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IDENTIFICATION OF A NOVEL INTERTYPIC RECOMBINANT SPECIES D HUMAN ADENOVIRUS IN A PEDIATRIC STEM CELL TRANSPLANT RECIPIENT

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Abstract

Background—Human adenoviruses (HAdV) are known opportunistic pathogens in hematopoietic stem cell transplant (SCT) recipients. The detection of HAdV infection in children after SCT has been implicated as a determinant of poor outcome but specific associations between HAdV species or individual HAdV types and disease are poorly understood.

Objectives—Characterization of a HAdV-D strain isolated from multiple clinical specimens of an 11-year-old female recipient of a matched unrelated donor peripheral SCT for T-cell lymphoma and case report.

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Conflict of interests
None declared.

Ethical approval
The study was approved by the Institutional Ethics and Review Committees of the Children's Hospital of Philadelphia. Because the personal and demographic data for the clinical specimens were deidentified prior to the study, the protocol was exempt of IRB review at the Lovelace Respiratory Research Institute.

Study Design—Archived HAdV PCR-positive plasma, urine, and stool specimens were processed for virus isolation and detailed molecular typing. Complete genomic sequencing was carried out on 2 isolates.

Results—The patient tested positive for HAdV DNA by real-time PCR of a stool specimen at 44 days after initiation of a SCT conditioning regimen. In the subsequent 3 months, HAdV was detected in plasma, urine and stool specimens in association with symptoms of gastroenteritis and hemorrhagic cystitis. A novel HAdV-D with a HAdV20-like hexon gene was isolated from both urine and stool specimens. All isolates yielded identical restriction profiles with endonucleases *Bam*HI, *BglII, BstEII, HindIII, PstI and SmaI*. Analysis of 2 complete genomic sequences further identified the virus as a novel intertypic recombinant HAdV-D (P20/H20/F42) closely related to HAdV42.

Conclusions—This case highlights the identification of a previously unknown HAdV-D from an immunocompromised host. In this patient, the course of adenovirus infection is compatible with reactivation of a latent virus or a primary opportunistic infection. Adenoviremia in this patient resolved without definitive adenovirus-directed antiviral therapy.

BACKGROUND

Human adenovirus (HAdV) is a known opportunistic pathogen in hematopoietic stem cell transplant (HSCT) recipients and other immunocompromising conditions [1-3]. A poor prognosis has been attributed to HAdV infections in pediatric SCT recipients [4,5].

The currently recognized 51 HAdV serotypes and the numerous “types” described based on genomics criteria are classified within 7 species (A-G) [6]. Despite the general conservation in the genetic organization of the viral genome and replication strategies, the spectrum of virus-host interactions and associated diseases varies with adenovirus species and across adenovirus types in immunocompetent hosts [7] so it is reasonable to assume that this is also the case with immunocompromised individuals. The detection of adenovirus infection in children post hematopoietic stem cell transplant (SCT) has been implicated as a risk factor for poor outcome [8,9] but the actual knowledge regarding specific associations between adenovirus species or individual types and disease is still poor [10,11].

OBJECTIVES

We report the characterization of a novel HAdV-D isolated from multiple clinical specimens of a pediatric female patient after a matched unrelated donor peripheral SCT. This work is part of an ongoing collaborative effort to establish more comprehensive data on the impact of HAdV infection in pediatric patients undergoing SCT and to contribute to the characterization of the still poorly defined epidemiology of adenovirus infections in pediatric SCT recipients.

STUDY DESIGN

Adenovirus detection in clinical specimens

For real-time TaqMan® PCR-based detection of adenovirus, DNA was extracted from 200 μl of each clinical specimen using an automated MagNAPure LC instrument and a total

nucleic acid isolation kit from Roche Diagnostics, Indianapolis, IN. An in-house quantitative real-time TaqMan® PCR was performed in 50 μl volumes using the primers and probe targeting the hexon gene developed by Heim and colleagues [12].

Virus isolation and initial rounds of molecular typing

One PCR-positive plasma specimen, three PCR-positive urine specimens and six PCR-positive stool/abdominal fluid specimens were inoculated onto monolayers of A549 cells. Intracellular viral DNA was isolated from infected A549 cells for restriction enzyme analysis as previously described [13]. Initial typing at the species level was accomplished by analysis of *Bam*HI digestion profiles. A more detailed characterization was subsequently carried out by digestion with *Bgl*II, *Bst*EII, *Hind*III, *Pst*I and *Sma*I. Molecular typing was carried out by PCR amplification and sequencing of hypervariable regions 1-7 of the hexon gene [14] and the fiber gene followed by BLAST analysis using the GenBank/NCBI database as a reference. Primers used for amplification and sequencing of the hexon and fiber genes are shown in Table 1. Molecular type identities were assigned based on the identity of the closest match.

Complete genomic sequencing

Viral DNAs purified from one of the stool isolates and from one of the urine isolates were further processed for complete genomic sequencing at the Applied Genomics Technologies Core, Wadsworth Center. Next generation whole genomic sequencing was performed on a Life Technologies Ion Torrent Personal Genome Machine (PGM). Shearing and library generation of viral DNA was performed using the Ion Xpress Plus Fragment Library kit and barcoded using Ion Xpress Barcode adapters. Templates were prepared using the Template kit v2 on the Ion OneTouch 200. Sequencing was performed on the Ion PGM using the Ion PGM 200 Sequencing kit on either a 314 or 316 chip. Sequence gaps in the pVII coding region and E3 region were filled by Sanger sequencing using primers listed in Table 1. Annotated complete genomic sequences were deposited in Genbank under accession numbers KJ626291 and KJ626292.

Genomic sequence data analysis

The complete genomic sequences of species D HAdV8 (AB448767), HAdV15 (JN226748), HAdV20 (JN226749), HAdV22 (FJ619037), HAdV28 (FJ824826), HAdV29 (JN226754), and HAdV42 (JN226761), and the genomic sequence from stool isolate 21-10-VT9763 (KJ626292) and urine isolate 21-10-VT10230 (KJ626291) were aligned in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 using Clustal W multiple alignment tool [15]. The multiple alignment was further analyzed with SimPlot recombination analysis [16] using the genome of isolate 21-10-VT9763 as a query sequence. SimPlot settings were set at a window of 200 base pairs with a step of 20 base pairs. Additionally, a phylogenetic tree was constructed in MEGA 6.0 using the Neighbor-Joining method with 500 bootstrap replicates [17]. Evolutionary distances were computed using the Maximum Composite Likelihood method [18]. The data set included a total of 34,572 base pairs from 14 different taxa. Virtual restriction enzyme analysis of viral genomes was carried out using Geneious v6.1.6 (Biomatters, Ltd., New Zealand).

RESULTS

Case Report

The patient is an 11-year-old female that received a matched unrelated T and B cell-depleted donor peripheral SCT for myelodysplastic syndrome secondary to treatment for T-cell lymphoma. Busulfan and thiotepa were administered as myeloablative therapy for preparation for the SCT and cyclophosphamide was administered for prophylaxis against graft versus host disease. Her initial post-transplant course was complicated by CMV viremia detected by routine surveillance testing and by skin graft versus host disease (GVHD). She was started on foscarnet for the CMV infection and tacrolimus and prednisone were initiated for GVHD. Engraftment studies revealed 100% donor engraftment by day +22 and she was discharged home on day +26 to continue on valgancyclovir, tacrolimus and prednisone. She presented to the outpatient clinic three days after discharge with progression of her skin GVHD to grade IV and was readmitted. Her immune suppression was escalated to include mycophenolate mofetil (MMF), intravenous methylprednisolone and continued tacrolimus. Her clinical course was complicated by poor oral intake and loose stools. Plasma specimens were repeatedly negative for adenovirus by PCR testing but a stool specimen tested positive by PCR on day +36. She was discharged home on tacrolimus, MMF and prednisone for GVHD therapy but again was readmitted on day +71 with generalized weakness and increased sleepiness. She was subsequently diagnosed with HHV-6-associated encephalopathy and treated with IV ganciclovir for three weeks at which time she was transitioned to oral valganciclovir. She also developed hemorrhagic cystitis, hepatitis and abdominal pain with bloody diarrhea. During this hospital course she was found to have adenoviremia on day +75, and then adenoviuria on day +99 and stool specimens remained PCR positive for adenovirus. A rectal biopsy on day +118 was found to be adenovirus PCR positive but pathology was consistent with GVHD. Figure 1 depicts the course of adenovirus infection providing cycle times (Ct) for PCR positivity from plasma, stool, and urine specimens and from a rectal biopsy tissue. Viral loads were modest in the plasma with all Ct values above 40. The Ct in stool peaked on day +117 at 28.66 and in urine on day +110 at 25.79. Urine specimens were also noted to be positive for BK virus and thus low dose cidofovir was initiated once every two weeks for two doses starting on day +102. Plasma adenovirus testing was negative on day +121 and she was transferred to a rehabilitation center on day +131. In retrospective review, adenovirus was considered as a probable cause for her hepatitis and as a possible cause for her cystitis and colitis. Because the patient had BK viruria and pathology-proven intestinal GVHD a definitive attribution to adenovirus for cystitis and colitis could not be concluded. Her adenoviremia resolved in temporal association to initiation of cidofovir. However, it was thought that this temporal decrease was not causative as only low dose cidofovir was administered to treat BK viruria and this may not have been sufficient for adenoviremia therapy.

Virus isolation and molecular typing

HAdV was successfully isolated from 5 of 6 available stool specimens (days +36, +85, +96, +112, and +117) and from the 3 available urine specimens (days +99, +110 and +117) within 2 passages in A549 cultures indicating a relatively high infectious viral load. Virus could not be recovered from the only plasma specimen available for further studies (day

+81, Ct= 41.45) or from the rectal biopsy (day +117, Ct= 32.52) after 5 serial passages in A549 cells.

Restriction enzyme analysis (REA) of purified viral DNA with a panel of 6 restriction enzymes (Figure 2) showed all virus isolates to be identical. REA data provided enough evidence to identify the virus as a HAdV-D by comparison with published profiles [19]. HAdV-D-specific primer pairs were subsequently used to amplify and sequence the hexon hypervariable regions 1-7 and the fiber gene. Blast analysis of sequence data identified all isolates as intertypic recombinants with a HAdV20-like hexon gene (H20) and a HAdV42-like fiber gene (F42) displaying high sequence identity to the HAdV15 fiber gene. Analysis of virtual restriction enzyme digests showed distinct profiles with BstEII to be readily distinguishable from those of the closely related HAdV15, HAdV20 and HAdV42 (Figure 3). The recombinant nature of the virus genome provided a strong rationale for expansion of the characterization of the virus by complete genomic sequencing. Among other pieces of valuable information regarding the genetic make-up of the isolated virus strain, the molecular identity of the penton base was established as HAdV20-like (P20) (Table 2). The initial observations of hexon and fiber gene molecular identities were further confirmed with the analysis of the corresponding complete gene sequences (Table 2), allowing us to complete the basic desirable molecular typing data set and to designate the strain as USA/CHOP21/2010/[P20H20F42].

Phylogenetic analysis of complete genomes showed the intertypic recombinant P20H20F42 to be most closely related to HAdV42 and HAdV15 (Figure 3, Table 2). Interestingly, when the identical sequence of the entire genomes of stool isolate 21-10-VT9763 and urine isolate 21-10-VT10230 was analyzed in comparison to those of closely related HAdV-D genomic sequences (HAdV15, 20, 22, 28, 29 and 42) the virus displayed the highest similarity to HAdV20 in all genes except for the E3 cassette and L5/fiber which most closely resembled the corresponding regions of HAdV42 and HAdV15 as shown in Figure 5 and Table 2.

DISCUSSION

A novel intertypic recombinant HAdV-D was isolated from multiple urine and stool specimens collected from an 11-year-old peripheral SCT recipient. The viral infection was first detected by PCR in a stool specimen collected 36 days after transplant. Adenovirus DNA was detected in plasma, stool and urine specimens over a period of 120 days after transplant. The course of the adenovirus infection is suggestive of a transient reactivation of a latent virus. This is pure speculation as the lack of access to pretransplant patient serum to determine the presence of pre-existing serotype-specific neutralizing antibodies precluded any further investigation of the natural history of infection by the detected virus [20]. Alternatively, the detected virus could represent a primary opportunistic infection.

With the exception of the oculopathogenic types, the vast majority of known HAdV-Ds represent fecal or urinary isolates from immunocompromised or asymptomatic individuals. Shedding in stool for prolonged periods of time has been well documented for adenoviruses not recognized as causative agents of gastroenteritis [21-24]. Interestingly, recent studies conducted using PCR-based detection and typing procedures in sub-Saharan and other

African communities, reported a high prevalence of HAdV-D DNA in diahrreal and non-diahrreal stools of patients of various age groups [25-27]. The isolation of a novel HAdV-D from the stools of 4 children with acute gastroenteritis in Bangladesh was also recently reported [28]. However, the role of the detected viruses in the etiology of gastroenteritis could not be demonstrated. Taken together, the data from these studies and the reported detection of adenovirus DNA representing various species in the gut-associated lymphoid tissue reported by Roy and colleagues [29] suggest that, like HAdV-C [30,31], other HAdV species including HAdV-D may also be capable of establishing latent/persistent infections in the gut with intermittent shedding.

Intertypic recombination is well-recognized as frequently occurring among HAdVs and has been extensively described for species HAdV-Ds [32-345]. Not surprisingly, given the lessons learned from serology-based studies and recent genomic sequencing efforts targeting HAdV-Ds, the strain isolated from our SCT patient represents a new example of a genome with an array of genes not previously reported.

A number of recently characterized HAdV-Ds have been described exclusively by bioinformatics analysis of complete genomic sequences and designated as “new phylogenetic types” with numbers consecutively added to the existing list of 51 serotypes [36-41]. In addition, the sequences for many non-designated “NEW” intertypic recombinant HAdV-Ds have been deposited to Genbank under accession numbers (KF268322, KF2683227, KF268332, KF268334, KF268335, among others). Some of these “new” HAdV-Ds were originally isolated from fecal samples [28,39,42].

In the absence of standardized protocols to conduct molecular typing and designate viruses based on complete genomic sequences or partial sequence data for genes encoding structural proteins, we chose not to pursue a unique number to designate the novel HAdV-D virus and to use, instead, a strain designation based on the place and year of circulation and the molecular identity of the major capsid proteins penton, hexon and fiber: USA/CHOP21/2010/[P20H20F42]. Interestingly, had molecular typing been approached exclusively by amplification and sequencing of the hexon gene, as reported in a number of studies published in the last decade [43-49], this virus would have been identified as HAdV20. Its role as a causative agent of the patient’s cystitis, hepatitis and/or colitis is unclear as BK viruria and GVHD were concomitantly diagnosed. Further, it is not clear that the resolution of adenoviremia was causatively or temporally related to low dose cidofovir administration. The context of isolation of the majority of the HAdV-Ds identified to the present suggests that many of them are likely well-adapted, non-pathogenic viruses in immunocompetent hosts. Phylogenetic analysis of complete genomic sequences showed the isolated novel virus described in this study to be most closely related to HAdV42, an example of a HAdV-D originally isolated from the stool of a healthy child [50].

It is clear that the study of adenovirus infections in immunocompromised patients will continue to provide the opportunity to identify previously uncharacterized HAdVs. The need for standardized operating procedures regarding identification and designation for reporting is imperative and so is the need to standardize attribution of clinical symptoms. Our experience highlights the value of extending molecular typing efforts beyond the hexon gene

to identify recombinant HAdVs and the importance of having complete genomic sequences to draw accurate conclusions on phylogenetic relationships of intertypic recombinant viruses.

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Highlights

- A novel HAdV-D was isolated from multiple specimens of a pediatric stem cell transplant recipient.
- The virus has a HAdV20-like penton base gene, a HAdV20-like hexon gene and a HAdV42-like fiber gene.
- The course of the adenovirus infection is compatible with a reactivation of a latent virus.

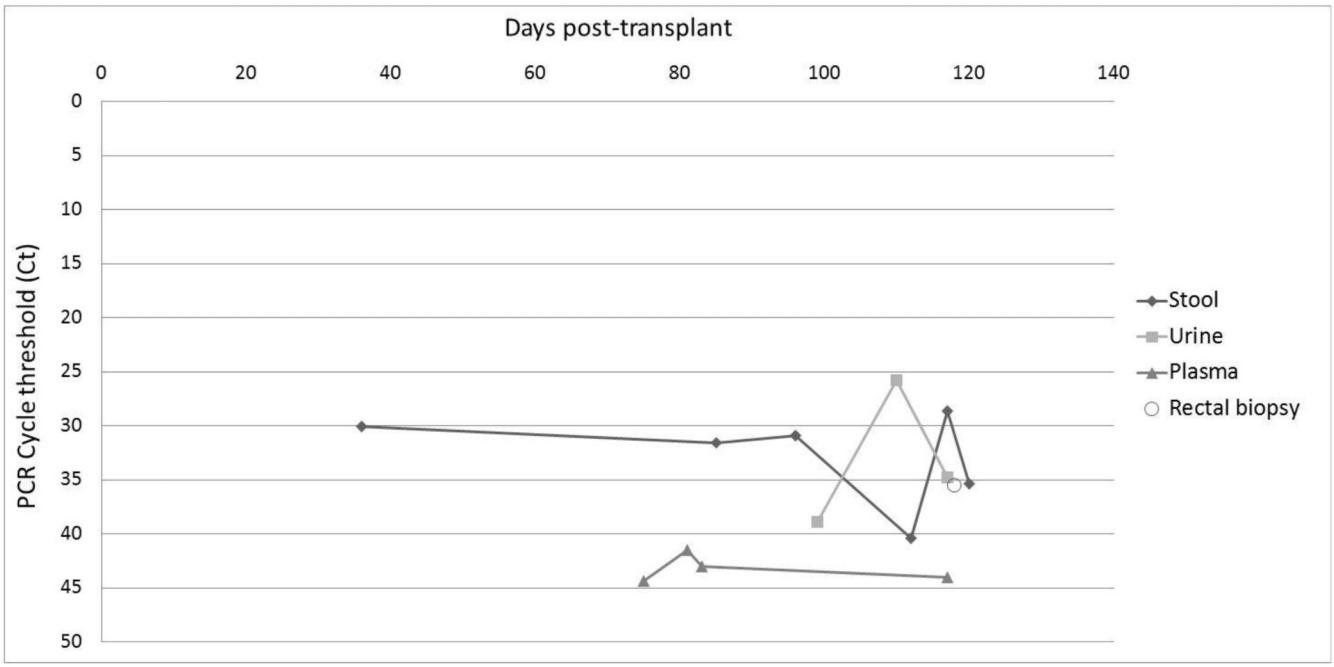

Figure 1.

Course of adenovirus infection as detected by quantitative real time PCR in diverse clinical specimens after transplantation. Cycle threshold (Ct) values for adenovirus positivity in plasma (▲), stool (◆), urine (■) and rectal biopsy tissue (○).

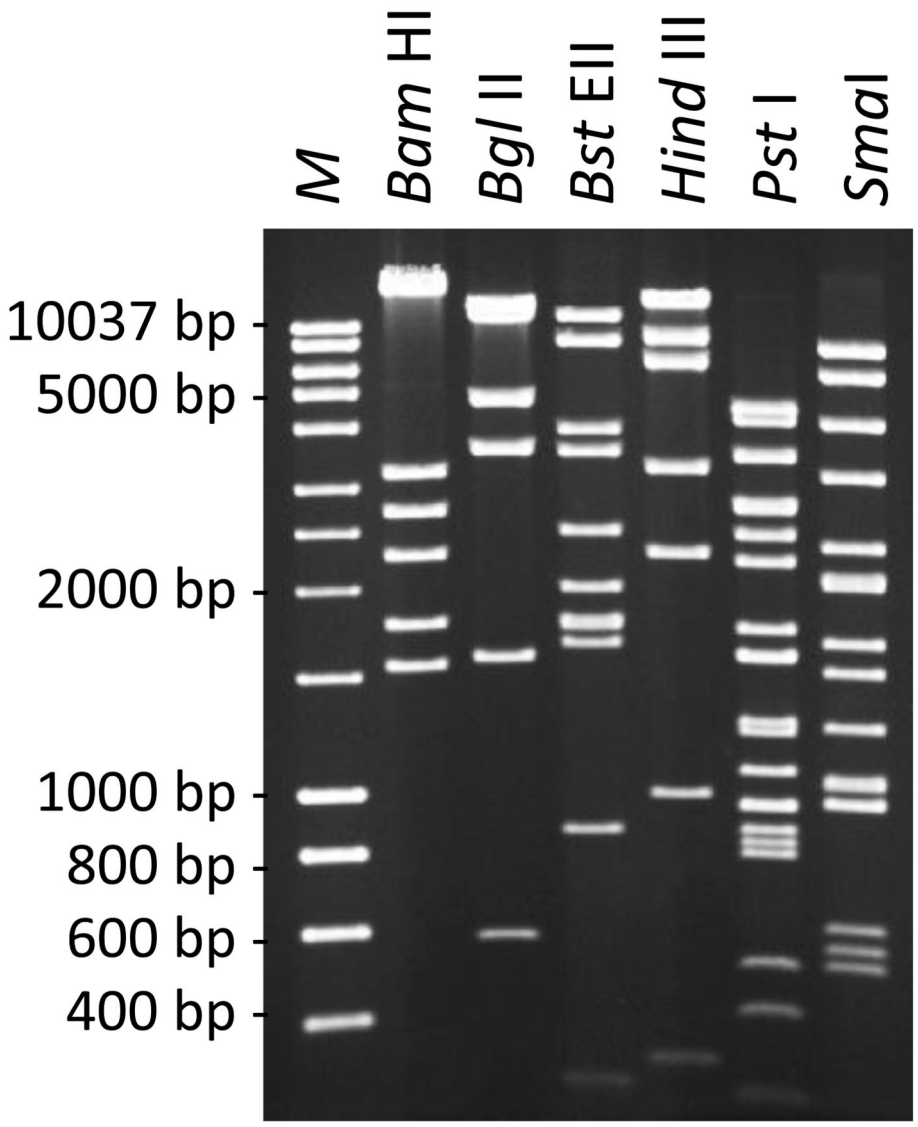

Figure 2. Restriction enzyme analysis of viral genomic DNA

Restriction profiles of genomic DNA from urine isolate 21-10-VT9763 with *Bam*HI, *Bgl*II, *Bst*EII, *Hind*III, *Pst*I and *Sma*I. M: Hyper Ladder™ I (400bp-10037bp, Bioline)

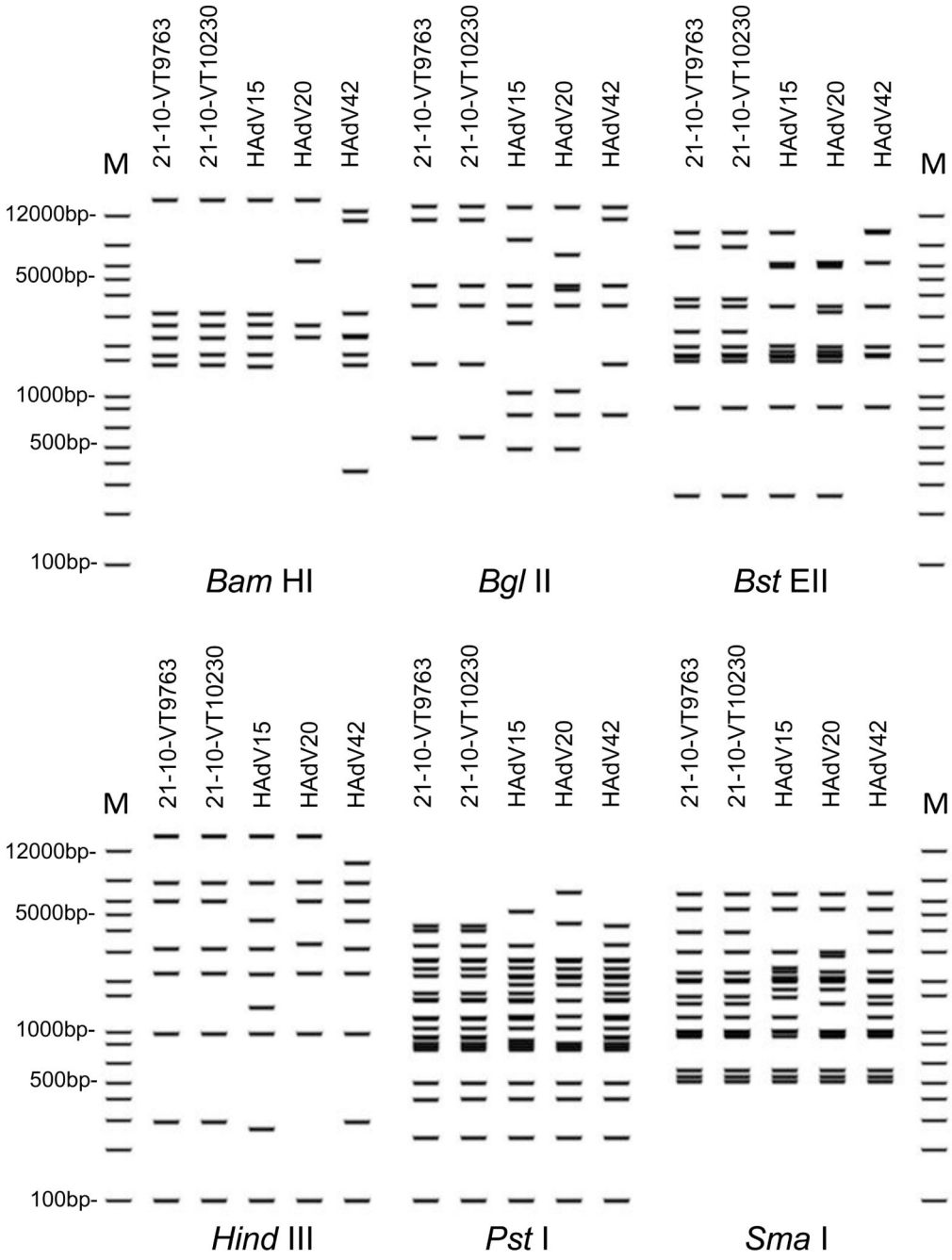

Figure 3. Virtual restriction enzyme analysis of viral genomic DNA

*Bam*HI, *Bgl*II, *Bst*EII, *Hind*III, *Pst*I and *Sma*I digests of genomic DNA of urine isolate 21-10-VT9763 (KJ626291) and stool isolate 21-10-VT10230 (KJ626292) in comparison to those of genomic DNA of HAdV15 (JN226748), HAdV20 (JN226749) and HAdV42 (JN226761). M: virtual 1Kb Plus™ ladder (100bp-12000bp, Life Technologies).

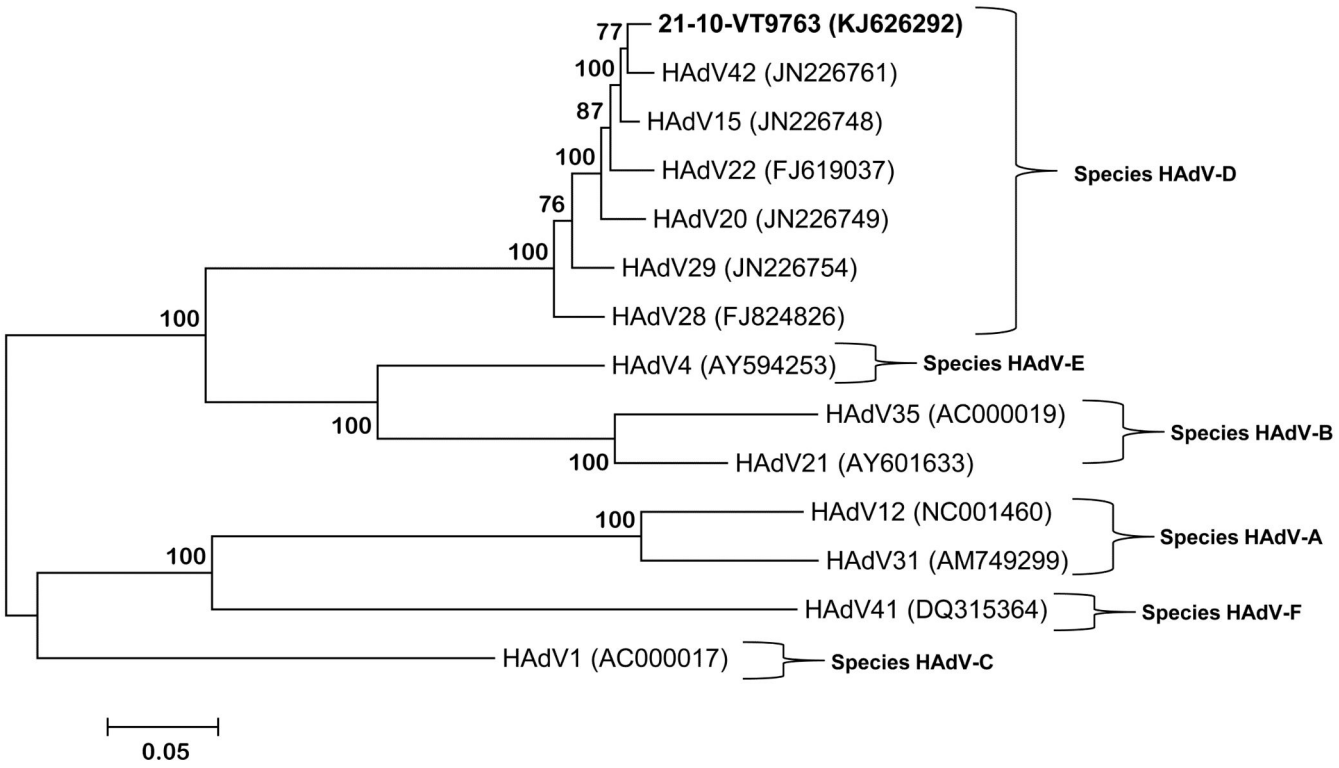

Figure 4. Phylogenetic analysis of complete HAdV genomic sequences

The phylogenetic tree was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model, with 500 bootstrap replicates. Bootstrap values less than 70 are not displayed on the tree. Evolutionary analyses were conducted in MEGA6. Genbank accession numbers for the genomic sequences included in the analysis are shown between brackets.

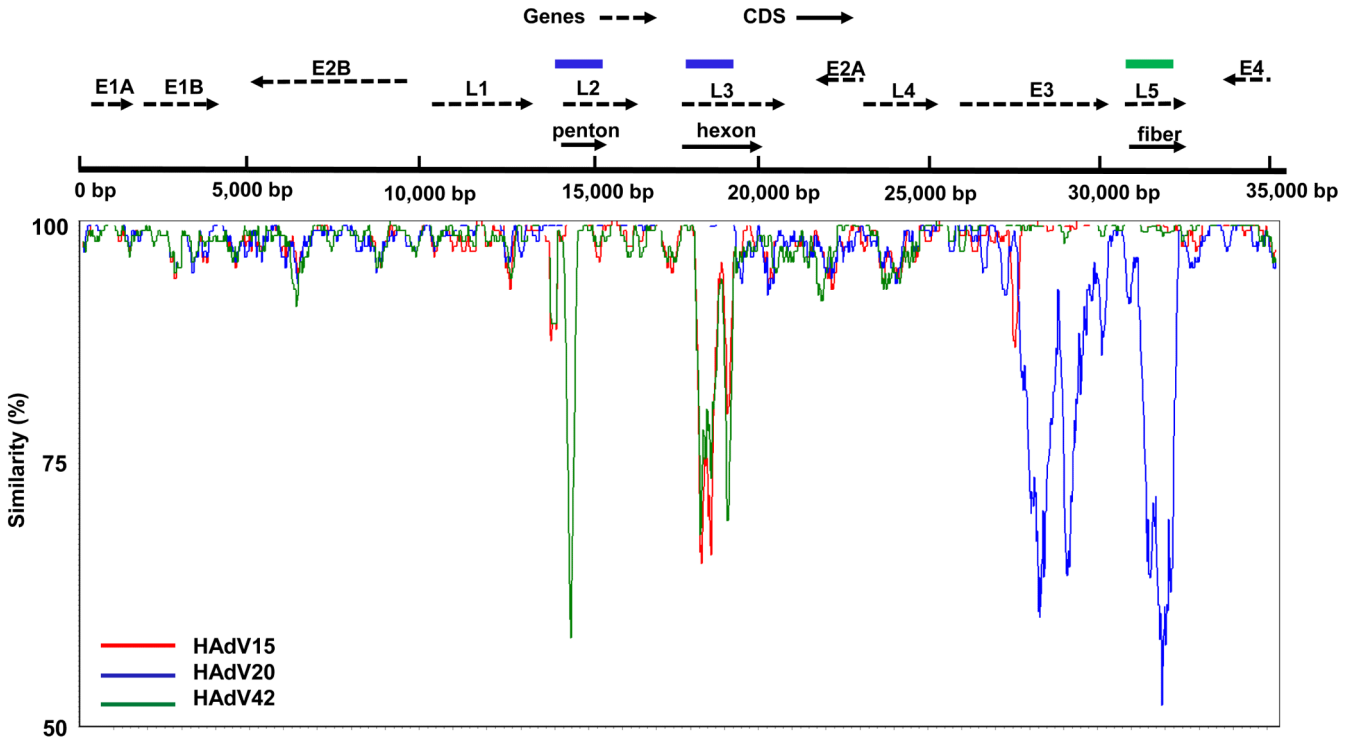

Figure 5. Analysis of sequence similarities between isolate 21-10-VT9763 and closely related HAdV-D types HAdV15, HAdV20, and HAdV42

Plots of similarity were generated by SimPlot [17]. Each curve is a comparison between the genome being analyzed and the genome of isolate 21-10-VT9763. Each point plotted is the percent identity within a sliding window of 200 bp wide centered on the position plotted, with a step size between points of 20 bp (GapSrip:On, J-C Correction: On). The horizontal bars above the curves are a cartoon of the coding regions of the HAdV-D genome. The colors indicate the type to which that part of the genome is most similar to, based on the plot below.

Table 1

Primers used for the amplification and sequencing of regions of the novel HAdV-D genome

REGION	PCR and sequencing primers	Amplicon
Hexon gene partial cds.	FW: 5′-TATGTGCCTTACCGCCAGAGAAC-3′ RV: 5′-CCGGCGTGTACTTGTAGGAGTC-3′ Internal FW: 5′-ACCAGATACIIIAGCATGTGGAACTCTG-3′ Internal RV: 5′-CCACATGCTAAAGTATCTGGTTCTGTCAC-3′	1472 bp SEQ
Fiber gene partial cds.	FW: 5′-GAACTTCCTCCACACCTTGAAAG-3′ RV: 5′-CCGTGCTGGTGTAAAAATCAATAAAG -3′ Internal FW: 5′-GACAACTCCAGACACATCTCCAAATTG-3′ Internal RV: 5′-CAAIIIGGAGATGTGTCTGGAGTTGTCC-3′	1209 bp SEQ
pVII	FW: 5′-CGCCCACCAICACCACCGII-3′ RV: 5′-CTCTTCGCGCGACCGCTTCC -3′ Internal FW: 5′-CCGCGCGTGCTCTCTAGTCG-3′ Internal RV: 5′-GCTCGTCTGCGAGTCCTGCC -3′	1251bp SEQ
E3	FW 5′-AACATATTGAAGCGGTTGGGTAIIIG-3′ RV 5′-TCTGCATGTTCAACTTTTTCTCCATCGCAG-3′	1099 bp

Table 2

Analysis of sequence similarities between the novel HAdV-D strain and closely related HAdV-D types at the whole genome level and also for individual genes and corresponding polypeptides

	% Similarity							
	Nucleic Acid					Amino Acid		
	Whole Genome	E3	Penton Base	Hexon	Fiber	Penton Base	Hexon	Fiber
HAdV15	97.4	97.5	98.2	91.6	97	98.5	92.9	97.3
HAdV20	95.8	86.5	99.7	98.4	68.5	99.8	99.7	64.5
HAdV22	95.4	87.6	92	90	90.8	91.2	90.7	87.7
HAdV28	92.6	76.4	97.9	90.7	67	98.8	92.3	64.9
HAdV29	94.3	93	92.4	91.1	64.1	91.7	92.6	56.3
HAdV42	97.5	99	92.2	91.1	99	91.2	93.8	99.2