The critical assessment of bovine adenoviruses (BAdV) as indicators of environmental fecal contamination requires improved knowledge of their prevalence, shedding dynamics, and genetic diversity. We examined DNA extracted from bovine and other animal waste samples collected in Wisconsin for atadenoviruses and mastadenoviruses using novel, broad-spectrum PCR primer sets. BAdV were detected in 13% of cattle fecal samples, 90% of cattle urine samples, and 100% of cattle manure samples; 44 percent of BAdV-positive samples contained both Atadenovirus and Mastadenovirus DNA. Additionally, BAdV were detected in soil, runoff water from a cattle feedlot, and residential well water. Overall, we detected 8 of 11 prototype BAdV, plus bovine, rabbit, and porcine mastadenoviruses that diverged significantly from previously reported genotypes. The prevalence of BAdV shedding by cattle supports targeting AdV broadly as indicators of the presence of fecal contamination in aquatic environments. Conversely, several factors complicate the use of AdV for fecal source attribution. Animal AdV infecting a given livestock host were not monophyletic, recombination among livestock mastadenoviruses was detected, and the genetic diversity of animal AdV is still underreported. These caveats highlight the need for continuing genetic surveillance for animal AdV and for supporting data when BAdV detection is invoked for fecal source attribution in environmental samples. To our knowledge, this is the first study to report natural BAdV excretion in urine, BAdV detection in groundwater, and recombination in AdV of livestock origin.

Adenoviruses (AdV) infecting humans (HAdV) and cattle (BAdV) have been proposed as library- and culture-independent source indicators of environmental fecal contamination (16, 17, 26). These double-stranded DNA viruses are hypothesized to have evolved with their named hosts (13). Accordingly, natural infections are thought to be limited to single or closely related host species (46), although evidence exists for broader host ranges for some AdV (18, 21). The family Adenoviridae is divided into four genera, including Mastadenovirus (MaAdV) and Atadenovirus (AtAdV). The genus Mastadenovirus comprises BAdV type 1 (BAdV-1), -2, -3, -9, and -10 (historically classified as group I BAdV) and a large variety of mammalian AdV, including all 52 accepted human AdV (6, 20). The genus Atadenovirus includes BAdV-4 through -8 and -10 (historically, group II), as well as AdV infecting ducks, goats, sheep, deer, and reptiles (6, 24). With occasional exceptions (1, 23, 36), BAdV infections are largely asymptomatic (23, 33).

The wide genetic diversity among known BAdV and imperfect knowledge of the host specificity of different BAdV genotypes preclude the design of a single fecal-source-tracking (FST) PCR assay that amplifies all BAdV while confidently excluding all AdV infecting noncattle hosts. In lieu of an ideal assay, the use of narrowly focused assays, each targeting a

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MATERIALS AND METHODS

**Virus stock preparation.** Enteric human HAdV-41 (ATCC VR-930) and bovine BAdV-1 (ATCC VR-313) and BAdV-2 (ATCC VR-314) were cultured in human embryonic kidney 293A cells (ATCC CRL-1573) and Madin-Darby bovine kidney cells (ATCC CCL-22), respectively. Cell monolayers demonstrating >75% cytopathic effect were lysed (3 times by freeze-thaw), and virus stocks were prepared from clarified cell culture supernatants.
Oligonucleotide design. Representative HAdV (i.e., drawn from all six viral species) and all unique animal MaAdV and AtAdV hexon gene sequences available as of January 2010 were obtained from GenBank and aligned by genus using ClustalW, executed in BioEdit (version 7.0.9.0) (12). Genus-specific, broad-spectrum primers were designed to overlap areas of strong nucleic acid identity among livestock AtAdV or MaAdV sequences and to amplify a range of known and novel AdV (Table 1). When configuring broad-spectrum primers, consensus/degenerate base composition was chosen to favor low primer degeneracy and amplification of livestock targets over expanded inclusiveness; accordingly, all primers were configured with degeneracy of ≤8. Oligonucleotide melting temperatures for perfect and mismatched annealing with potential targets were predicted in silico using the MelTcAle spreadsheet (34); oligonucleotides were screened for dimer and hairpin formation using AutoDimer (41).

Primers were tested empirically for their ability to generate amplicons of the expected sizes from (i) DNA extracts from HAdV-41, BAdV-1, and BAdV-2, (ii) purified plasmid DNA from BAdV-4, -6, -7, and -8 and Odoloculis (genus of North American deer) AdV (OdAdV) (kindly provided by H. Lehmkuhl), and (iii) DNA extracts from nonbovine animal fecal samples and HAdV-positive wastewater treatment plant influent samples. Detection limits for AtAdV and MaAdV seminested PCR assays were determined by spiking composite BAdV-negative fecal DNA extracts (~100 ng DNA per PCR) with serial dilutions of BAdV-1 DNA and AtAdV plasmid DNA, quantified fluorometrically (Qubit system; Invitrogen). The expected target ranges of primer pairs were determined in silico using the Specificity Check feature of Primer-BLAST (National Center for Biotechnology Information) and are available upon request.

Sample collection. Samples of dairy and beef cattle (Bos primigenius taurus) feces (n = 32) and urine (n = 21) were acquired from individual animals between August 2008 and January 2010; 11 dairy cows provided paired fecal and urine samples. Six additional manure samples (i.e., mixed wastes, including feces and urine, from multiple animals) from four separate locations were obtained: three bedding samples (moist, soiled hay), two liquid manure lagoon samples, and a sand-separated, dewatered manure sample. Overall, cattle excreta samples between August 2008 and January 2010; 11 dairy cows provided paired fecal and urine samples. These and additional animal excreta and environmental samples that were available for comparison were examined (Table 2). All samples were collected in sterile containers, transported on ice, and stored for <1 week at 4°C or at ~80°C until analysis (except for one liquid manure sample, which was held at 4°C for approximately 1 year until analysis). Samples were acquired opportunistically (i.e., based on convenience and on need during hypothesis testing) and present a picture of viral DNA sequences as expected in a mix of livestock and nonlivestock species (except for MaAdF2 (8), which was employed at 1,600 nM total. For example, flanking primers MaAdF1 (D = 3) and MaAdR1 (D = 2) were used at 900 and 600 nM, respectively. Acetylated bovine serum albumin (Promega, Inc.) was included in in silico PCR reactions at a final concentration of 0.1 μg·μl⁻¹. Identical TD-PCR programs were used for both rounds of seminested amplification as follows: 94°C for 4 min, followed by 10 cycles of 94°C for 30 s, 65°C for 30 s (with a decrement of 1°C per cycle), and 72°C for 1 min. An additional 30 cycles were completed as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, finishing with a 72°C, 7-min extension. All PCR mixtures were prepared in a cooling block (4°C) before placement in the preheated (94°C) Eppendorf Mastercycler thermocycler. All bovine fecal DNA extracts were screened for inhibition by attempted amplification of a 10-fold dilution. PCR products were detected under UV light after agarose gel electrophoresis (2%) and ethidium bromide staining. Oligonucleotides were synthesized by the UW Biotechnology Center.

Cloning and sequencing. AdV seminested PCR amplicons at or near the expected target size were cloned using TOPO TA kits (Invitrogen) and sequenced using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems), M13F or M13R primers, and a magnetic bead clean-up (CleanSEQ; Agencourt Bioscience) at the UW Biotechnology Center. Prior to cloning, MaAdV amplicons were purified (Wizard SV gel and PCR clean-up; Promega, Inc.) from excised gel slices; AtAdV amplicons were cloned directly from each transformation reaction sequence were sequenced.

Phylogenetic analysis and recombination detection. Translated hexon amino acid sequences for six divergent MaAdV (detected during this study; ≤95% nucleotide identity with known AdV) and 38 representative MaAdV were aligned using ClustalW, back translated, and cropped to 749 bp (aligned). Multiple alignment gap penalties of 3 (Opening) and 1.8 (Extension) were employed (11), and the resulting alignment was inspected visually for obvious misalignment errors. A Bayesian phylogenetic tree was generated using MrBayes, version 3.1.2 (15). The analysis was run for 600,000 generations (nchains = 4 and temperature = 0.2) using the General-Time-Reversible model (rates = invgamma). One tree was cached every 100 generations, and posterior probabilities were determined from the final 75% of the stored trees. The resulting majority rule consensus tree was reconstructed using MEGA 4.0.2 (37).

Novel BAdV and selected known MaAdV partial and near-complete hexon gene sequences were screened using the Recombination Detection Program, version 3.44 (RDP3) (28). Default model parameters were used except as follows: (i) sequences were identified as linear, and recombination events were listed if detected by more than two methods (General options); (ii) internal and external reference sequences were considered (RDP3 options); and (iii) bootstrap cutoff and replicate values were increased to 95% and 500, respectively (Bootscan algorithm). We calibrated the program by detecting a previously reported recombination event involving HAdV-3, -4, and -16 (7); HAdV-16 was confirmed as a recombination with multiple-comparison-corrected (“global”) P
TABLE 2. Evaluation of samples for MaAdV and AtAdV by broad-spectrum seminested touchdown PCR

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample information and age of animal(s)</th>
<th>No. of samples with AdV/total no. of samples</th>
<th>Novel or recognized AdV (% identity to recognized AdV)</th>
<th>No. of samples with AdV/total no. of samples</th>
<th>Novel or recognized AdV (% identity to recognized AdV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine feces</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy calf</td>
<td>AARS-DCU; &lt;14 wk</td>
<td>0/9</td>
<td>MaAdV-Wl, MaAdV-Wl</td>
<td>0/9</td>
<td>MaAdV-Wl, MaAdV-Wl</td>
</tr>
<tr>
<td>Adult¹</td>
<td>UW Dairy and AARS-BNC; 1–10 yr</td>
<td>4/23</td>
<td>MaAdV-Wl, MaAdV-Wl</td>
<td>0/23</td>
<td>MaAdV-Wl, MaAdV-Wl</td>
</tr>
<tr>
<td>Bovine urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef cow</td>
<td>AARS-BNC; −15 mo</td>
<td>2/3</td>
<td>MaAdV-10 (≥97)</td>
<td>3/3</td>
<td>MaAdV-4 (99), MaAdV-6 (98)</td>
</tr>
<tr>
<td>Dairy cow</td>
<td>UW Dairy (n = 17) and farm A (n = 1); 2.5–9 yr</td>
<td>13/18</td>
<td>MaAdV-10 (≥97)</td>
<td>18/11</td>
<td>MaAdV-4 (99), MaAdV-6 (98)</td>
</tr>
<tr>
<td>Bovine manure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy lagoon slurry</td>
<td>Farms B and C</td>
<td>2/2</td>
<td>MaAdV-1 (99), MaAdV-10 (98)</td>
<td>2/2</td>
<td>BAdV-4 (98)</td>
</tr>
<tr>
<td>Dairy manure</td>
<td>Farm D, sand separated</td>
<td>1/1</td>
<td>MaAdV-10 (98)</td>
<td>1/1</td>
<td>BAdV-6 (98), BAdV-8 (98)</td>
</tr>
<tr>
<td>Beef cattle bedding</td>
<td>AARS-BNC, moist, soiled hay from three separate pens; −15 mo</td>
<td>3/3</td>
<td>MaAdV-10 (99), MaAdV-Wlc, MaAdV-2 (99), MaAdV-Wl</td>
<td>3/3</td>
<td>MaAdV-4 (99), MaAdV-5 (97), BAdV-7 (99)</td>
</tr>
<tr>
<td>Bovine influenced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field mud⁵</td>
<td>Water station, farm E</td>
<td>0/2</td>
<td></td>
<td>2/2</td>
<td>BAdV-8 (99)</td>
</tr>
<tr>
<td>Field runnel⁶</td>
<td>Drainage ditch, farm E</td>
<td>1/1</td>
<td>BAdV-2 (98)</td>
<td>1/1</td>
<td>BAdV-5 (99), BAdV-8 (99)</td>
</tr>
<tr>
<td>Groundwater⁷</td>
<td>Private well (600 ml)</td>
<td>1/1</td>
<td>BAdV-10 (99)</td>
<td>1/1</td>
<td>BAdV-6 (99)</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human sewage⁸</td>
<td>NSWTF, 24-h composites</td>
<td>5/5</td>
<td>HAdV-31 (99–100), HAdV-41-WI (96)</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Pig feces</td>
<td>AARS-P, 1 sow and 1 piglet</td>
<td>0/2</td>
<td></td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Pig wash water⁴</td>
<td>AARS-P, newborn to finished</td>
<td>1/1</td>
<td>OAdV-6 (64)⁵</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Deer feces</td>
<td>From separate animals</td>
<td>0/4</td>
<td></td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Rabbit feces</td>
<td>One fecal pellet</td>
<td>1/1</td>
<td>OAdV-3, OAdV-5, OAdV-2 (71)</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>Dog feces</td>
<td>3–4 yr</td>
<td>0/2</td>
<td></td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Goose feces</td>
<td>10-scat composite</td>
<td>0/1</td>
<td></td>
<td>0/1</td>
<td></td>
</tr>
</tbody>
</table>

a Abbreviations: AARS, Arlington Agricultural Research Station (WI); DCU, Dairy Calf Unit; BNC, Beef Nutrition Center; P, Porcine Research Center; UW Dairy, University of Wisconsin—Madison Dairy; NSWTF, Nine Springs Wastewater Treatment Facility (Madison, WI).

b Percent nucleotide identity with previously identified AdV.

c Bovine cows (n = 5), Arlington Agricultural Research Station, AARS; dairy cows (n = 18), UW Dairy.

d Nucleotide sequence not recognized (BLASTn); % identity provided for protein sequence (BLASTx).

e Collected approximately 3 and 6 m from a water station.

f Collected from a muddy pool adjacent to a large cattle lot.

g Collected March 2010 near Eau Claire, WI.
h Collected May, June, and October 2009 and January and February 2010. HAdV-31 was detected once, and HAdV-41-WI was detected in all samples.
i Pen wash water recirculated throughout the facility, sampling newborn to finished swine.

RESULTS

Development, specificity, and sensitivity of PCR assays. We designed novel, broad-spectrum primer sets (Table 1) targeting the 5′ region of the AdV hexon gene; AtAdV primers amplify most of conserved region one (C1), while MaAdV primers amplify most of C1 and all of variable region one (V1) (7). Amplification of plasmid DNA (BAdV-4, -6, -7, and -8 and OdAdV) and genomic DNA extracts (BAdV-1, -2, and HAdV-2, -41) demonstrated the utility of the primer sets employed; in silico primer evaluation against NCBI GenBank databases (last consulted December 2010) supported amplification of the intended livestock targets and, for the MaAdV assay, some additional AdV, including several HAdV. The application of seminested TD-PCR reduced the occurrence of nonspecific amplification for excreta samples that were determined to be AdV positive. In these samples, AdV amplification was strong and clearly distinguished from AdV-negative samples by gel electrophoresis.

The sensitivities of the seminested AdV assays were determined against serial dilutions of BAdV-1 DNA, extracted from values of 2 × 10⁻⁹ and 8 × 10⁻¹² for hexon gene alignments of representative genotypes from (i) all six HAdV species and (ii) HAdV E and HAdV B, respectively. Accordingly, a proposed recombination event was considered statistically significant when the corresponding global P value was <10⁻⁸ and when Boot-scans and outgroup-rooted neighbor-joining trees (1,000 replicates) both provided clear support for incongruent phylogeny for regions within versus outside inferred breakpoints (4, 27, 48).

Nucleotide sequence accession numbers. Nucleotide sequences reported in this paper were deposited in GenBank under accession numbers JF690045 to JF699138.
TABLE 3. Summary of detection of recognized and novel BAdV genotypes by sample type

<table>
<thead>
<tr>
<th>Virusa</th>
<th>Manure</th>
<th>Bedding</th>
<th>Feces</th>
<th>Urine</th>
<th>Bovine influenced</th>
<th>Total no. of samples of indicated type with virus</th>
<th>No. of locations with virus detected (n = 9 locations)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAdV-1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BAdV-2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BAdV-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BAdV-10</td>
<td>2</td>
<td>1</td>
<td></td>
<td>15</td>
<td>1</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>BAdV-WIa</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>BAdV-WIb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BAdV-WIc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BAdV-4</td>
<td>2</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td></td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>BAdV-5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BAdV-6</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>BAdV-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BAdV-8</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong>a</td>
<td><strong>3</strong></td>
<td><strong>4</strong></td>
<td><strong>4</strong></td>
<td><strong>15</strong></td>
<td><strong>2</strong></td>
<td><strong>28</strong></td>
<td><strong>8</strong></td>
</tr>
</tbody>
</table>

a Novel BAdV genotypes detected in this study are designated with the suffix WI.
b Bovine or bovine-manure-influenced samples were acquired from nine separate locations in WI; no BAdV were detected at the AARS dairy calf unit.
c The AtAdV-positive urine sample, from farm A, was not sequenced; therefore, the detection frequency is underestimated (by one) for at least one of these BAdV.
d Total number of individual BAdV detected (by sample type) for MaAdV and AtAdV.

cell culture supernatant, or BAdV-5 and BAdV-8 plasmid DNA. DNA spiked into reaction mixtures with or without the inclusion of BAdV-negative fecal DNA extract (~100 ng DNA) was detectable to 10 to 100 genome equivalents (GE) per 5-μl sample in 50-μl reaction mixtures. This detection limit corresponds to DNA concentrations in excreta of between 10^3 and 10^4 GE g^-1.

AdV occurrence in source materials. We detected 8 of 11 prototype BAdV (~97% nucleotide identity) and three MaAdV that diverged from previously reported genotypes (Table 2). Divergent MaAdV sequences, designated BAdV-Wisconsin (WI) “a” through “c” (BAdV-WIa, -b, and -c), were detected in adult dairy cow feces (BAdV-WIa and BAdV-WIb) and beef cattle bedding (BAdV-WIa and BAdV-WIc). BAdV-WIa was detected in samples collected at different times and locations (separated by 11 months and 37 km). Based on protein identity (BLASTx), BAdV-WIa and BAdV-WIb were most similar to ovine AdV (OAdV) 1 (88% and 85%, respectively), while BAdV-WIc shared 93% identity with BAdV-10.

At least one BAdV was detected at six of seven locations from which bovine excreta were sampled. The overall prevalence of BAdV in feces was low; no BAdV were detected in fecal samples (9/21) collected from dairy calves, and only MaAdV (BAdV-WIa and BAdV-WIb) were detected in the remaining fecal samples (4/23) surveyed. No BAdV were amplified from 10-fold dilutions of BAdV-negative fecal DNA extracts. In contrast with the results for fecal samples, 90% (19/21) of cattle urine samples and 100% (6/6) of cattle manure samples (viz., one manure, two lagoon slurry, and three bedding samples) collected were positive for at least one BAdV.

All manure samples (n = 6) and 10 of the 21 urine samples were positive for both MaAdV and AtAdV. Focusing on the 11 cows from which paired urine and fecal samples were collected, five distinct BAdV were detected, two AtAdV (BAdV-4 and -6) and three MaAdV (BAdV-10, -Wla, and -Wlb). Nine animals shed at least one BAdV, five shed two BAdV (three cows through both urine and feces), and one shed three different BAdV (one via feces, BAdV-WIa, and two via urine, BAdV-10 and BAdV-4). Identical results were obtained for these 11 fecal samples regardless of whether DNA was extracted directly from feces or from clarified fecal suspensions. Overall, BAdV-10 and, together, BAdV-4 and -6 demonstrated similar levels of prevalence (~70%) in urine samples and were the most frequently detected BAdV across the sample types (Table 3). In particular, BAdV-10 was detected in 19 samples, across four different matrices, and from seven of the nine locations where excreta or environmental (see below) samples were collected. The BAdV concentrations in manure and feces, determined by serial dilution, ranged from 10^4 to 10^5 copies per gram.

Nonbovine excreta samples were evaluated to explore the extended utility of the broad-spectrum primer sets. No amplification was observed with AtAdV primer sets when nonbovine source samples were assayed. In contrast, MaAdV were identified in human sewage, swine facility pen wash water, and wild Eastern cottontail rabbit (Sylvilagus floridanus) feces. The porcine AdV (PAdV-WI) and rabbit AdV (RabAdV-WI) detected were substantially divergent from previously identified MaAdV; BLASTx queries revealed that the highest identity for PAdV-WI was with OAdV-6 (64%) (GenBank accession no. ABG22145) and for RabAdV-WI was with OAdV-A (71%) genotypes. A novel variant of HAdV-41 (HAdV-41-WI) was detected in human sewage, swine facility pen wash water, and wild Eastern cottontail rabbit (Sylvilagus floridanus) feces. The porcine AdV (PAdV-WI) and rabbit AdV (RabAdV-WI) detected were substantially divergent from previously identified MaAdV; BLASTx queries revealed that the highest identity for PAdV-WI was with OAdV-6 (64%) (GenBank accession no. ABG22145) and for RabAdV-WI was with OAdV-A (71%) genotypes. A novel variant of HAdV-41 (HAdV-41-WI) was detected in all sewage samples analyzed; this virus shared 96% nucleotide and amino acid identity with HAdV-41 Tak (GenBank accession no. DQ315564). HAdV-31 (99 to 100% identity; GenBank accession no. DQ149611) was also detected in one sewage sample (October 2009).

Detection of AdV in environmental samples. Four environmental samples with a suspected bovine manure impact were examined (Tables 2 and 3): BAdV-6 and -10 were amplified from an Escherichia coli-positive, 600-ml residential well water sample submitted to the Wisconsin State Laboratory of Hy-
giene for bacterial source tracking; BAdV-2, -5, and -8 were detected in muddy, pooled runoff from a large, poorly vegetated dairy lot; and BAdV-8 was detected at the same farm in each of two soil samples collected near a cattle drinking water station. Viruses detected in environmental samples had ≥98% identity with prototype BAdV strains.

**Phylogenetic analysis.** Bayesian phylogenetic analysis of broad-spectrum MaAdV PCR amplicons (Fig. 1) confidently discriminated HAdV from AdV infecting nonhuman animals, including livestock. The phylogenies were similar to those constructed previously from nearly complete livestock and human MaAdV hexon gene sequences (7, 13, 24). BAdV-WIa and -Wib fell within a clade of livestock MaAdV (Fig. 1, clade L) comprised of PAdV-5, goat AdV 2 (CapAdV-2), OAdV-1, BAdV-1, and OAdV-A genotypes, while BAdV-Wlc was a sister taxon to BAdV-10. The AtAdV sequences detected here were highly similar to previously described genotypes; the phylogeny of AtAdV based on hexon gene sequences has been reported previously (24).

**Recombination detection.** To determine whether recombination may have influenced our phylogenetic analyses, we examined AdV hexon DNA sequence alignments using RDP3 (28). No significant recombination events were revealed for the novel BAdV identified here. However, a significant event involving OAdV-4, PAdV-5, and OAdV-3 was detected within our broad-spectrum amplicon (global P value, 6 × 10^-9). The parent/recombinant relationship of this event was uncertain.

Agent host specificity is a prerequisite for targeting in microbial-source tracking; we therefore also searched for signs of recombination in available near-complete hexon gene sequences for clade L livestock MaAdV and among closely related ruminant AtAdV (BAdV-4 through -8, OdAdV-1, GAdV-1, and OAdV-7). No recombination events were detected among the AtAdV examined. Conversely, strongly supported events involving OAdV-A viruses, BAdV-2, OAdV-5, and OAdV-3 (global P value, 2 × 10^-20) and OAdV-2, -4, and -5 (global P value, 7 × 10^-12) were revealed. The parent/recombinant relationships were uncertain, although BAdV-2 demonstrated the most apparent incongruent phylogeny in the first event. To our knowledge, evidence for natural recombination among livestock AdV has not previously been reported.

**FIG. 1.** Bayesian phylogenetic analysis (MrBayes version 3.1.2) (15) of existing and novel selected partial Mastadenovirus hexon gene nucleotide sequences (749 bp, aligned) discriminated AdV infecting humans and livestock to separate evolutionary clades. Novel genotypes identified in the current study are designated with the suffix WI (Wisconsin) and a star; a clade of closely related livestock-infecting AdV (clade L) is indicated by a vertical dashed line adjacent to the tree. Posterior probabilities (as percentages) are provided for each clade. The scale bar for branch length indicates the number of nucleotide changes per site. GenBank accession numbers for the viruses considered are as follows: bovine AdV-WIa (BAdV-WIa), JF690902; BAdV-Wib, JF699102; BAdV-Wlc, JF690904; PaAdV-WI, JF699045; RabAdV-WI, JF699046; HaAdV-41-WI, JF699084; BAdV-1, DQ630761; BAdV-2, DQ630762; BAdV-3, NC_001876; BAdV-10, AF282774; goat AdV 2 (CapAdV-2), DQ630766; cow AdV-1 (OAdV-1), DQ630754; OAdV-2, DQ630755; OAdV-3, DQ630756; OAdV-4, DQ630757; OAdV-5, DQ630758; OAdV-6, DQ630759; porcine AdV-3 (PAdV-3), AB026117; PAdV-WI, NC_002702; Alpaca AdV (AlpAdV), GQ499375; canine AdV-1 (CAdV-1), CAU55011; CAdV-2, AC_000002; equine AdV-1 (EqAdV-1), L79955; EqAdV-2, L80007; human AdV-1 (HaAdV-1), AB330082; HaAdV-2, NC_001405; HaAdV-3, CAAS4501; HaAdV-4, AP635606; HaAdV-5, AC_000008; HaAdV-9, AB330090; HaAdV-12, X73487; HaAdV-13, AB330094; HaAdV-14, DQ149612; HaAdV-18, DQ149610; HaAdV-19, DQ149618; HaAdV-22, DQ149620; HaAdV-24, DQ149622; HaAdV-31, DQ149611; HaAdV-40, L19443; HaAdV-41, X51783; simian AdV-7 (SAdV-7), DQ792570; SAdV-22, AYS30876; SAdV-25, AF394196; white sturgeon AdV (WsAdV), AJ495768.
**DISCUSSION**

**BAdV prevalence and shedding.** The critical assessment of BAdV, broadly and by genotype, as indicators of environmental contamination from cattle manure requires improved knowledge of their prevalence, shedding dynamics, and genetic diversity. Previously published PCR assays targeting BAdV suffer from poor sensitivity, limited breadth of detection (43, 45), or both (26), restricting their usefulness for broadly evaluating BAdV as indicator organisms. Here, the examination of bovine excreta samples using novel, broad-spectrum PCR primer sets revealed the shedding of most prototype BAdV (except BAdV-3, -9, and -Rus) and three divergent MaAdV by cattle in Wisconsin, significantly expanding the genetic diversity of BAdV detected to date by PCR (16, 26, 44, 45). Our finding that cattle populations commonly shed multiple BAdV augments extensive serologic data showing the widespread, asymptomatic exposure of cattle (i.e., presence of antibodies) to most BAdV types (e.g., see references 9, 10, and 22). In particular, our results are consonant with those of Lehmkhul et al. (22), who demonstrated the exposure of cattle from four Tennessee farms to BAdV serotypes 1 to 8 and 10, with the highest seroprevalences (>82%) observed for AtAdV and BAdV-10.

The detection of both recognized and novel BAdV at multiple sampling locations is not surprising; activities associated with livestock transport, commerce (e.g., passage through sale yards), and exhibition provide numerous routes for the exchange of microorganisms among cattle populations (9, 29, 38). However, cross-exposure of populations need not be invoked to explain our results. The beef and dairy cattle populations sampled here do not commingle, and yet, BAdV-4, -6, and -10 and PAdV-WIa were detected in both populations, suggesting some consistency in the profile of BAdV shed by cattle examined here and elsewhere (16, 26, 44).

BAdV fecal shedding frequencies determined using PCR have ranged widely, from 0% to 30% for samples collected from single animals (2, 43, 45; the current study) and from 50% to 75% for samples pooled from multiple animals (17, 26). Factors contributing to this variability may include the history and management practices of the cattle populations examined (35), the timing/season of sample collection, and the sensitivity of the detection strategy employed. Our finding of BAdV-4, -6, and -10 in urine was surprising and may partly explain the reported disparity between BAdV prevalence in fecal versus manure and environmental samples (2, 16, 44, 45). Urinary shedding of MaAdV has been observed in dogs (46), humans (8), and colostrum-deprived calves experimentally infected with OAdV-A viruses (3). To our knowledge, natural urinary shedding of BAdV has not previously been reported.

Our results demonstrate parallel, apparently asymptomatic infection of herds and individual animals with multiple BAdV. The significance of BAdV (individually and collectively) for livestock health remains unclear (10, 22, 36). BAdV-10, the most frequently detected BAdV in our study, is also the BAdV most closely associated with a well-defined clinical condition, enteric vascular disease (23, 36). BAdV-10 has been isolated worldwide from cattle with fatal cases of enteritis exhibiting this disease (1, 14, 23, 36). However, the widespread detection of BAdV-10 antibodies (1, 14, 23, 36) and the unexpected prevalence of silent BAdV-10 shedding (reported here) suggest that BAdV-10 infection rarely causes clinical or life-threatening disease.

**Novel MaAdV classification.** The primers developed here detected five novel MaAdV genotypes, exposing a small portion of the undocumented animal AdV genetic variability suggested by existing serological and restriction analysis data (e.g., see references 3, 18, 33, 40, and 46). Phylogenetic analyses support the provisional classification of BAdV-WIc as a *Bovine adenovirus C* (with BAdV-10) and both BAdV-WIa and -WIb as members of *Bovine adenovirus A* (with BAdV-1). Based on their genetic divergence from extant sequences, PAdV-WI and RabAdV-WI are confidently proposed as prototype members of two new *Mastadenoviridus* species. PAdV have been detected previously in porcine wastes (16, 17, 26); the relationship between PAdV-WI and PAdV-C, the only recognized PAdV species with no published hexon gene sequences, remains to be clarified. A RabAdV had been previously isolated but not sequenced (33).

**Suitability of BAdV as indicators of environmental fecal contamination.** The efficacy of any microbial target to accurately indicate the presence or source(s) of environmental contamination by animal excreta rests fundamentally on the microorganism’s host specificity, shedding dynamics, and phylogenetic relationship to other organisms—factors that are informed by sequence data obtained from potential host sources of contamination (e.g., see reference 35). Like most microbial indicators, AdV are imperfect targets, with benefits and drawbacks that are important to understand.

The prevalent shedding of multiple BAdV genotypes by cattle and the documented environmental stability of the AdV virion/genome (30) support targeting AdV broadly as conservative indicators of the presence of viral or fecal contamination in aqueous environments. Large-scale fecal contamination from livestock (and humans) is expected to originate in most instances from urine and feces that are aggregated naturally or during waste management, so that the environmental detection of AdV excreted in urine will likely still be protective. Our detection of HAdV in sewage, BAdV in surface water, groundwater, soil, and manure, and novel MaAdV from multiple host species supports the utility of our broad-spectrum PCR assays for detecting AdV and highlighting the presence (and, with DNA sequencing and phylogenetic analysis, suggesting a source) of fecal contamination in complex samples; these data also anecdotally demonstrate the persistence of AdV virions/genomes. In particular, one liquid manure sample determined to be BAdV positive was held at 4°C for nearly a year before our opportunistic analysis for BAdV.

On the other hand, findings reported here and elsewhere suggest that caution must be exercised if the detection of BAdV is invoked for fecal contamination source attribution. Our detection of novel Eastern cottontail rabbit and porcine MaAdV supports the notion that unsequenced AdV infecting wild and domestic animals likely contribute to environmental AdV pools; such viruses could, in principle, confound source attribution if amplified unexpectedly with an FST PCR assay of presumed specificity. Wider-than-reported AdV host ranges could similarly lead to inaccurate attribution of a contamination source(s). In fact, several lines of reasoning encourage informed skepticism of the purported host specificity of at least
some AdV. First, biological systems adopted for FST should ideally have perfect host species specificity. However, the AdV infection of a given livestock host were generally not monophyletic, and inferred phylogenies clearly show a history of common ancestry among AdV that currently infect different species, implying cross-species transmission events. Such events in primates, for example, may have contributed to the evolution of AdV now infecting humans (32). Second, mechanisms for AdV host adaption or cross-infection clearly exist, as demonstrated by OAdV-A genotypes, which have maintained the ability to infect both sheep and cattle (3). Additional evidence for expanded host ranges has been reported for several other AdV, including OAdV (47), BAdV-6 (5), canine AdV (18), and HAdV C viruses (19, 21, 31, 39). Finally, recombination events detected among livestock MaAdV provide further evidence of historical cross-host animal AdV infection and, for OAdV-A viruses, correlate with reports that recombination among HAdV belonging to the same species is common (25, 42).

Implications. On balance, the informed targeting of MaAdV and AtAdV during FST investigations has the potential to add clarity to contamination source(s) when used in a weight-of-evidence, or “toolbox,” approach. However, existing source tracking assays targeting well-conserved portions of the AdV hexon gene should be employed cautiously, preferably in conjunction with sequencing, and continually reevaluated against new source samples and growing public genetic databases. For improved confidence in assay specificity, we suggest that BAdV PCR assays designed for FST target individual genotypes and amplify (or anneal within) hexon hypervariable regions, which code for proteins that contribute strongly to type-specific antigenic reactivity (7). BAdV-10 and AtAdV-4 are strong candidates for variable-region targeted source tracking PCR assays. Both of these viruses are prevalent (26, 45; the current study) and, based on current knowledge, monophyletic with other BAdV. Additional research exploring animal AdV diversity and the validation of narrowly targeted BAdV PCR assays will further the interpretation/implementations of livestock AdV genetic targets for FST. Additional research comparing the fate and transport characteristics of BAdV with those of common zoonotic pathogens of livestock origin will clarify the diagnostic value of environmental BAdV detection for protecting human health.

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