ORIGINAL ARTICLE

Adenovirus Genotyping Based on Penton Gene Expression

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Abstract

Adenoviruses contain double-stranded DNA, and human adenoviruses are no exception. An individual's immune status and the type of infection can determine the severity of adenovirus infections. Many successful vaccines have been developed using vectors derived from adenoviruses, including the COVID-19 vaccines manufactured by AstraZeneca and Johnson & Johnson. Ervebo uses an adenovirus vector to deliver a gene for the Ebola virus glycoprotein, and the rabies vaccine Imovax delivers the rabies virus glycoprotein gene using an adenovirus vector. As part of the icosahedral virus particle, the penton protein is encoded by the penton gene of the adenovirus. The penton protein is also required to initiate virus entry into cells and attach to host cells. Adenovirus Hypervariable Regions (HVR) region limited typing was examined to determine the genotype of the virus. To construct phylogenetic trees for HAdV-D types 1 to 70, 9 isolates were typed and sequenced, and phylogenetic trees were constructed for the penton HVR loop regions that recorded novelty strains that had not been reported previously. DNA extraction and PCR were conducted in this study using phylogenetic analysis.

Keywords

Adenovirus, Genotype, Penton gene, Phylogenetic trees

INTRODUCTION

Human adenovirus (HAdV) is a type of virus that contains double-stranded DNA. It was first found in cell cultures made from adenoid tissue in 1953[1]. HAdV is a member of the Mastadenovirus genus and the Adenoviridae family[2]. It has an icosahedral structure and a genome size that ranges from 26 to 45 kilobases[2]. HAdV is a non-enveloped virus, meaning it does not have a fatty outer membrane[3]. More than 70 types of HAdV have been identified. The first 51 types were identified based on immunological and biological characteristics, while the rest were identified based on genome analysis[4]. These types are classified into seven species (subgroups A-G) based on genome sequencing, phylogenomic, and serological assays[4]. Species B of HAdV is further subdivided into two subspecies: subspecies B1, which includes HAdV types 3, 7, 16, 21, and 50, and subspecies B2, which includes HAdV types 11, 14, 34, 35, and 55[4].

The severity of adenovirus infections can vary depending on the type of infection and the person's immune status[5]. Most adenovirus infections are mild and resolve independently within a few weeks[6,7]. However, some can be severe, especially in young children, older adults, and people with weakened immune systems[5]. Adenovirus can cause various infections, such as respiratory, gastrointestinal, conjunctivitis, Urinary tract infection, liver, pancreas, and central nervous system[5,6,7].

Numerous efficacious vaccines have been formulated utilizing adenovirus-derived vectors. Examples include COVID-19 vaccines, such as those developed by AstraZeneca and Johnson & Johnson. Additionally, the Ervebo vaccine employs an adenovirus vector to administer the gene encoding the Ebola virus glycoprotein. Similarly, the Rabies vaccine, Imovax, utilizes an adenovirus vector to convey the gene responsible for the rabies virus glycoprotein expression. These applications underscore the versatility and efficacy of adenovirus vectors in developing vaccines targeting diverse viral pathogens[8,9,10]. However, adenovirus vectors can cause more severe side effects, such as Guillain-Barré syndrome[11]. Additionally, some people may already have immunity to adenoviruses, which could reduce the effectiveness of adenovirus vector vaccines[12].

The penton gene of adenovirus encodes the penton protein, a major capsid protein that forms the vertices of the icosahedral virus particle[13]. The penton protein is also involved in virus attachment to host cells and initiating virus entry into the cell[13]. The penton protein has three main functions[14]. The penton protein binds to specific receptors on the surface of host cells[14]. This binding helps the virus to attach to the cell and initiate infection[14,15]. The penton protein also plays a role in endocytosis, which is the process by which the virus is taken into the cell[14]. The penton protein helps to destabilize the cell membrane and facilitate the entry of the virus into the cell[15].

The HVR region is part of the penton gene in adenovirus[16]. The HVR region is a region of the penton gene that is highly variable between different strains of adenovirus[16]. This variability allows the virus to evade the host immune system and cause infection[16]. The HVR region is thought to be involved in the virus's ability to attach to host cells and enter the cell[17]. Mutations in the HVR region can affect the virulence of adenovirus. It can change rapidly from one strain of adenovirus to another. Mutations in the HVR can have a number of effects on the virus. For example, they can make the virus more or less infectious, or they can make the virus more or less resistant to the host cell's immune system. The HVR is also the part of the adenovirus genome that interacts with the host cell's immune system. Studies have shown that strains of adenovirus with mutations in the HVR region are more likely to cause severe and fatal infections[16,17].

In this study, we examined whether limited typing of the HVR region of adenovirus is sufficient to identify the correct genotype. Nine isolates were typed and sequenced. Phylogenetic trees for the penton HVR loop regions of HAdV-D types 1–70 were constructed.

METHODOLOGY

DNA Extraction

DNA extraction was executed using the QIAamp DNA Mini Kit following the manufacturer's protocols. Briefly, Q-Pro and AL were added to the sample in a microcentrifuge tube, followed by vortexing and incubation at 56°C for 15 minutes. The resultant mixture was subjected to centrifugation, and ethanol was subsequently added. The resulting solution was transferred to a QIAamp spin column tube and centrifuged for 1 minute. The filtrate was discarded, and the column was repositioned into a new collection tube. Subsequently, AW1 was added, followed by centrifugation for 1 minute. After discarding the filtrate,

the column was transferred to a new collection tube, and AW2 was added with centrifugation for 3 minutes. Finally, elution was performed with AE, followed by centrifugation for 1 minute. The eluted DNA was stored at -20°C.

Polymerase Chain Reaction (PCR)

Conventional PCR was performed to amplify the partial penton region, which includes the HVR region. The forward primer was ACTACCAAAACGACCACAGC, and the Reverse primer was CCTCATACATGATTCTGAAGC. The master mix was prepared as follows: 5 µl of 10 X PCR buffer, 1 µl of 0.2 mM dNTPs, 0.5 µl of 0.2 µM forward and reverse primers, 0.25 µl of 1.25 units/µl Taq DNA polymerase, 37.75 µl of sterile distilled water, 5 µl of DNA template or control to the PCR tube and the final volume was 45 µl. The conventional PCR cycling program was initiated with 15 minutes of activation at 95°C. It is followed by 40 cycles of 20 seconds at 94°C (denaturation), 20 seconds at 57°C (annealing), and 40 seconds at 72°C (elongation). The last step was 5 minutes of incubation at 72°C for the final extension before cooling at 4°C. The amplicons were run on 2% agarose E-gel to check the molecular weight of the amplicons compared with a DNA easy ladder. It is a double-strand DNA that contains fragments of different sizes ranging from 100 bp to 2,500 bp.

Phylogenetic Analysis

The penton nucleotide sequences (918 bases referring to HAdV-D9) and deduced amino acids were used to construct phylogenetic trees of species D human adenoviruses, and the HVR regions of the penton were analyzed. BioEdit v7.0.5 was used to edit the sequences. ClustlX v1.83 was used to calculate the sequence alignments. Phylo-win software v2 was used to construct the trees. Bootstrap analysis was made with 1000 pseudoreplicates. The phylogenetic trees were drawn by TreeView v1.6.6.

RESULTS

PCR Amplification of Clinical Isolate

The DNA was extracted from the nine isolates and subsequently amplified using the partial penton region and two primers to generate a fragment of the penton region of species D HAdV. The size of the amplicons and positive controls were 600 bp and 400 pb, as expected, as shown in Figure 1.

Typing and Phylogenetic Analysis of Adenovirus Isolates

Nine clinical isolates from AIDS patients were typed using sequencing and phylogenetic analysis of the hexon and fiber regions used in this study. These isolates were found to be from HAdV-D species, with the same typing results in these two regions. The results of the previous typing are shown in Table 1.

Phylogenetic Analysis Reveals Genetic Relationships of New Adenovirus Types with Clinical isolates

HAdV-D49, with 0.6% nucleotide divergence, is closest to sample 3 and 4 isolates. HAdV-D19, HAdV-D48, and HAdV-D49 have identical amino acid sequences in

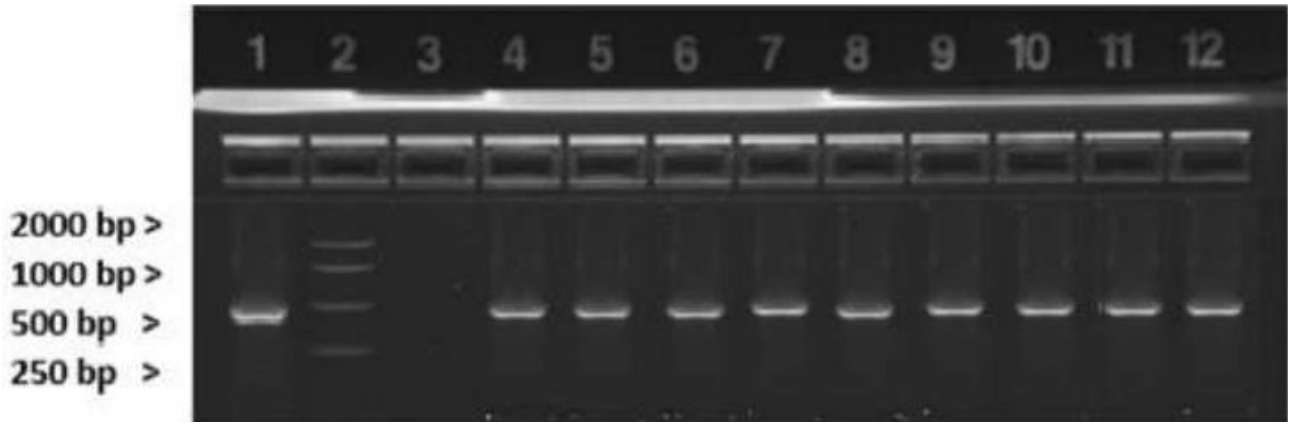

Figure 1. Two percent E-agarose gel of HVR region. Lane 1: Positive Control; Lane 2: Quantitative DNA easy ladder; Lane 3: Negative Control; Lane 4: Sample 1; Lane 5: Sample 2; Lane 6: Sample 3; Lane 7: Sample 4; Lane 8: Sample 5; Lane 9: sample 6; Lane 10: Sample 7; Lane 11: Sample 8; Lane 12: Sample 9.

Table 1. **The HAdV-D types of samples for Hexon and Fibre genes**

Sample No	HAdV-D Type
1	51
2	49
3	9
4	9
5	23
6	47
7	47
8	43
9	.26

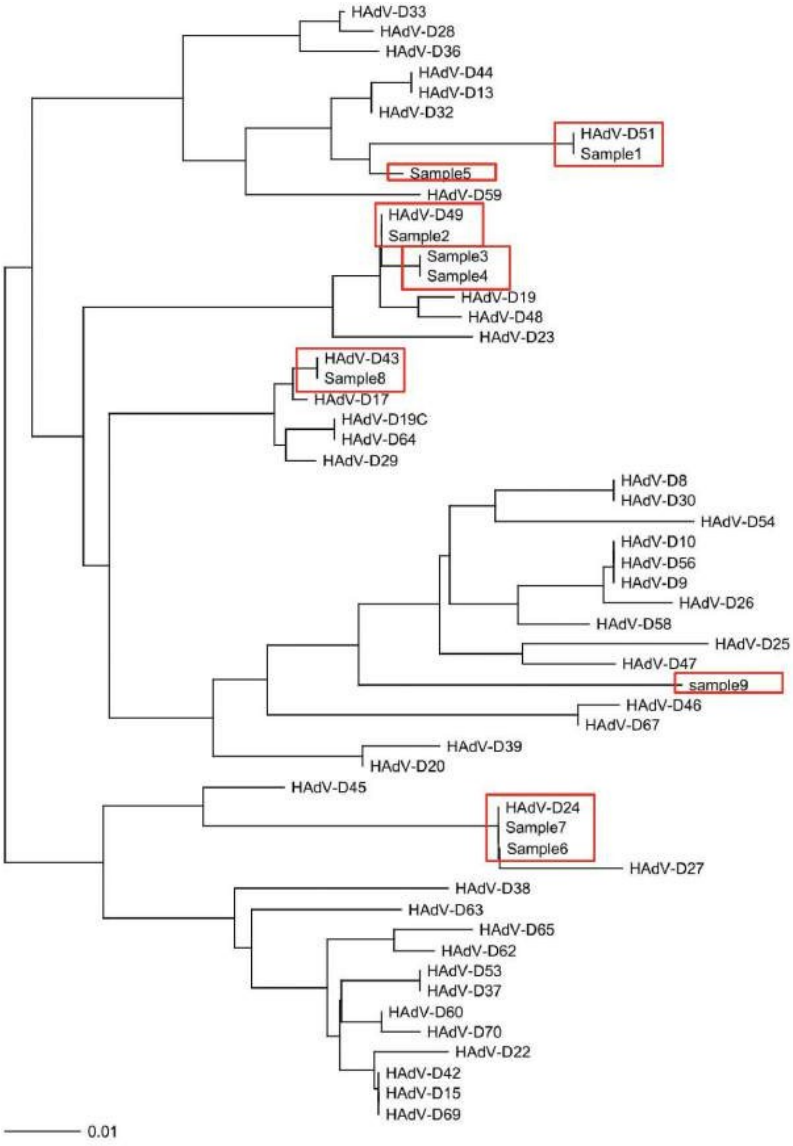

Figure 2. **Nucleotide phylogenetic tree of all clinical isolates. The tree was built from the Penton HVR region of prototype reference sequences and isolate sequences.**

this region and cluster with these two isolates. In the nucleic acid phylogenetic tree, sample 5 clustered with HAdV-D 13, 32, 44, 51, and 59, and the closest type was HAdV-D32, with 1% divergence. Based on the amino acid phylogenetic tree, this sample clustered with the same types above with 1.5% divergence to HAdV-D 13, 32, 44. Samples 6 and 7 have identical sequences to HAdV-D24 in this region. In terms of nucleotide divergence and amino acid divergence, HAdV-D9, 10, 47, and 56 were the closest types to sample 9 isolates.

Figures 2 and 3 are presented. The tree was built from the penton HVR region from prototype reference sequences and isolated sequences for nucleotide and amino acid phylogenetic trees, respectively. Figures 4 and 5 show nucleotide and amino acid alignment of the HVR region of the penton of HAdV for samples 3, 4, and 5, while samples 6, 7, and 9 are illustrated in Figures 6 and 7. The summary of the nucleotide and amino acid sequence's identity of samples 1-9 HVR region are described in Tables 2 and 3, respectively.

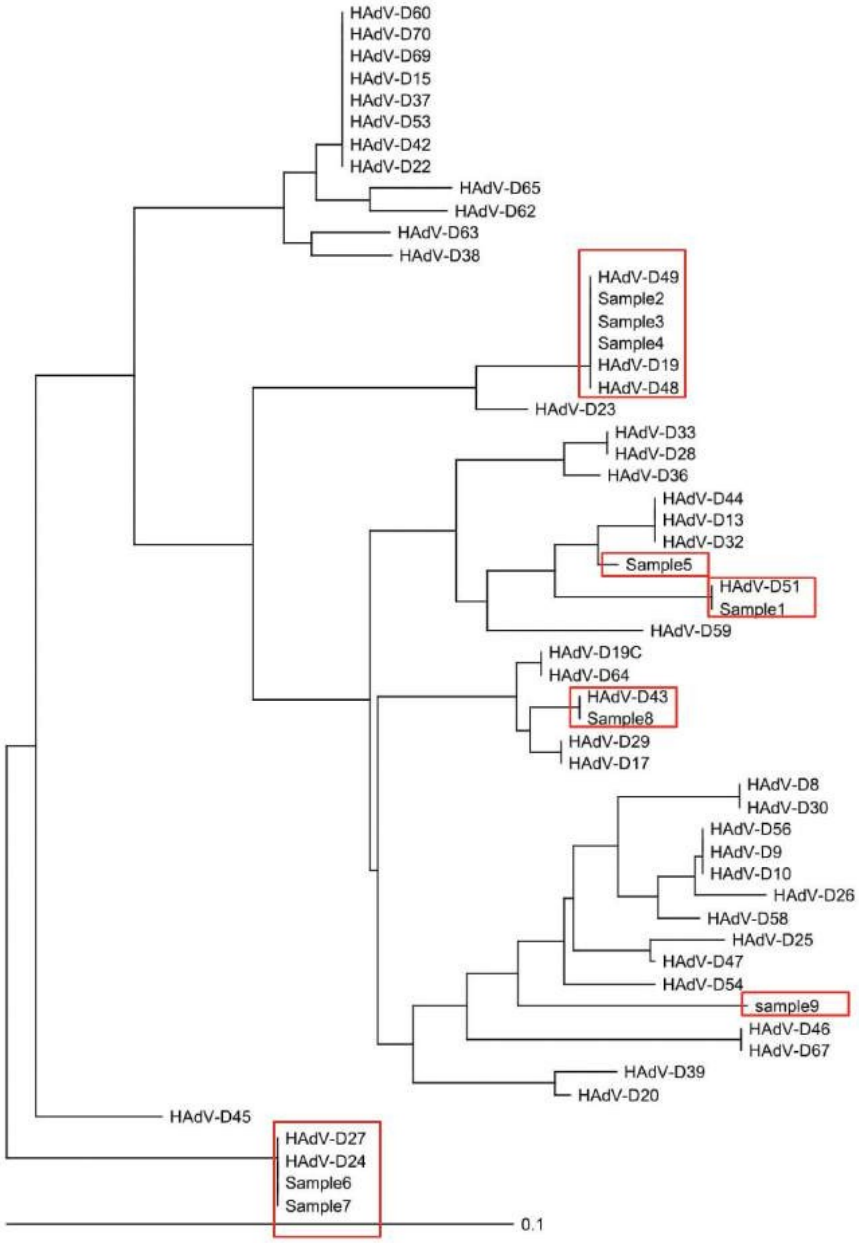

Figure 3. Amino acid phylogenetic tree of all clinical isolates. The tree was built from the Penton HVR region of prototype reference sequences and isolate sequences.

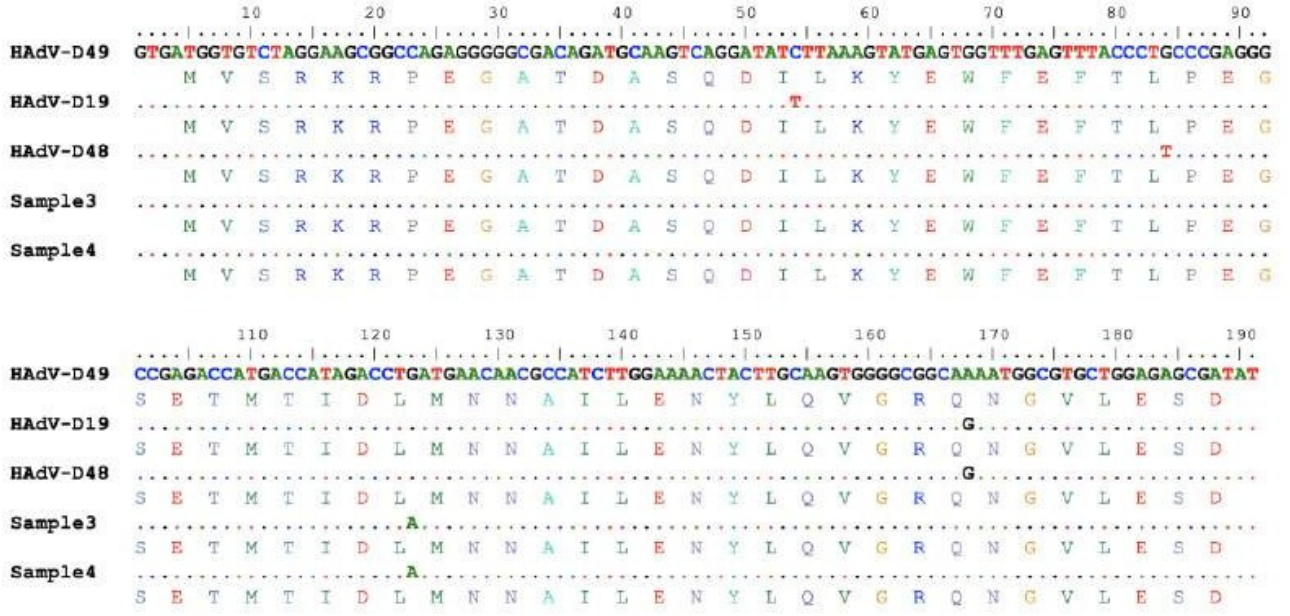

Figure 4. **Nucleotide and amino acid alignment of the HVR region of the penton of HAdV-D 19, 48, and 49 with samples 3 and 4.**

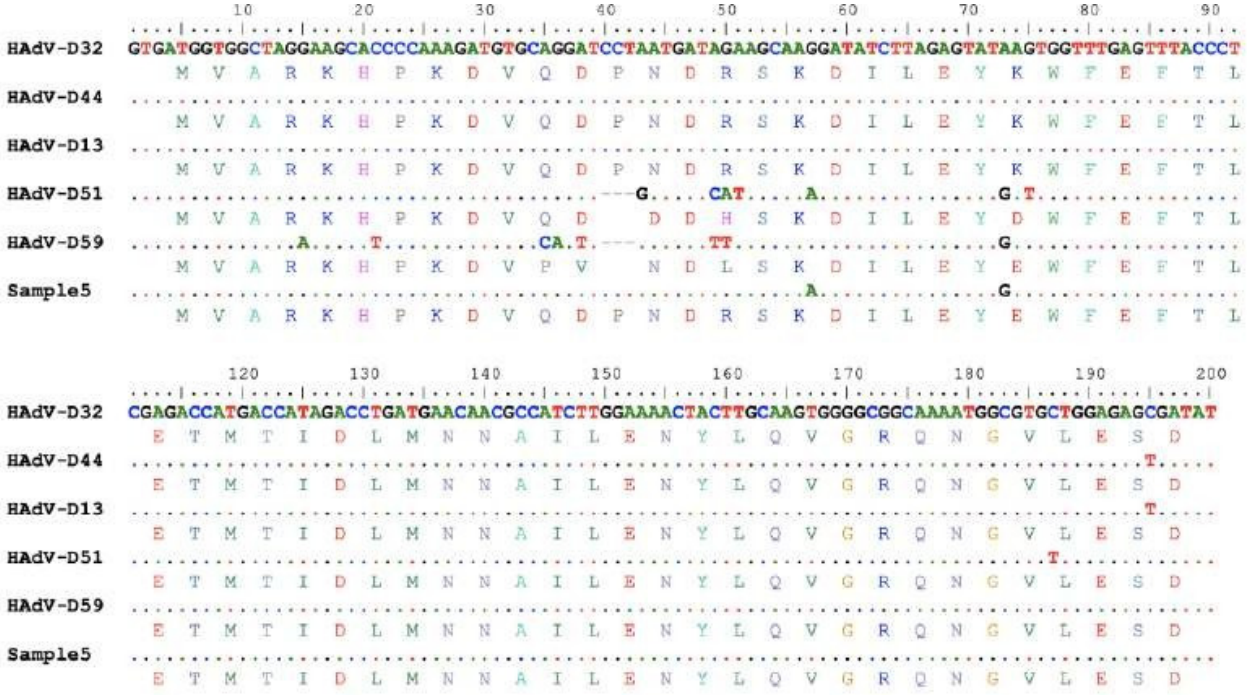

Figure 5. **Nucleotide and amino acid alignment of the HVR region of the penton of HAdV-D 13, 32, 44, 51, and 59 with samples 5.**

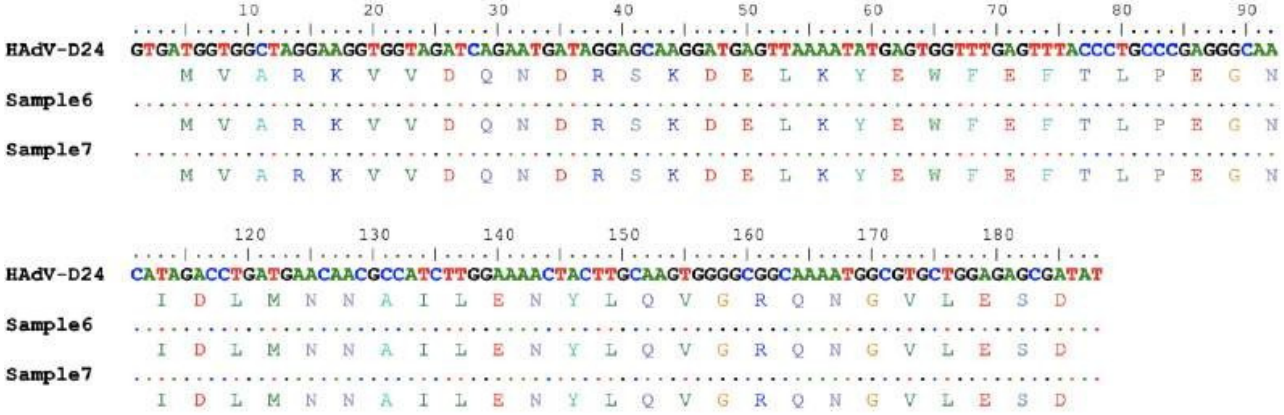

Figure 6. **Nucleotide and amino acid alignment of the HVR region of the penton of HAdV-D 24 with samples 6 and 7.**

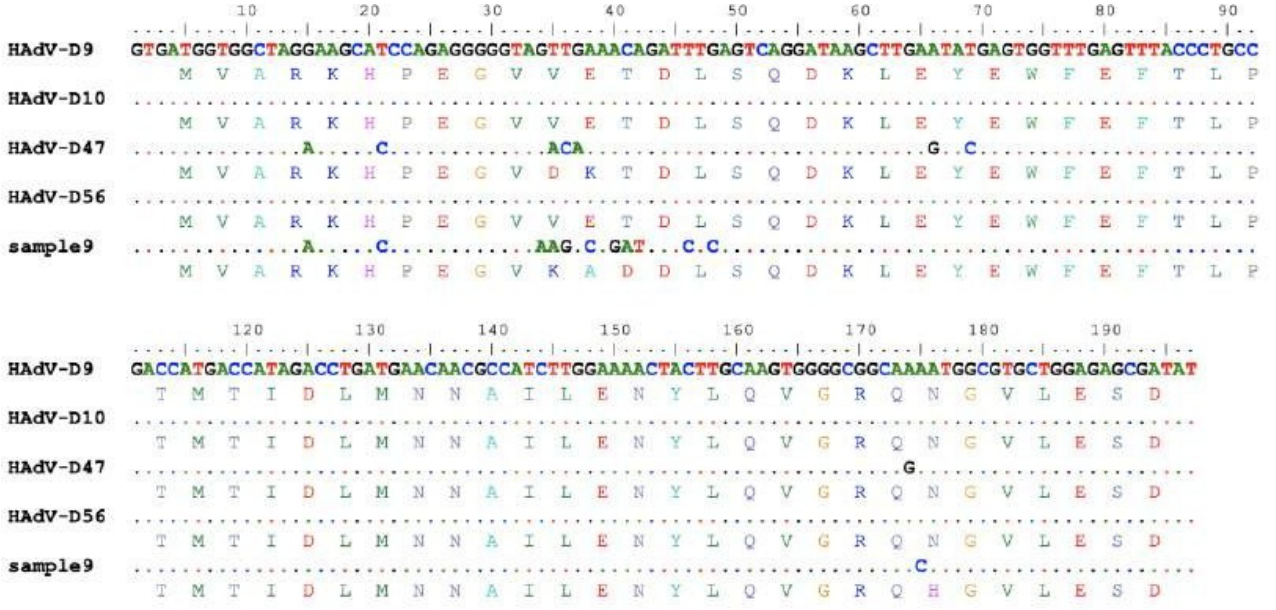

Figure 7. **Nucleotide and amino acid alignment of the HVR region of the penton of HAdV-D 9, 10, 47, and 56 with samples 9.**

Table 2. Nucleotide sequence identity of samples 1-9 HVR region compared to adenovirus references. Types with the highest similarity are highlighted. The table shows that the nine samples have varying degrees of similarity to the different adenovirus references. For example, sample 1 has the highest similarity to Ad5, while sample 9 has the highest similarity to Ad3.

Sample4	Sample3	Sample2	sample9	Sample8	Sample5	Sample1	Sample7	Sample6	Seq->
0.868	0.868	0.873	0.873	0.918	0.89	0.878	0.873	0.873	HAdV-D15
0.868	0.868	0.873	0.873	0.918	0.89	0.878	0.873	0.873	HAdV-D69
0.868	0.868	0.873	0.873	0.918	0.89	0.878	0.873	0.873	HAdV-D42
0.857	0.857	0.862	0.868	0.908	0.88	0.868	0.868	0.868	HAdV-D22
0.873	0.873	0.868	0.868	0.913	0.885	0.873	0.868	0.868	HAdV-D53
0.873	0.873	0.868	0.868	0.913	0.885	0.873	0.868	0.868	HAdV-D37
0.868	0.868	0.873	0.873	0.918	0.89	0.878	0.873	0.873	HAdV-D60
0.862	0.862	0.868	0.868	0.913	0.885	0.873	0.868	0.868	HAdV-D70
0.873	0.873	0.878	0.883	0.918	0.895	0.883	0.868	0.868	HAdV-D65
0.878	0.878	0.883	0.888	0.923	0.9	0.893	0.873	0.873	HAdV-D62
0.866	0.866	0.871	0.827	0.857	0.87	0.857	0.952	0.952	HAdV-D45
0.856	0.856	0.861	0.842	0.837	0.855	0.842	1	1	HAdV-D24
0.841	0.841	0.846	0.827	0.822	0.84	0.827	0.984	0.984	HAdV-D27
0.86	0.86	0.865	0.86	0.9	0.985	0.94	0.85	0.85	HAdV-D44
0.86	0.86	0.865	0.86	0.9	0.985	0.94	0.85	0.85	HAdV-D13
0.865	0.865	0.87	0.865	0.905	0.99	0.945	0.855	0.855	HAdV-D32
0.857	0.857	0.862	0.878	0.888	0.955	1	0.842	0.842	HAdV-D51
0.868	0.868	0.873	0.888	0.939	0.925	0.928	0.847	0.847	HAdV-D33
0.868	0.868	0.873	0.883	0.934	0.925	0.928	0.847	0.847	HAdV-D28
0.862	0.862	0.868	0.878	0.939	0.92	0.913	0.842	0.842	HAdV-D36
0.878	0.878	0.883	0.878	0.923	0.945	0.939	0.852	0.852	HAdV-D59
0.893	0.893	0.898	0.903	1	0.895	0.888	0.837	0.837	HAdV-D43
0.898	0.898	0.903	0.908	0.994	0.9	0.893	0.842	0.842	HAdV-D17
0.893	0.893	0.898	0.893	0.984	0.895	0.888	0.837	0.837	HAdV-D19C
0.893	0.893	0.898	0.893	0.984	0.895	0.888	0.837	0.837	HAdV-D64
0.903	0.903	0.908	0.903	0.989	0.905	0.898	0.847	0.847	HAdV-D29
0.883	0.883	0.888	0.923	0.923	0.875	0.868	0.852	0.852	HAdV-D8
0.883	0.883	0.888	0.923	0.923	0.875	0.868	0.852	0.852	HAdV-D30
0.873	0.873	0.878	0.913	0.903	0.86	0.857	0.847	0.847	HAdV-D54
0.888	0.888	0.893	0.928	0.913	0.86	0.857	0.862	0.862	HAdV-D10
0.888	0.888	0.893	0.928	0.913	0.86	0.857	0.862	0.862	HAdV-D56
0.888	0.888	0.893	0.928	0.913	0.86	0.857	0.862	0.862	HAdV-D9
0.888	0.888	0.893	0.918	0.903	0.85	0.847	0.852	0.852	HAdV-D26
0.888	0.888	0.893	0.918	0.918	0.86	0.857	0.852	0.852	HAdV-D58
0.868	0.868	0.873	0.923	0.908	0.865	0.868	0.852	0.852	HAdV-D25
0.883	0.883	0.888	0.928	0.923	0.86	0.857	0.842	0.842	HAdV-D47
0.873	0.873	0.878	0.898	0.908	0.88	0.888	0.857	0.857	HAdV-D46
0.878	0.878	0.883	0.903	0.913	0.885	0.893	0.862	0.862	HAdV-D67
0.994	0.994	1	0.868	0.898	0.87	0.862	0.861	0.861	HAdV-D49
0.984	0.984	0.989	0.862	0.888	0.86	0.852	0.856	0.856	HAdV-D19
0.979	0.979	0.973	0.862	0.893	0.855	0.847	0.846	0.846	HAdV-D23

Table 3. Amino acid sequence identity of samples 1-9 HVR compared to adenovirus reference types. Types with the highest similarity are highlighted. The table shows the percentage identity of amino acid sequences in nine human adenovirus (HAdV) hypervariable region (HVR) amplicons compared to five reference adenovirus HVR sequences.

Sample7	Sample6	Sample8	Sample4	Sample3	Sample2	sample9	Sample1	Sample5	Seq->
0.848	0.848	0.863	0.848	0.848	0.848	0.878	0.833	0.865	HAdV-D65
0.848	0.848	0.878	0.863	0.863	0.863	0.878	0.863	0.865	HAdV-D62
0.833	0.833	0.848	0.848	0.848	0.848	0.818	0.833	0.85	HAdV-D63
0.818	0.818	0.863	0.863	0.863	0.863	0.833	0.833	0.835	HAdV-D38
0.848	0.848	0.878	0.848	0.848	0.848	0.848	0.848	0.865	HAdV-D53
0.848	0.848	0.878	0.848	0.848	0.848	0.848	0.848	0.865	HAdV-D37
0.848	0.848	0.878	0.848	0.848	0.848	0.848	0.848	0.865	HAdV-D15
0.848	0.848	0.878	0.848	0.848	0.848	0.848	0.848	0.865	HAdV-D69
0.848	0.848	0.878	0.848	0.848	0.848	0.848	0.848	0.865	HAdV-D60
0.848	0.848	0.878	0.848	0.848	0.848	0.848	0.848	0.865	HAdV-D70
0.835	0.835	0.895	0.805	0.805	0.805	0.835	0.94	0.985	HAdV-D44
0.835	0.835	0.895	0.805	0.805	0.805	0.835	0.94	0.985	HAdV-D13
0.835	0.835	0.895	0.805	0.805	0.805	0.835	0.94	0.985	HAdV-D32
0.818	0.818	0.893	0.818	0.818	0.818	0.863	1	0.94	HAdV-D51
0.818	0.818	0.924	0.833	0.833	0.833	0.878	0.939	0.91	HAdV-D33
0.818	0.818	0.924	0.833	0.833	0.833	0.878	0.939	0.91	HAdV-D28
0.818	0.818	0.939	0.833	0.833	0.833	0.878	0.924	0.91	HAdV-D36
0.848	0.848	0.909	0.833	0.833	0.833	0.878	0.924	0.94	HAdV-D59
0.818	0.818	0.924	0.863	0.863	0.863	0.924	0.863	0.865	HAdV-D8
0.818	0.818	0.924	0.863	0.863	0.863	0.924	0.863	0.865	HAdV-D30
0.818	0.818	0.909	0.878	0.878	0.878	0.939	0.863	0.865	HAdV-D56
0.818	0.818	0.909	0.878	0.878	0.878	0.939	0.863	0.865	HAdV-D9
0.818	0.818	0.909	0.878	0.878	0.878	0.939	0.863	0.865	HAdV-D10
0.803	0.803	0.893	0.893	0.893	0.893	0.924	0.848	0.85	HAdV-D26
0.803	0.803	0.909	0.863	0.863	0.863	0.924	0.863	0.85	HAdV-D58
0.818	0.818	0.893	0.863	0.863	0.863	0.924	0.863	0.865	HAdV-D25
0.818	0.818	0.909	0.878	0.878	0.878	0.939	0.863	0.865	HAdV-D47
0.803	0.803	0.909	0.863	0.863	0.863	0.924	0.863	0.85	HAdV-D54
0.803	0.803	0.909	0.848	0.848	0.848	0.893	0.878	0.865	HAdV-D46
0.803	0.803	0.909	0.848	0.848	0.848	0.893	0.878	0.865	HAdV-D67
0.843	0.843	0.863	1	1	1	0.863	0.818	0.82	HAdV-D49
0.843	0.843	0.863	1	1	1	0.863	0.818	0.82	HAdV-D19
0.843	0.843	0.863	1	1	1	0.863	0.818	0.82	HAdV-D48
0.812	0.812	0.893	0.968	0.968	0.968	0.863	0.833	0.82	HAdV-D23
0.818	0.818	1	0.863	0.863	0.863	0.893	0.893	0.88	HAdV-D43
0.818	0.818	0.984	0.863	0.863	0.863	0.893	0.893	0.88	HAdV-D64
0.848	0.848	0.924	0.848	0.848	0.848	0.909	0.909	0.895	HAdV-D20
1	1	0.818	0.843	0.843	0.843	0.803	0.818	0.85	HAdV-D24
1	1	0.818	0.843	0.843	0.843	0.803	0.818	0.85	HAdV-D27
0.92	0.92	0.833	0.843	0.843	0.843	0.787	0.833	0.85	HAdV-D45

DISCUSSION

The hexon, fiber, and penton proteins are human adenovirus's primary antigenic determinants. Each capsid gene contains at least one hypervariable region, and most amino acid substitutions occur within these regions[18]. However, homologous recombination is a key mechanism of evolution for species D adenoviruses, and the recombination rate in this species is higher than in other adenovirus species[19]. New species D adenovirus types could emerge through recombination, altering virulence, tropism, or transmission ability. For example, HAdV-D53 results from recombination between types 37, 22, and 8 and a previously unknown type[20]. Another example is HAdV-D70, which results from recombination between types 29, 58, 13, and a previously unknown type[4].

Analysis of the partial penton region containing the HVR region revealed that only three isolates (1, 2, and 8) had identical typing results for the main structural proteins encoded by this region. The remaining six isolates had a novelty of penton types despite having the same type in the HVR region, suggesting that they have not been reported. Samples 3 and 4 had identical sequences in the studied region of the genome, as did samples 6 and 7.

None of the adenovirus isolates with conflicting typing results between hexon/fiber and penton had an identical penton type to any existing HAdV types 1–70. The divergence between these isolates and the closest type was between 1.2% and 3.5%. However, the two isolates 6 and 7 had an amino acid sequence in the penton region that was identical to HAdV-D24, which indicates that these samples are variants of this type in the penton region.

Adenovirus isolates 3, 4, 5, and 9 had penton sequences significantly different from the closest known types. Nucleotide divergence ranged from 2.6% to 3.5%, while amino acid divergence ranged from 1% to 4%. These divergences are higher than the divergence between some existing penton types, suggesting that the sequences of these isolates may represent new penton types. This could be the result of recombination events within the penton gene itself.

Most previously documented adenovirus recombination events involved the hexon gene from one type and the fiber gene from another. The resulting strains are called intermediate strains. However, other recombination events have been reported, such as recombination between the penton gene from one type and the hexon/fiber genes from another (Robinson et al., 2009). This type of recombination usually occurs as part of a multi-recombination event involving the hexon and fiber genes. However, in the current study, all isolates had the same type in this region.

Adenovirus isolates 3 and 4 have penton genes closely related to those of HAdV-D 19 and 23 but with 2.6% nucleotide divergence and 1% amino acid divergence. This suggests that these isolates carry a new variant of the penton gene that has not been reported before. The divergence between the penton genes of isolates 3 and 4 is like that between the penton genes of HAdV-D 19 and 23.

Analysis of isolate 5 showed that it clustered differently from other isolates and had a sequence similar to the HVR of type 32, with nucleotide and amino acid divergences of 1% and 1.5%, respectively. There was no evidence of recombination within the penton of samples 6 and 7, as these isolates clustered with HAdV-D 24 and appeared to be variants of this penton type with similar nucleotide sequences and identical amino acid sequences. Sample 9 clustered with types 58 and 65 but high divergences of 3.2% and 3.3%, respectively. Analysis of the HVR region of sample 9 showed an unidentified sequence at this region with a different clustering pattern and a divergence of 7.2% from the closest type.

Adenovirus species D is a promising vector for gene therapy. Still, it is important to understand adenovirus's stability and its evolution mechanisms to develop safe and effective vectors. Recombination between wild-type adenovirus and adenovirus vectors is a potential concern, so it is important to be able to accurately type adenovirus isolates and avoid novelty strains. The results of this study suggest that neither serology nor limited sequencing of one or two regions of the adenovirus genome is sufficient for typing. Full genome sequencing is the best way to type adenoviruses.

Conflicts of Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agreed with the manuscript's contents, and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Disclosure

The authors did not receive any form of commercial support, either in the form of compensation or financial assistance, for this case report. The authors have no financial interest in any of the products, devices, or drugs mentioned in this article.

Ethical Approval

The study was approved by the Ethics Committee of the KAUH in Jeddah, Kingdom of Saudi Arabia, also known as the Institutional Review Board of Hospitals.

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