A.D. Blackwood, W. Chu, S.C. Jiang, G.L. Lovelace, M.D. Sobsey, J.R. Stewart, D.A. Wait. 2003. Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial course tracking comparison study. J. Water Health **1**:195–207.

Peixoto, C., T.B. Ferreira, M.J.T. Carrondo, P.E. Cruz, P.M. Alves. 2006. Purification of adenoviral vectors using expanded bed chromatography. J. Virol. Meth. **132**:121–126

Piranha, J.M., A. Pacheco, R.C. Gamba. 2006. Faecal Contamination (Viral and Bacteria) Detection in Groundwater used for Drinking Purposes in São Paulo, Brazil. Geomicrobiology Journal. **23**:279–283.

Polaczyk, A.L., J. Narayanan, T.L. Cromeans, D. Hahn, J.M. Roberts, J.E. Amburgey, V.R. Hill. 2008. Ultrafiltration-based techniques for rapid and simultaneous concentration of multiple microbe classes from 100-L tap water samples. J. Microbiol. Meth. **73**:92–99.

Polaczyk, A.L., J.M. Roberts, V.R. Hill. 2007. Evaluation of 1MDS electropositive microfilters for simultaneous recovery of multiple microbe classes from tap water. J. Microbiol. Meth. **68**:260-266.

Pond, K.R., R. Rangdale, W.G. Meijer, J. Brandao, L. Falcamacro, A. Rince, B. Masterson, J. Greaves, A. Gawler, E. McDonnell, A.A. Cronin, S. Pedley. 2004. Workshop Report: Developing Pollution Source Tracking for Recreational and Shellfish Waters. Environmental Forensics. **5**:237 – 247.

Rangdale, R E., W. Meijer, A. Rincé, J. Brandão, L. Falcão, J. Machado, C. Nogueira, M.L. Rosado, C. Veríssimo. 2003. Development of Methods for Pollution Source Tracking-Consolidated Literature Review. Accessed September 2008. http://randd.defra.gov.uk/Document.aspx?Document=WT03012_2082_FRP.pdf.

Read, S.J. 2001. Recovery efficiencies of nucleic acid extraction kits as measured by quantitative LightCycler™ PCR. J. Clin. Pathol: Mol. Pathol. **54**:86–90.

Rozen, S., H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. In: S. Krawetz and S. Misener, eds. Bioinformatics Methods and Protocols in the series Methods in Molecular Biology. Humana Press, Totowa, NJ, 365-386. http://fokker.wi.mit.edu/primer3/.

Sambrook, J. Russell, D.W. 2001. Molecular cloning: a laboratory manual, 3rd ed. New York: Cold Spring Harbor Laboratory.

Schutz, E., N. von Ahsen. 1999. Spreadsheet Software for Thermodynamic Melting Point Prediction of Oligonucleotide Hybridization with and without Mismatches. BioTechniques **27**:1218-1224.

Schwab, K.J., R. De Leon, M.D. Sobsey. 1995. Concentration and Purification of Beef Extract Mock Eluates from Water Samples for the Detection of Enteroviruses, Hepatitis A Virus, and Norwalk Virus by Reverse Transcription-PCR. Appl. Environ. Microbiol. **61**:531–537

Scott, T. M., J. B. Rose, T. M. Jenkins, S. R. Farrah, J. Lukasik. 2002. Microbial source tracking: current methodology and future directions. Appl. Environ. Microbiol. **68**:5796–5803.

Shieh, Y.S., R.S. Baric, M.D. Sobsey. 1997. Detection of Low Levels of Enteric Viruses in Metropolitan and Airplane Sewage. Appl. Environ. Microbiol. **63**:4401–4407.

Sobsey, M.D., J.S. Glass. 1984. Influence of water quality on enteric virus concentration by microporous filter methods. Appl. Environ. Microbiol. **47**:956-960.

Tepper, F., L. Kaledin. 2006. A high-performance electropositive filter. Bioprocess International. **4**:64-68.

Toogood, C.I.A., R.T. Hay. 1988. DNA Sequence of the Adenovirus Type 41 Hexon Gene and Predicted Structure of the Protein. J. gen. Virol. **69**:2291-2301.

Trilisky, E.I., A.M. Lenhoff. 2007. Sorption processes in ion-exchange chromatography of viruses. J. Chromatogr. A. **1142**:2–12.

U.S. Environmental Protection agency. 2003. Workshop to Develop a Protocol for Reliable Genetic Methods for the Detection of Viruses, for Use in EPA's Water Programs. http://www.epa.gov/safewater/ucmr/ucmr1/pdfs/meeting_ucmr1_january2003.pdf. Accessed September 2008.

U.S. Environmental Protection agency (US EPA 2001a). 2001. Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure. EPA 821-R-01-029. http://www.epa.gov/nerlcwww/1602ap01.pdf. Accessed September 2008.

U.S. Environmental Protection agency (US EPA 2001b). 2001. USEPA Manual of Methods for Virology, Chapter 14. EPA 600/4-84/013 (N14). http://www.epa.gov/nerlcwww/chapt14.pdf. Accessed September 2008.

Vallone, P.M., J.M. Butler. 2004. AutoDimer: a screening tool for primer-dimer and hairpin structures. BioTechniques **37**:226-231.

van Heerden, J., M.M. Ehlers, A. Heim, W.O.K. Grabow. 2005. Prevalence, quantification and typing of adenoviruses detected in river and treated drinking water in South Africa. J. Appl. Microbiol. **99**:234-242.

Winona, L.J., A.W. Ommani, J. Olszewski, J.B. Nuzzo, K.H. Oshima. 2001. Efficient and predictable recovery of viruses from water by small scale ultrafiltration systems. Can J. Microbiol. **47**:1033–1041.

World Health Organization. 2004. http://www.who.int/water_sanitation_health/dwq/en/9241546301_chap8.pdf. Accessed October 2005

Woods, L.W., H.D. Lehmkuhl, P.K. Swift, P.H. Chiu, R.S. Hanley, R.W. Nordhausen, M.H. Stillian, M.L. Drew. 2001. Experimental adenovirus hemorrhagic disease in white-tailed deer fawns. Journal of Wildlife Diseases, **37**:153–158.

Xagoraraki, I., D.H.-W. Kuo, K.Wong, M. Wong, J.B. Rose. 2007. Occurrence of Human Adenoviruses at Two Recreational Beaches of the Great Lakes. Appl. Environ. Microbiol. **73**: 7874–7881.

APPENDIX A:

Papers Presented

1. Sibley, S.D.; Pedersen, J.A. Efficacy of nanoaluminum oxide fiber filters for concentrating bacteriophage and adenovirus from water for fecal source tracking. American Water Resources Association, 32nd Annual Wisconsin Section Meeting, 6-7 March 2008, Brookfield, WI.
2. Sibley, S.D.; Pedersen, J.A. Recovery of sorbed bacteriophages and adenovirus from nano-aluminum oxide fiber filters. Q456. 2008 General Meeting, American Society for Microbiology, Boston, MA, 1-5 June 2008.

Appendix B: Supplemental Information.

Table B1: Settings for BLAST searching sequences for evaluating oligonucleotide specificity.

Enter Query Sequence
Enter Sequence of interest

Choose Search Set:
Database: "Others"
Organism: "Adenoviridae (taxid:10508)"
Entrez Query: "adenovirus AND hexon"

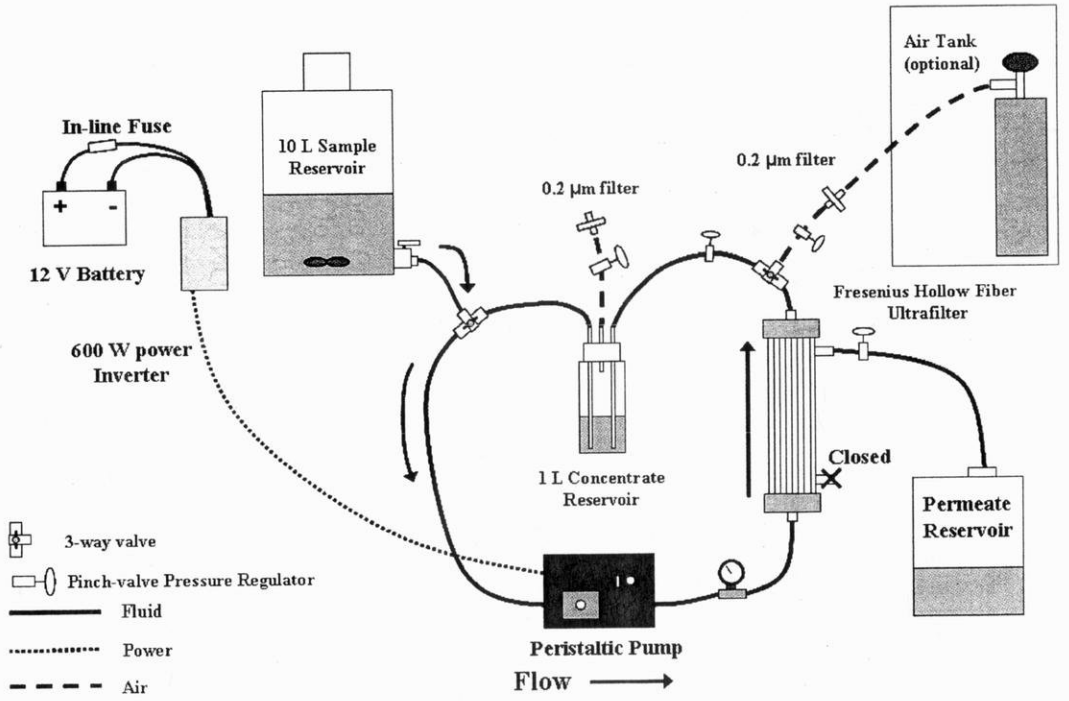
Program Selection:
Optimize for: "Somewhat similar sequences (blastn)"

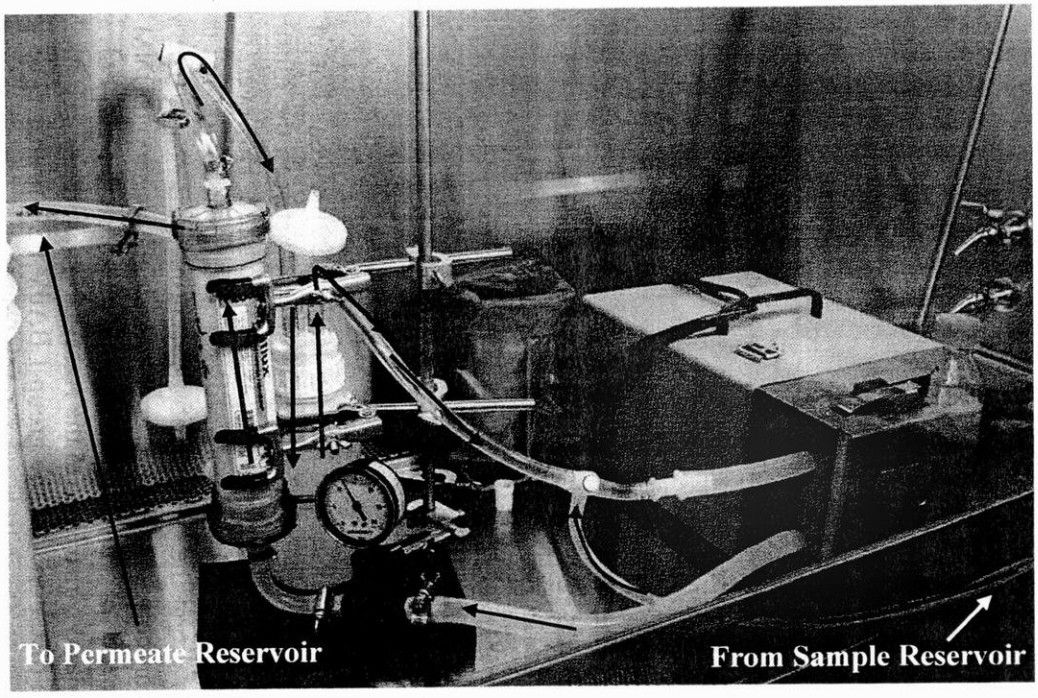
Algorithm parameters
General Parameters:
Max Target Sequences: 100
Short Queries: deselect "Automatically adjust parameters for short input sequences."
Expected Threshold: 100
Word Size: 7

Scoring Parameters:
Match/Mismatch Score: 1,-1
Gap Costs: Existence: 5; Extension: 2.

When BLASTing primers containing inosines, replace 'I' with 'N' to obtain consistent results with these settings. (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Figure B1: Optimized Hollow Fiber Ultrafiltration (HFUF): the systems requires user manipulation at the beginning (for rinsing blocking agent from the filter and allowing sample to fill concentrate reservoir) and the conclusion of filtration (elution). However, once sample filtration has begun, the system operates independent of user manipulation.





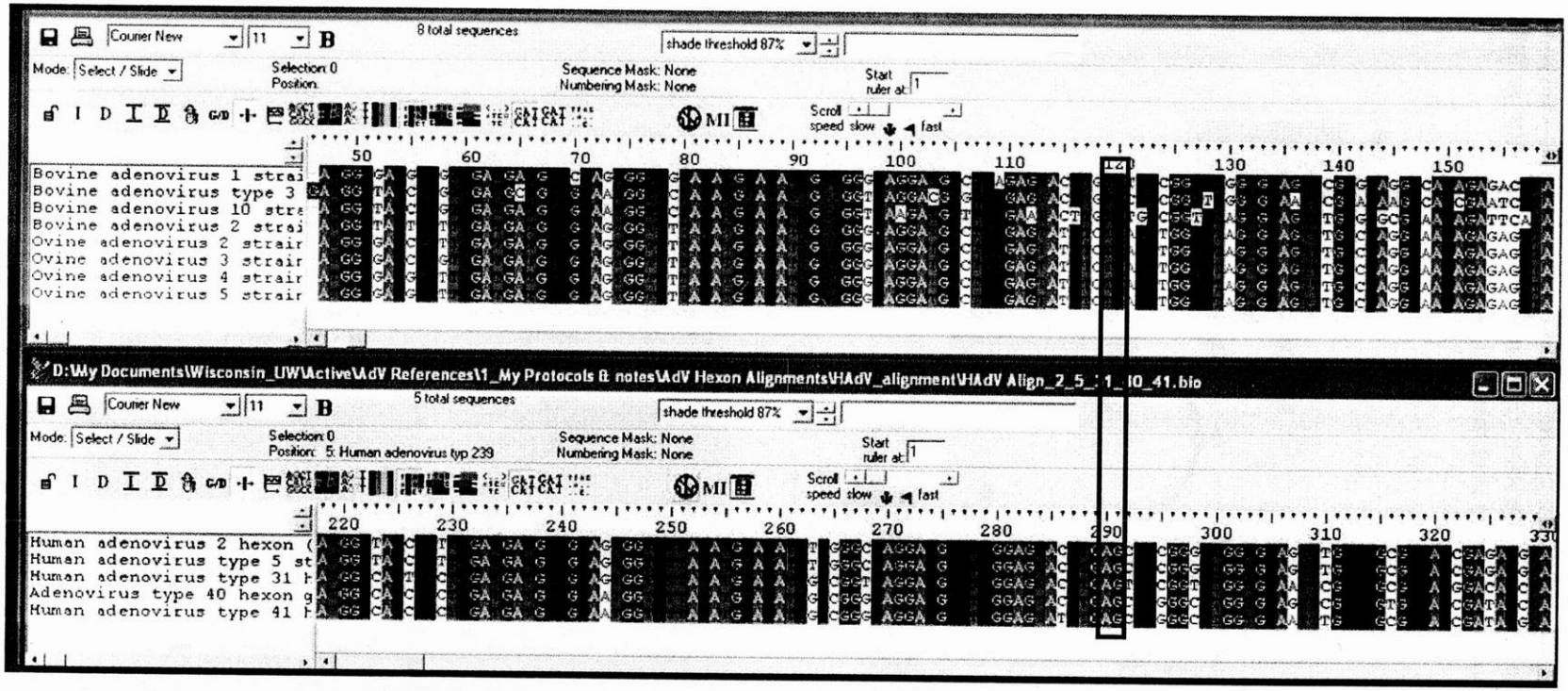


Figure B2: BioEdit (v. 7.0.9.0, Hall, 1999) screen shot of juxtaposed group I (GI) BAdV (above) and HAdV (below) multiple-sequence alignments. The large similarity between the illustrated portion of the hexon gene sequences of these viruses, all belonging to the genus *Mastadenovirus*, is demonstrated. However, 3 sets of conserved (among specific groups) dual nucleotide mismatches (DNM; one set shown) were discovered that allowed for the specific amplification of group I BAdV vs. HAdV. Taq polymerase extends from the 3' end of PCR primers. 3' base pair must be complementary with their DNA targets for efficient DNA extension/copying. The presence of a single conserved DNM can reduce amplification efficiency by many orders of magnitude, while the presence of 3'DNMs was shown here to inhibit Taq Polymerase extension completely. A single 3'DNM is highlighted above. Two additional downstream (to the right) DNPs were utilized, the proximal one for the reverse HAdV fecal source tracking (FST) primer and the distal one for the group I BAdV FST primer. The sequence data surrounding these DNPs was scrutinized as potential PCR primer sites. One site was suitable for HAdV; two sites were possible for BAdV based on the inherent variability of the collective sequences around the 3' DNMs. Those options with the lowest possible degeneracies were synthesized and evaluated.

Appendix C: Primer Maps

HAdV 41 primer map and hexon gene sequence translation: The FASTA format sequence shown begins at the first consensus bases for the majority of *Mastadenovirus*, including group I bovine and human adenovirus, hexon gene alignments, which represents the beginning of the open reading frame for the hexon protein gene (Toogood and Hay, 1988). Primers generated to validate the 3'DNM assay are outlined below. All bp numbers refer to positions along the HAdV 41 hexon gene as submitted in GenBank (**accession number: D13781**). Note: the 3' dual nucleotide mismatch (3'DNM) primers 3'DNM1 and 3'DNM2b exactly overlap with the eventual HAdV fecal source tracking (FST) primers. Cloning primers C3 and C4 were used for generating "true" purified-PCR-product qPCR standards, while primers 3' on-purpose mismatch T1 and T2 (3'OPMM-T1 and 3'OPMM-T2) were used to introduce mismatched into the "mismatch template."

```
                                                                {3'OPMM-A1}
                                                          [3'OPMM-T1]
                                                          [GGCAG{GACGCC
   1 ATGGCCACCCCCTCGATGATGCCGCAATGGTCTTACATGCACATCGCCGGGCAG(GACGCC
                    C1                                              3'DNM1

     TCGGAGTATCTGTC}CCCGG]
  61 TCGGAGTATCTGAG)CCCGGGC<CTGGTGCAATTTGCCCGCGCCAC>CGATACGTACTTCAGC
                           <HAdV sense FST probe>

 121 CTGGGGAACAAGTTCAGAAATCCCACTGTGGCTCCGACCCACGATGTAACCACAGACAGG


                 {3'OPMM-A2}
           [3'OPMM-T2]
           [GACTG{CAGCTGCGATTCGTGCCAGT}CGACC]
 181 TCACAGCGACTG(ACGCTGCGATTCGTGCCAGT)CGACCGCGAGGACACCGCTTATTCTTAC
                  3'DNM2                                          C2

 241 AAAGTGCGCTTTACGCTGGCCGTGGGCGACAACCGGGTGTTGGACATGGCCAGCACCTAC


 301 TTTGACATCCGCGGCGTGCTGGATCGTGGCCCCAGCTTTAAACCCTACTCCGGAACCGCC
                             (3'DNM exercised by BAdV group I RVS primer)
```

C1: Final left cloning primer (233-250): 5'-ATG ATGCCGCAATGGTCT-3'
C2: Final right cloning primer (473-454): 5'-GCGTAAAGCGCACTTTGTAA-3'

(3'DNM1): Left HAdV 41 3' mismatch primer (272-291): 5'-GACGCCTCGGAGTATCTG**AG**-3'
(3'DNM2): Right HAdV 41 3' mismatch primer (427-410):5'-TGGCACGAATCGCAGC**GT**-3'

{3'OPMM-T1}: Left HAdV 41 3' on-purpose mismatch template primer:
5'-GGCAGGACGCCTCGGAGTATCTG**TC**CCCGG-3'
[3'OPMM-A1]: Left HAdV 41 3' on-purpose mismatch amplification primer:
5'-GACGCCTCGGAGTATCTG**TC**-3'
{3'OPMM-T2}: Right HAdV 41 3' on-purpose mismatch template primer:
5'-GGTCGACTGGCACGAATCGCAGC**TG**CAGTC-3'
[3'OPMM-A2]: Right HAdV 41 3' on-purpose mismatch amplification primer:
5'-ACTGGCACGAATCGCAGC**TG**-3'

<HAdV sense FST probe>: FAM-5′-CTGGTGCA**I**TT(T/C)GCCCG(C/T)GCCAC-3′-BHQ

Sequence translation:
Forward Frame 1: http://www.vivo.colostate.edu/molkit/translate/

```
  1   M  A  T  P  S  M  M  P  Q  W  S  Y  M  H  I  A  G  Q  D  A
  1   ATGGCCACCCCCTCGATGATGCCGCAATGGTCTTACATGCACATCGCCGGGCAGGACGCC

 21   S  E  Y  L  S  P  G  L  V  Q  F  A  R  A  T  D  T  Y  F  S
 61   TCGGAGTATCTGAGCCCGGGCCTGGTGCAATTTGCCCGCGCCACCGATACGTACTTCAGC

 41   L  G  N  K  F  R  N  P  T  V  A  P  T  H  D  V  T  T  D  R
121   CTGGGGAACAAGTTCAGAAATCCCACTGTGGCTCCGACCCACGATGTAACCACAGACAGG

 61   S  Q  R  L  T  L  R  F  V  P  V  D  R  E  D  T  A  Y  S  Y
181   TCACAGCGACTGACGCTGCGATTCGTGCCAGTCGACCGCGAGGACACCGCTTATTCTTAC

 81   K  V  R  F  T  L  A  V  G  D  N  R  V  L  D  M  A  S  T  Y
241   AAAGTGCGCTTTACGCTGGCCGTGGGCGACAACCGGGTGTTGGACATGGCCAGCACCTAC

101   F  D  I  R  G  V  L  D  R  G  P  S  F  K  P  Y  S  G  T  A
301   TTTGACATCCGCGGCGTGCTGGATCGTGGCCCCAGCTTTAAACCCTACTCCGGAACCGCC

121   Y  N  S  L  A  P  K  T  A  P  N  P  C  E  W
361   TATAACTCCCTGGCTCCTAAAACAGCACCCAATCCATGCGAATGG
```

BAdV 1 primer map and Hexon gene sequence translation: The sequence shown begins at the first consensus bases for the majority of *Mastadenovirus*, including group I bovine and human adenovirus, hexon gene alignments, which represents the beginning of the open reading frame for the hexon protein gene (Toogood and Hay, 1988). Primers generated to validate the 3'DNM assay are outlined below. All bp numbers refer to positions along the BAdV 1 hexon gene (accession number: DQ630761). Note: the 3' dual nucleotide mismatch (3'DNM) primers 3'DNM1 and 3'DNM2b exactly overlap with the eventual BAdV fecal source tracking (FST) primers. Cloning primers C1 and C2 were used for generating and "true" purified-PCR-product qPCR standards, while primers 3' on-purpose mismatch T1 and T2 (3'OPMM-T1 and 3'OPMM-T2) were used to introduce mismatched into the "mismatch template."

```
                                                                {3'OPMM-A1}
                                                          [3'OPMM-T1]
                     <C1>                                 [GGCAG{GATGCC
   1 ATGGCGACGCCGTC<GATGATGCCCCAGTGGTC>GTACATGCACATCGCCGGGCAG(GATGCC
                                                            3'DNM1

     TCAGAGTACCTGAG}TCCCG]
  61 TCAGAGTACCTGTC)TCCCGGCCTGGTGCAGTTCGCGCAGGCCACAGAGACCTACTTTAAG

 121 CTGGGTAACAAGTTTAGAAACCCCACTGTGGCTCCAACGCATGACGTCACCACAGAGCGG

 181 TCACAGCGGCTG(CAGCTGCGATTTGTTCCAGT)TGACCGTGAAGACACGCAGTACACTCAC
                  3'DNM2a

 241 AAGACCAGATTTCAGTTGGCTGTGGGCGACAACCGAGTACTTGACATGGCGAGCACTTAC

                                                        {3'OPMM-A2}
                                                   [3'OPMM-T2]
                                                   [CATAC{TCCGGCACGGCA
 301 TTTGACATCCGCGGTACTTTGGACAGAGGTCCAAGCTTTAAGCCATAC(AGCGGCACGGCA
                                                     3'DNM2b

     TACAAC}GCTCT
 361 TACAAC)GCTCTAGCCCCTAAGGGGTCTATCAAT<AACACTTTCGTATCCGTGGC>TGGAAAC
                                      <C2>

 421 AACAACGCCAAAGCT
```

C1: Final left cloning primer (15-32): 5'-CGTCGATGATGCCCCAGT-3'
C2: Final right cloning primer (413-394): 5'-GCCACGGATACGAAAGTGTT-3'

(3'DNM1): Left BAdV1 3' mismatch test primer (55-74):
5'-GAT GCC TCA GAG TAC CTG **TC**-3'
(3'DNM2a): BAdV 1 3' mismatch test primer Right option A (212-193):
5'-ACT GGA ACA AAT CGC AGC **TG**-3'
(3'DNM2b): BAdV 1 3' mismatch test primer (349-366) Right option B:
5'-GTT GTA TGC CGT GCC G**CT**-3'

{3'OPMM-T1}: Left BAdV 1 3' on-purpose mismatch template primer:
5'-GGCAGGATGCCTCAGAGTACCTG**AG**TCCCG-3'
[3'OPMM-A1]: Left BAdV 1 3' on-purpose mismatch amplification primer:
5'-GATGCCTCAGAGTACCTG**AG**-3'
{3'OPMM-T2}: Right BAdV 1 3' on-purpose mismatch template primer:

5′-AGAGCGTTGTATGCCGTGCCG**GA**GTATG-3′

[3′OPMM-A2]: Right BAdV 1 3′ on-purpose mismatch amplification primer:

5′-GTTGTATGCCGTGCCG**GA**-3′

Sequence translation:

Forward Frame 1: http://www.vivo.colostate.edu/molkit/translate/

```
   1  M  A  T  P  S  M  M  P  Q  W  S  Y  M  H  I  A  G  Q  D  A
   1  ATGGCGACGCCGTCGATGATGCCCCAGTGGTCGTACATGCACATCGCCGGGCAGGATGCC

  21  S  E  Y  L  S  P  G  L  V  Q  F  A  Q  A  T  E  T  Y  F  K
  61  TCAGAGTACCTGTCTCCCGGCCTGGTGCAGTTCGCGCAGGCCACAGAGACCTACTTTAAG

  41  L  G  N  K  F  R  N  P  T  V  A  P  T  H  D  V  T  T  E  R
 121  CTGGGTAACAAGTTTAGAAACCCCACTGTGGCTCCAACGCATGACGTCACCACAGAGCGG

  61  S  Q  R  L  Q  L  R  F  V  P  V  D  R  E  D  T  Q  Y  T  H
 181  TCACAGCGGCTGCAGCTGCGATTTGTTCCAGTTGACCGTGAAGACACGCAGTACACTCAC

  81  K  T  R  F  Q  L  A  V  G  D  N  R  V  L  D  M  A  S  T  Y
 241  AAGACCAGATTTCAGTTGGCTGTGGGCGACAACCGAGTACTTGACATGGCGAGCACTTAC

 101  F  D  I  R  G  T  L  D  R  G  P  S  F  K  P  Y  S  G  T  A
 301  TTTGACATCCGCGGTACTTTGGACAGAGGTCCAAGCTTTAAGCCATACAGCGGCACGGCA

 121  Y  N  A  L  A  P  K  G  S  I  N  N  T  F  V  S  V  A  G  N
 361  TACAACGCTCTAGCCCCTAAGGGGTCTATCAATAACACTTTCGTATCCGTGGCTGGAAAC

 141  N  N  A  K  A  F  A  Q  A  P  Q  S  A  T  V  D  G  T  T  G
 421  AACAACGCCAAAGCTTTTGCGCAAGCCCCTCAGTCGGCAACAGTAGACGGAACTACGGGC
```

Table C1: Codon table.

	T	C	A	G
T	TTT Phe F	**----TCT Ser S----**	TAT Tyr Y	TGT Cys C
	TTC Phe F	**----TCC Ser S----**	TAC Tyr Y	TGC Cys C
	TTA Leu L	**----TCA Ser S----**	TAA Ochre (*Stop*)	TGA Opal (*Stop*)
	TTG Leu L	**----TCG Ser S----**	TAG Amber (*Stop*)	TGG Trp W
C	CTT Leu L	CCT Pro P	CAT His H	CGT Arg R
	CTC Leu L	CCC Pro P	CAC His H	CGC Arg R
	CTA Leu L	CCA Pro P	CAA Gln Q	CGA Arg R
	CTG Leu L	CCG Pro P	CAG Gln Q	CGG Arg R
A	ATT Ile I	ACT Thr T	AAT Asn N	**----AGT Ser S----**
	ATC Ile I	ACC Thr T	AAC Asn N	**----AGC Ser S----**
	ATA Ile I	ACA Thr T	AAA Lys K	AGA Arg R
	ATG Met M (*start*)	ACG Thr T	AAG Lys K	AGG Arg R
G	GTT Val V	GCT Ala A	GAT Asp D	GGT Gly G
	GTC Val V	GCC Ala A	GAC Asp D	GGC Gly G
	GTA Val V	GCA Ala A	GAA Glu E	GGA Gly G
	GTG Val V	GCG Ala A	GAG Glu E	GGG Gly G

Appendix D: Fecal Source Tracking primers and TaqMan probes designed for the specific amplification of HAdV, group I BAdV and group II BAdV. Anchor primers designed to amplify all HAdV and/or BAdV (for use when targeting source samples). 'Fluor" indicates the position of a user-defined fluorescent molecule; 'BHQ,' black hole quencher. I, inosine. An asterix has been placed next to the TaqMan probes that were labeled with HEX (and, thus, did not generate an amplification signal). These probes must re-made and reevaluated.

BAdV group I FST primers: Group I BAdV share the same RVS primer, but required different FWD primers based on greater similarity between particular BAdV with other livestock AdV than between all the group I BAdV.
BAdV2/OAdV2-5 FWD 3' DNM primer: 5'-GATGCCTCCGAGTATCTCTC-3'
BAdV 1, 3 and PAdV 3&5 FWD 3' DNM primer: 5'-GATGC(C/G)TC(C/A)GAGTACCTGTC-3'
BAdV 1, 3 & 10; PAdV 3&5 FWD 3' DNM primer: GATGC(C/G/T)TCCGAGTAC(C/T)TGTC
BAdV RVS 3' DNM primer (BAdV 1, 2, 3 10): 5'- GTTGTAIGC(G/T/C)GTGCCGCT-3'

BAdV group I FST probe:
***BAdV group I FST antisense probe (BAdV 1, 2 and 3)**:
Fluor-5'-CG(A/G)ATGTCAAAGTAIGTGCT(G/C)GCCATGTCCA-3'-BHQ

HAdV FST primers: HAdV are divided into subgroups based on genetic similarity; these subgroups were aggregated when possible to facilitate the amplification of as many HAdV as possible with a single primer.
HAdV FST FWD primer (species A, B, C, D, E, F): 5'-ACGC(C/T)TCGGAGTA(T/C)CTGAG-3'
HAdV species A,D,E,F RVS 3' DNM primer: 5'-IGGCAC(G/A)AAICGCAGCGT-3'
HAdV species C RVS 3' DNM primer: 5'-AGGGATGAACCGCAGCGT-3'
HAdV species B RVS 3' DNM primer: 5'-GGGCACGAAGCGCA(A/G)CAT-3'

HAdV FST probes:
HAdV sense FST probe: Fluor-5'-CTGGTGCAITT(T/C)GCCCG(C/T)GCCAC-3'-BHQ
***HAdV 40/41 antisense FST probe:** Fluor-5'-TCGCTG(C/T)GACCTGTCTGTGGTTACATC-3'-BHQ

Anchor primers: Anchor primers are designed to amplify all HAdV or group I BAdV in source materials. These sets share the same FWD primer.
FWD Anchor primer: 5'-CAITGG(T/G)CITACATGCACATC-3'
RVS HAdV anchor primer: 5'-ACIGTGGGITT(C/T)CT(G/A)AACTTGTT-3'
RVS BAdV group I anchor primer: 5'- ACIGT(C/T/G)GGGTTTCTAAA(C/T)TTGTT-3'

MAdV Primers
FWD: 5'-GGCCAACACTACCGACACTTG-3'
RVS: 5'-TTGTCCTGTGGCATTTGA-3'

MAdV Probes:
***MAdV Probe A**: Fluor-5'- CGCCAATGTGGCTCAGTATATGCCGG -3'–BHQ
***MAdV Probe B**: Fluor-5'-GGAAAGGGAAACATGGCTGCCATGG-3'–BHQ

BAdV group II Anchor primers:
Atadenovirus FWD Anchor: 5'-CACAT(T/C)GC(G/T)GGTAGAAATGC-3'
Atadenovirus RVS Anchor A: 5'-CCATGG(A/C/T)AC(G/A)CTIGAATCC-3'
Atadenovirus RVS Anchor B: 5'-GCTTGATTATAA(T/C)TIGC(T/A)GCCATTTG-3'

BAdV group II FST primers:
BAdV group II FST FWD: 5'-G(A/G)AATGCTAC(T/A)AATGATC-3'
BAdV group II FST RVS A: 5'-GCTTT**I**A(A/C)TCT(A/G)TTAAA(A/G)CTCC-3'
BAdV group II FST RVS B: 5'-CCATGG(C/T)AC**I**CT(A/T)GAATCC-3'

BAdV group II probes:
FST Probe BAdV 4, 5 & 8: 5'-TTTGC(A/T)GACTATTTGGGAGCTGTTAA(C/T)AAT-3'
FST Probe BAdV 6 & 7: 5'-TTTGC(A/T)GATTACTTAGG(A/T)GCAGT**I**AATAATCTT-3'

Deer AdV FWD primer:
FST deer AdV FWD: 5'GAAATGCCACCAATGATC-3 (OdAdV)

Deer AdV probe:
Deer AdV Probe: Fluor-5'-TTTGCTGATTTCCTTGGCGCTGTAAATAAT-3'