ORIGINAL ARTICLE

Recombination Dynamics Divergent of the RGD Region of Penton, Hexon, and Fibre Genes for Adenovirus

Basem A. Jawa[1], Khulud A. Alhazmi[2], Rehab G. Alqurashi[3], Ghazi S. Alkhaldi[4], Karem A. Ibrahem[5], Rashad G. Rawzi[1], Fawzi S. Almatrafi[1], Mohammed A. Moazen[1], Mohammed A. Kurdi[4], Fouad A. Bantn[1], Ahmed A. Khan[4], Fahad T. Faqeeh[1], Ahmad A. Saati[4], Eman A. Mulla[4], Edhah S. Alsaeedi[4], Zainab A. Alkhayri[4], Zain Z. Alzubaidi[4], Mazin S. Alqurashi[1], Thamer N. Alotaibi[1], Alaa M. Alsaggaf[6], Mohamed A. Bazaid[4], Faisal H. Alzahrani[1], Shirin M. Kaki[1], Abeer H. Najjar[7], Ahmad M Sait[9,10] and Hammad H. Alsuwat[8]

[1]Department of Laboratory and Blood Bank, Alnoor Specialist Hospital, Makkah Health Cluster, Makkah, Saudi Arabia
[2]Department of Microbiology and Parasitology, Faculty of Medicine, Umm Alqura University, Makkah, Saudi Arabia
[3]Infection prevention and control, Makkah medical cluster, Ministry of Health, Makkah, Saudi Arabia
[4]Depratment of Laboratory, Regional Lab, Ministry of Health, Makkah, Saudi Arabia.
[5]Department of Clinical Microbiology and Immunology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[6]Department of Laboratory and Blood Bank, King Salman Bin Abdulaziz Medical City, Madinah Health Cluster, Madinah, Saudi Arabia
[7]Department of Poison Control Forensic Chemistry, Ministry of Health, Makkah, Saudi Arabia
[8]Department of Laboratory and Blood Bank, Asir Health Cluster, Asir, Saudi Arabia
[9]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[10]Regenerative Medicine Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah 21589, Saudi Arabia

Correspondence
Dr. Basem A. Jawa
Department of Laboratory and Blood Bank, Alnoor Specialist Hospital, Ministry of Health, Makkah 21955, Saudi Arabia
e-M: bajawa@moh.gov.sa

Submission: 26 Oct. 2024
Accepted: 15 Nov. 2024

Citation
Jawa BA, Alhazmi KA, Alqurashi RG, Alkhaldi GS, Ibrahem KA, Rawzi RG, Almatrafi FS, Moazen MA, Kurdi MA, Bantn FA, Khan AA, Faqeeh FT, Saati AA, Mulla EA, Alsaeedi ES, Alkhayri ZA, Alzubaidi ZZ, Alqurashi MS, Alotaibi TN, Alsaggaf AM, Bazaid MA, Alzahrani FH, Kaki SM, Najjar AH. Recombination dynamics divergent of the RGD region of penton, hexon, and fibre genes for adenovirus. JKAU Med Sci 2024; 31(2): 21-34. DOI: 10.4197/Med.31-2.3.

Abstract

A Recent research on adenoviruses has mainly concentrated on their potential as vectors for gene therapy and vaccines, with less attention given to their evolutionary patterns and genome stability. Traditional typing methods, which rely on serological assays, have proven inadequate for classifying adenoviruses due to their genetic diversity. To address this, sequencing is often limited to key regions such as the hexon and fiber genes. However, recent studies suggest that relying solely on these regions may not fully capture the genetic landscape of adenoviruses. Notably, multiple recombination events have been identified within the adenovirus genome, raising concerns about the reliability of partial sequencing approaches for precise typing. In this study, we sequenced the penton gene in nine adenovirus isolates with previously characterized hexon and fiber types. Our analysis revealed that six of these isolates had penton sequences distinct from their assigned hexon and fiber types, suggesting recombination between adenovirus types. Additionally, four of these isolates displayed penton gene sequences that diverged significantly from known adenovirus types. Further examination uncovered potential recombination within the penton gene itself, particularly between the hypervariable region and the RGD (Arg-Gly-Asp) loop. These findings underscore the high prevalence of recombination in species D adenoviruses, not only between the hexon, fiber, and penton genes but also within the penton gene itself. This points to the limitations of hexon/fiber typing alone and emphasizes the need for full-genome sequencing to ensure accurate adenovirus classification. These insights are critical for advancing adenovirus-based vector development, as they contribute to a better understanding of adenovirus genetic stability and evolution.

Keywords

Adenovirus genome recombination, Adenovirus vector development, Full-genome sequencing, Evolutionary diversity

INTRODUCTION

Adenoviruses (AdVs) are double-stranded DNA viruses belonging to the family Adenoviridae, which encompass a wide range of subtypes infecting various species, including humans, animals, and birds[1]. Human adenoviruses (HAdVs) are significant pathogens responsible for a variety of clinical manifestations, including respiratory infections, gastroenteritis, conjunctivitis, and in some cases, severe disease in immunocompromised individuals[2]. The substantial genomic diversity among HAdVs necessitates precise and robust methods for classification and typing, which are crucial for epidemiological surveillance, clinical diagnostics, and the development of targeted therapeutic interventions[3].

Historically, adenovirus classification has relied on serological typing based on neutralization assays[4]. However, these methods are time-consuming, labor-intensive, and sometimes need more sensitivity and specificity[5]. The advent of molecular techniques, particularly polymerase chain reaction (PCR) and sequencing, has revolutionized adenovirus typing, providing more precise and rapid identification of HAdV genotypes[6]. Among the various genomic regions analyzed, the hypervariable region (HVR) of the penton gene has emerged as a particularly promising target for molecular typing due to its high sequence variability[7]. The penton base protein, a crucial structural component of the adenovirus capsid, plays a pivotal role in virus-host interactions, including cell entry and immune evasion[8]. The HVR within the penton gene exhibits significant sequence diversity, reflecting the evolutionary pressures exerted by host immune responses and contributing to the genetic heterogeneity observed among adenovirus subtypes[8].

Consequently, the HVR region serves as a valuable marker for distinguishing between closely related adenovirus strains and for identifying novel genotypes[9]. The significance of this research extends beyond mere classification. Accurate typing of adenoviruses is essential for understanding the epidemiology of HAdV infections, particularly in outbreak settings where rapid identification of the causative agent can inform public health responses[10]. Furthermore, the identification of novel variants and recombination events can provide insights into adenovirus evolution and pathogenesis, with implications for vaccine design and antiviral strategies[11].

The penton base protein is a crucial component of the adenovirus capsid, forming the penton complex along with the fiber protein. It plays a pivotal role in virus attachment and entry into host cells, interacting with integrins to mediate internalization[12]. The penton base gene exhibits significant genetic variability, particularly in its hypervariable regions (HVRs), which are subject to immune selection pressures. The HVRs within the penton base gene are characterized by high sequence diversity, making them ideal targets for molecular typing. This variability reflects the evolutionary pressures exerted by host immune responses and contributes to the antigenic diversity of HAdVs[13]. Studies have shown that the penton HVRs can distinguish between closely related adenovirus types and identify novel variants[14,15].

Adenovirus classification has been based on serological methods, particularly neutralization assays, which identify virus types based on their reactivity with specific antisera[16]. While serological typing has been instrumental in the initial classification of AdVs, it has several limitations, including the requirement for virus culture, cross-reactivity between closely related types, and limited sensitivity. The advent of molecular techniques has revolutionized adenovirus typing. Polymerase chain reaction (PCR) and sequencing have emerged as the gold standards for accurate and rapid identification of HAdV genotypes[17]. Molecular typing targets various genomic regions such as the hexon, fiber, and penton genes, which encode key structural proteins of the virus capsid.

The objective of this study is to investigate the utility of limited typing of the hypervariable region (HVR) of the penton gene in adenovirus for identifying virus genotypes. Specifically, the study aims to assess whether sequencing and phylogenetic analysis of the penton HVR region are sufficient to classify subtypes of human adenovirus (HAdV). This involves examining the nucleotide and amino acid sequences of the penton HVR region from clinical isolates and comparing them with known reference sequences to determine their relatedness and potential novelty. The study also aims to evaluate the divergence between isolates and known types, assess potential recombination events within the penton gene, and discuss the implications of these findings for adenovirus typing and vaccine development.

METHODOLOGY

DNA EXTRACTION

The extraction of DNA was conducted using the QIAamp (QIAGEN, a biotechnology company based in Germany) DNA Mini Kit, in accordance with the manufacturer's instructions. Initially, Q-Pro and AL buffers were added to the sample contained within a microcentrifuge tube. The mixture was vortexed thoroughly and subsequently incubated at 56°C for 15 minutes to facilitate cell lysis. After incubation, the tube was centrifuged and ethanol was added to the mixture to precipitate the DNA. The resulting solution was then transferred to a QIAamp spin column and centrifuged for 1 minute to allow the DNA to bind to the silica membrane within the column. The filtrate was discarded, and the column was placed into a new collection tube. To wash the DNA, AW1 buffer was added to the column, which was then centrifuged for 1 minute. This step was followed by another wash with AW2 buffer, after which the column was centrifuged for 3 minutes to ensure the thorough removal of contaminants. The final elution of the purified DNA was performed by adding AE buffer to the column and centrifuging for 1 minute. The eluted DNA was then stored at -20°C for subsequent use.

POLYMERASE CHAIN REACTION (PCR)

Conventional PCR was employed to amplify the partial penton region, encompassing the hypervariable region (HVR). The specific primers used for amplification were designed as follows: the forward primer sequence was TTCGCAAGAAGCAACCTTT, and the reverse primer sequence was TCTTGCATGAGGTCCGG. The PCR master mix (Thermo Fisher Scientific, United States) was prepared with the following components: 5 µl of 10X PCR buffer, 1 µl of 0.2 mM dNTPs, 0.5 µl of 0.2 µM forward primer, 0.5 µl of 0.2 µM reverse primer, 0.25 µl of 1.25 units/µl Taq DNA polymerase, and 37.75 µl of sterile distilled water. To this mixture, 5 µl of the DNA template or a control sample was added, making the final reaction volume 50 µl.

The PCR cycling program began with an initial denaturation step at 95°C for 15 minutes to activate the Taq DNA polymerase. This was followed by 40 cycles of amplification, each consisting of 20 seconds at 94°C for denaturation, 20 seconds at 57°C for primer annealing, and 40 seconds at 72°C for elongation. The final extension step involved a 5-minute incubation at 72°C to ensure complete extension of the amplified products. After PCR amplification, the amplicons were analyzed by electrophoresis on a 2% agarose E-gel. The molecular weights of the PCR products were determined by comparison with a DNA ladder, which contained fragments ranging from 100 bp to 2500 bp.

forward primer	TTCGCAAGAAGCAACCTTT,
reverse primer	TCTTGCATGAGGTCCGG

PHYLOGENETIC ANALYSIS

The nucleotide sequences of the penton region, specifically the 918 bases corresponding to the HVR of HAdV-D9, along with the deduced amino acid sequences, were used to construct phylogenetic trees to elucidate the evolutionary relationships among species D human adenoviruses. Sequence editing was performed using BioEdit version 7.0.5. Multiple sequence alignments were calculated using ClustalX version 1.83 to ensure precise alignment of the sequences.

Phylogenetic trees were constructed utilizing the Phylo-Win software version 2. To assess the robustness of the phylogenetic groupings, bootstrap analysis was performed with 1,000 pseudoreplicates. The phylogenetic trees were subsequently visualized and drawn using TreeView version 1.6.6. This comprehensive analysis allowed for the detailed examination of the genetic relationships and evolutionary patterns among the adenovirus isolates under study, particularly within the hypervariable regions of the penton gene.

RESULTS

PCR AMPLIFICATION OF CLINICAL ISOLATE

The DNA from the nine adenovirus isolates was extracted, and a specific region of the penton gene was amplified using PCR. Two primers were designed to generate around 600-base pair fragments of the penton gene. The amplified products, along with positive control fragments of 400 base pairs, were visualized on a gel electrophoresis image (Figure 1).

TYPING AND PHYLOGENETIC ANALYSIS OF ADENOVIRUS ISOLATES

Nine clinical samples from individuals with AIDS were analyzed using DNA sequencing and the phylogenetic analysis of the hexon and fiber genes. These isolates were identified as belonging to the HAdV-D species,

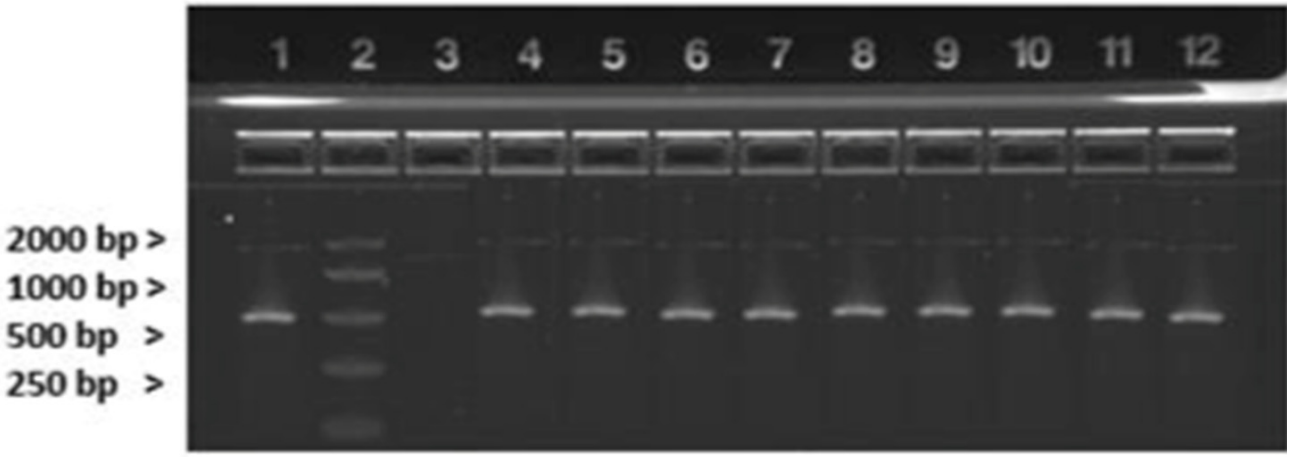

Figure 1. **Two percent E-agarose gel of RGD region. The size of the region is 551 base pairs. Lane 1: Positive Control. Lane 2: Quantitative DNA easy ladder. Lane 3: Negative Control. Lane 4: Sample 1. Lane 5: Sample 2. Lane 6: Sample 3. Lane 7: Sample 4. Lane 8: Sample 5. Lane 9: sample 6. Lane 10: 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

with consistent typing results for both the hexon and fiber regions. The previous typing results are summarized in Table 1.

PHYLOGENETIC ANALYSIS REVEALS GENETIC RELATIONSHIPS OF NEW ADENOVIRUS TYPES WITH CLINICAL ISOLATES

The phylogenetic analysis of the penton gene revealed the presence of multiple distinct clades within the HAdV-D species. Notably, several clinical isolates from AIDS patients clustered closely with specific reference strains, indicating their classification within established adenovirus types. However, the tree also highlighted the presence of several isolates that did not cluster with any known HAdV type, suggesting potential novel or recombinant strains. These findings underscore the genetic diversity within the HAdV-D species and emphasize the need for comprehensive genomic analysis to accurately characterize and classify adenoviruses, particularly in immunocompromised individuals, as shown in Figures 2 and 3.

The phylogenetic analysis of the penton gene revealed a complex pattern of divergence within the HAdV-D species. While some clinical isolates clustered closely with established reference strains, indicating a high degree of sequence similarity, others exhibited substantial divergence. This divergence may be attributed to several factors, including genetic recombination, rapid evolution, and potential adaptation to specific host environments. Further genomic analysis is necessary to elucidate the precise mechanisms underlying this divergence and its implications for adenovirus epidemiology and pathogenesis.

The table presents the nucleotide sequence and amino acids identity of the RGD region in nine clinical adenovirus samples compared to various reference adenovirus types. The highest similarity scores for each sample are highlighted, as shown in Tables 2 and 3.

The nucleotide and amino acid alignment of the RGD region of the penton gene revealed a high degree of sequence conservation among HAdV-D23, HAdV-D63, and two clinical samples. While nucleotide differences were observed, particularly in the hypervariable regions, the amino acid sequence remained largely conserved, suggesting strong functional constraints.

The presence of a few amino acid variations in the RGD loop region of the clinical samples may indicate potential recombination events or unique adaptations. Further analysis is required to elucidate the functional implications of these sequence variations and their impact on viral pathogenesis and host interactions, as illustrated in Figures 4, 5, 6, and 7.

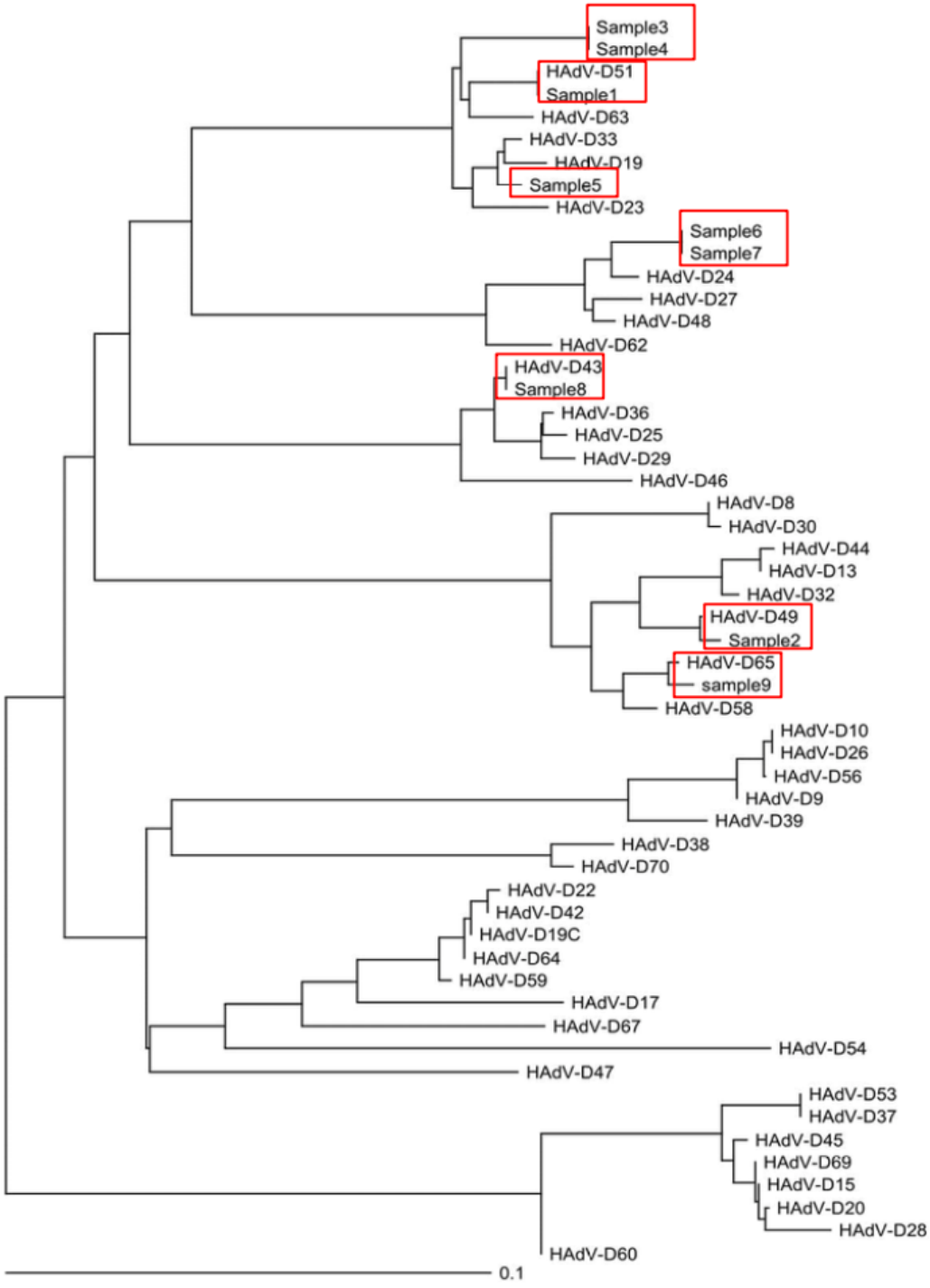

Figure 2. Nucleotide phylogenetic tree of all clinical isolates. The tree was built from the penton RGD region from prototype reference sequences and isolates sequences.

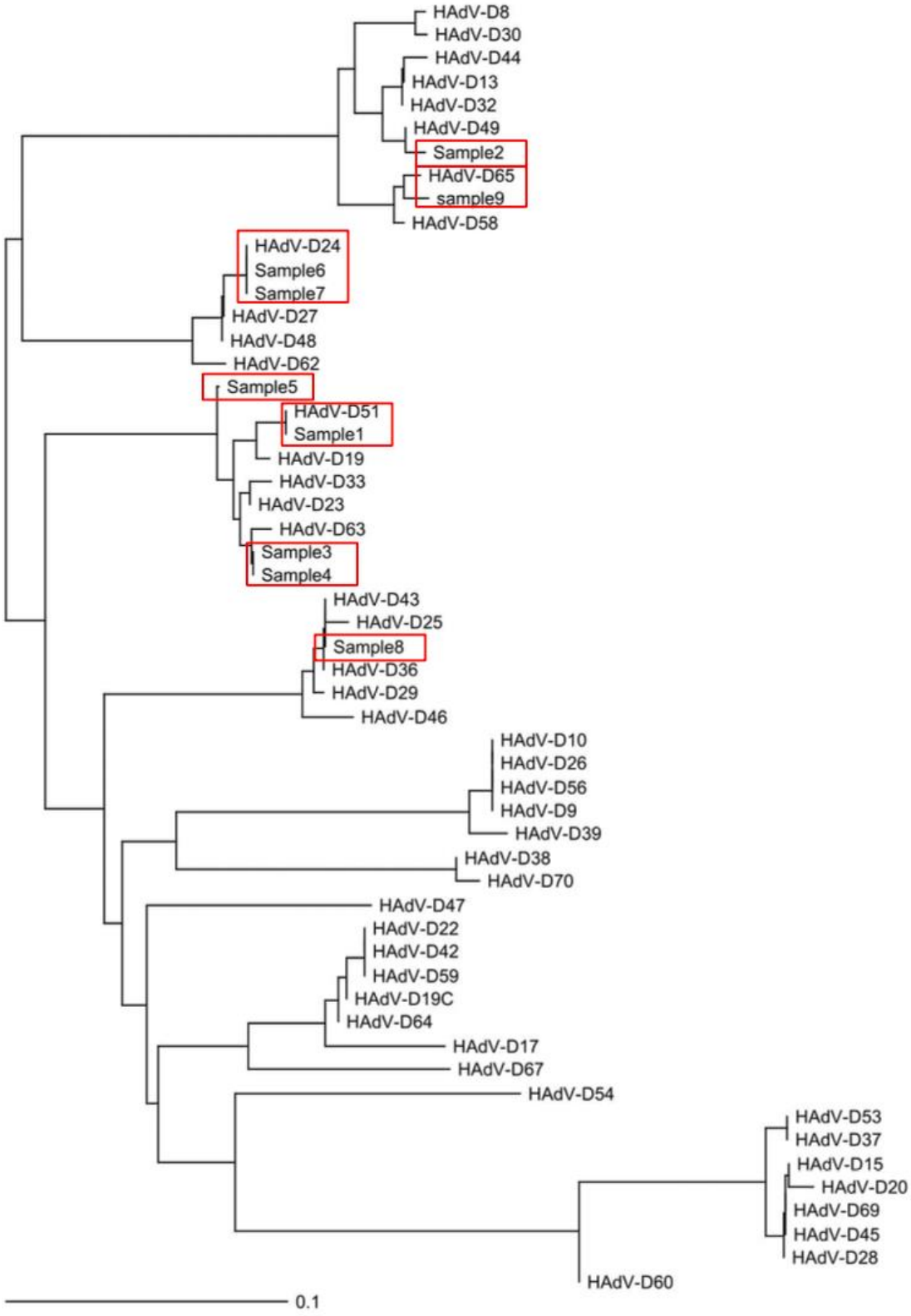

Figure 3. Amino acid phylogenetic tree of all clinical isolates. The tree was built from the penton RGD region from prototype reference sequences and isolates sequences.

Table 2. Nucleotide sequences identity of samples 1-9 RGD region comparing to adenovirus references. Types with highest similarity are highlighted.

Sample8	Sample1	Sample5	Sample4	Sample3	Sample7	Sample6	sample9	Sample2	Seq->
0.805	0.818	0.801	0.813	0.813	0.808	0.808	0.795	0.793	HAdV-D22
0.807	0.82	0.803	0.816	0.816	0.811	0.811	0.797	0.795	HAdV-D42
0.793	0.808	0.791	0.804	0.804	0.794	0.794	0.783	0.781	HAdV-D19C
0.793	0.808	0.791	0.804	0.804	0.794	0.794	0.783	0.781	HAdV-D64
0.814	0.827	0.81	0.818	0.818	0.816	0.816	0.802	0.8	HAdV-D59
0.789	0.806	0.784	0.797	0.797	0.787	0.787	0.781	0.781	HAdV-D17
0.789	0.813	0.791	0.792	0.792	0.801	0.801	0.788	0.783	HAdV-D67
0.779	0.764	0.756	0.766	0.766	0.778	0.778	0.741	0.744	HAdV-D54
0.803	0.778	0.761	0.773	0.773	0.757	0.757	0.755	0.753	HAdV-D10
0.803	0.778	0.761	0.773	0.773	0.757	0.757	0.755	0.753	HAdV-D26
0.803	0.778	0.761	0.773	0.773	0.754	0.754	0.758	0.755	HAdV-D56
0.808	0.783	0.766	0.778	0.778	0.759	0.759	0.762	0.76	HAdV-D9
0.822	0.787	0.766	0.778	0.778	0.766	0.766	0.769	0.765	HAdV-D39
0.803	0.794	0.777	0.785	0.785	0.792	0.792	0.783	0.781	HAdV-D38
0.81	0.799	0.782	0.794	0.794	0.799	0.799	0.79	0.788	HAdV-D70
0.829	0.811	0.803	0.808	0.808	0.778	0.778	0.753	0.76	HAdV-D47
0.802	0.79	0.767	0.781	0.781	0.795	0.795	0.937	0.951	HAdV-D8
0.8	0.79	0.767	0.779	0.779	0.793	0.793	0.934	0.948	HAdV-D30
0.797	0.772	0.755	0.781	0.781	0.783	0.783	0.944	0.958	HAdV-D44
0.8	0.774	0.758	0.783	0.783	0.786	0.786	0.946	0.96	HAdV-D13
0.802	0.781	0.765	0.79	0.79	0.788	0.788	0.948	0.962	HAdV-D32
0.816	0.786	0.769	0.786	0.786	0.788	0.788	0.958	0.995	HAdV-D49
0.811	0.797	0.779	0.786	0.786	0.8	0.8	0.993	0.955	HAdV-D65
0.82	0.795	0.779	0.79	0.79	0.8	0.8	0.979	0.96	HAdV-D58
0.801	0.856	0.842	0.839	0.839	0.98	0.98	0.793	0.786	HAdV-D24
0.801	0.854	0.839	0.846	0.846	0.964	0.964	0.79	0.788	HAdV-D27
0.803	0.858	0.844	0.842	0.842	0.968	0.968	0.8	0.793	HAdV-D48
0.827	0.873	0.846	0.856	0.856	0.952	0.952	0.816	0.804	HAdV-D62
0.848	0.968	0.961	0.954	0.954	0.849	0.849	0.79	0.776	HAdV-D33
0.843	0.971	0.956	0.947	0.947	0.842	0.842	0.79	0.776	HAdV-D19
0.831	0.956	0.947	0.961	0.961	0.844	0.844	0.781	0.772	HAdV-D23
0.843	1	0.937	0.959	0.959	0.844	0.844	0.795	0.781	HAdV-D51
0.845	0.973	0.944	0.959	0.959	0.846	0.846	0.802	0.793	HAdV-D63
1	0.843	0.829	0.831	0.831	0.799	0.799	0.809	0.811	HAdV-D43
0.985	0.831	0.815	0.817	0.817	0.794	0.794	0.802	0.804	HAdV-D36
0.983	0.829	0.813	0.815	0.815	0.792	0.792	0.8	0.802	HAdV-D25
0.981	0.829	0.813	0.815	0.815	0.792	0.792	0.8	0.802	HAdV-D29
0.95	0.808	0.792	0.806	0.806	0.785	0.785	0.781	0.786	HAdV-D46
0.768	0.761	0.747	0.752	0.752	0.721	0.721	0.737	0.737	HAdV-D53
0.768	0.761	0.747	0.752	0.752	0.721	0.721	0.737	0.737	HAdV-D37
0.672	0.691	0.711	0.684	0.684	0.65	0.65	0.648	0.648	HAdV-D60

Table 3. Amino acid sequences identity of samples 1-9 RGD compared to adenovirus reference types. Types with the highest similarity are highlighted.

Sample8	Sample5	Sample1	Sample4	Sample3	Sample7	Sample6	sample9	Sample2	Seq->
0.783	0.807	0.821	0.828	0.828	0.828	0.828	0.77	0.77	HAdV-D22
0.783	0.807	0.821	0.828	0.828	0.828	0.828	0.77	0.77	HAdV-D42
0.783	0.807	0.821	0.828	0.828	0.828	0.828	0.77	0.77	HAdV-D59
0.776	0.8	0.814	0.821	0.821	0.821	0.821	0.763	0.763	HAdV-D64
0.762	0.785	0.8	0.807	0.807	0.807	0.807	0.75	0.756	HAdV-D17
0.755	0.8	0.807	0.807	0.807	0.792	0.792	0.75	0.75	HAdV-D67
0.763	0.756	0.777	0.777	0.777	0.777	0.777	0.93	0.958	HAdV-D8
0.763	0.756	0.777	0.777	0.777	0.777	0.777	0.93	0.958	HAdV-D30
0.777	0.743	0.756	0.763	0.763	0.777	0.777	0.951	0.972	HAdV-D44
0.784	0.75	0.763	0.77	0.77	0.784	0.784	0.958	0.979	HAdV-D13
0.784	0.75	0.763	0.77	0.77	0.784	0.784	0.958	0.979	HAdV-D32
0.784	0.75	0.763	0.77	0.77	0.777	0.777	0.958	0.993	HAdV-D49
0.77	0.756	0.77	0.77	0.77	0.777	0.777	0.986	0.951	HAdV-D65
0.77	0.756	0.77	0.777	0.777	0.784	0.784	0.986	0.958	HAdV-D58
0.776	0.828	0.842	0.85	0.85	1	1	0.777	0.77	HAdV-D24
0.783	0.835	0.85	0.857	0.857	0.992	0.992	0.784	0.777	HAdV-D27
0.783	0.835	0.85	0.857	0.857	0.992	0.992	0.784	0.777	HAdV-D48
0.783	0.828	0.842	0.85	0.85	0.971	0.971	0.791	0.784	HAdV-D62
0.832	0.95	0.971	0.992	0.992	0.842	0.842	0.77	0.763	HAdV-D63
0.825	0.957	0.964	0.985	0.985	0.842	0.842	0.756	0.75	HAdV-D33
0.832	0.964	0.971	0.992	0.992	0.85	0.85	0.763	0.756	HAdV-D23
0.825	0.942	1	0.978	0.978	0.842	0.842	0.763	0.756	HAdV-D51
0.832	0.957	0.985	0.978	0.978	0.835	0.835	0.756	0.75	HAdV-D19
1	0.825	0.825	0.825	0.825	0.776	0.776	0.763	0.777	HAdV-D43
0.993	0.818	0.818	0.818	0.818	0.769	0.769	0.756	0.77	HAdV-D25
1	0.825	0.825	0.825	0.825	0.776	0.776	0.763	0.777	HAdV-D36
0.993	0.825	0.825	0.825	0.825	0.776	0.776	0.763	0.777	HAdV-D29
0.979	0.811	0.811	0.811	0.811	0.762	0.762	0.756	0.77	HAdV-D46
0.804	0.767	0.762	0.769	0.769	0.748	0.748	0.756	0.756	HAdV-D10
0.804	0.767	0.762	0.769	0.769	0.748	0.748	0.756	0.756	HAdV-D26
0.804	0.767	0.762	0.769	0.769	0.748	0.748	0.756	0.756	HAdV-D56
0.804	0.767	0.762	0.769	0.769	0.748	0.748	0.756	0.756	HAdV-D9
0.804	0.76	0.755	0.762	0.762	0.741	0.741	0.75	0.75	HAdV-D39
0.79	0.785	0.774	0.774	0.774	0.788	0.788	0.756	0.77	HAdV-D38
0.783	0.778	0.767	0.767	0.767	0.781	0.781	0.75	0.763	HAdV-D70
0.839	0.821	0.814	0.835	0.835	0.785	0.785	0.722	0.729	HAdV-D47
0.79	0.771	0.778	0.778	0.778	0.778	0.778	0.701	0.708	HAdV-D54
0.734	0.704	0.69	0.697	0.697	0.69	0.69	0.694	0.701	HAdV-D69
0.734	0.704	0.69	0.697	0.697	0.69	0.69	0.694	0.701	HAdV-D45
0.657	0.705	0.671	0.671	0.671	0.657	0.657	0.659	0.666	HAdV-D60

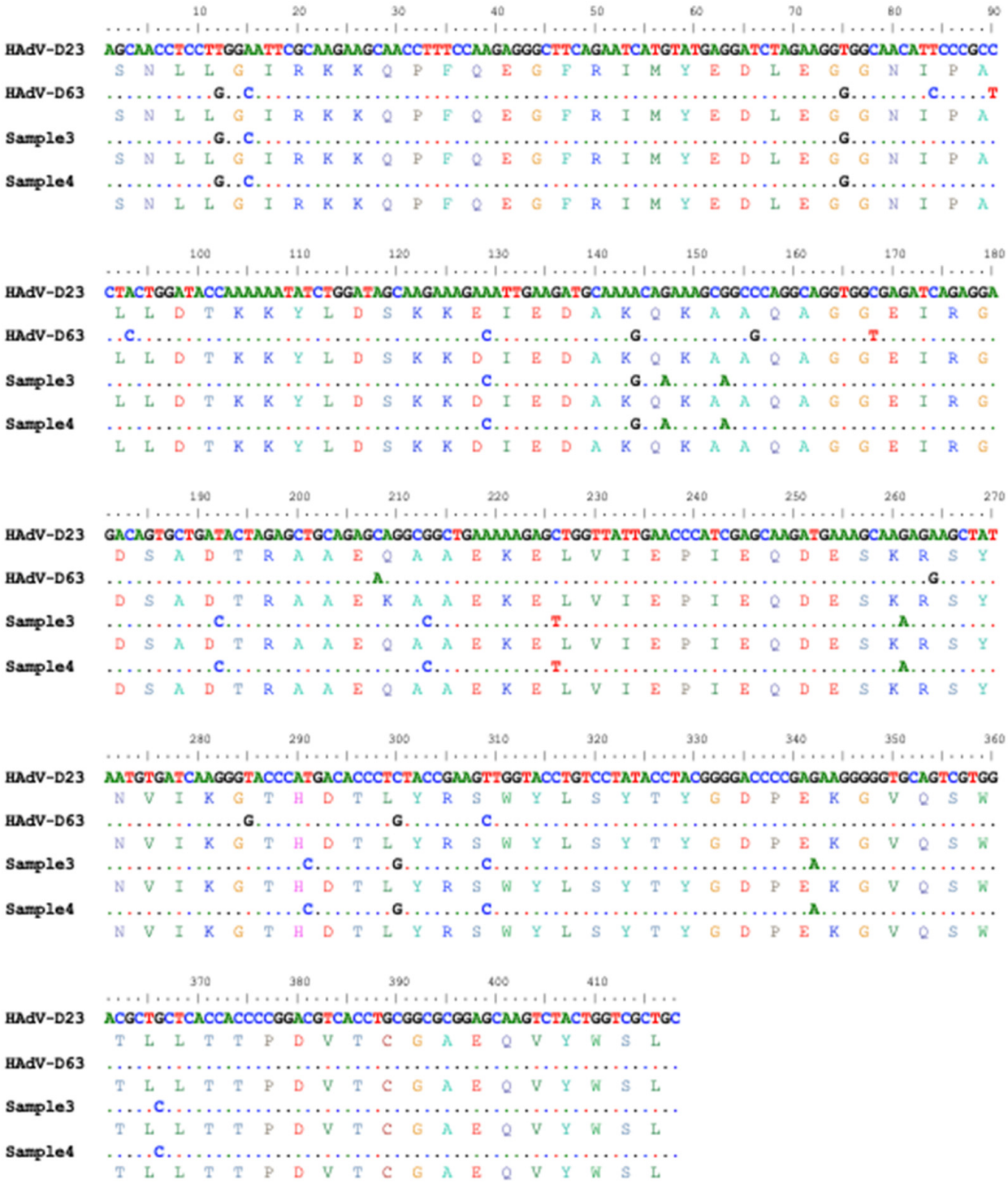

Figure 4. **Nucleotide and amino acid alignment of the RGD region of the penton of HAdV-D 19, 48, and 49 with samples 3 and 4.**

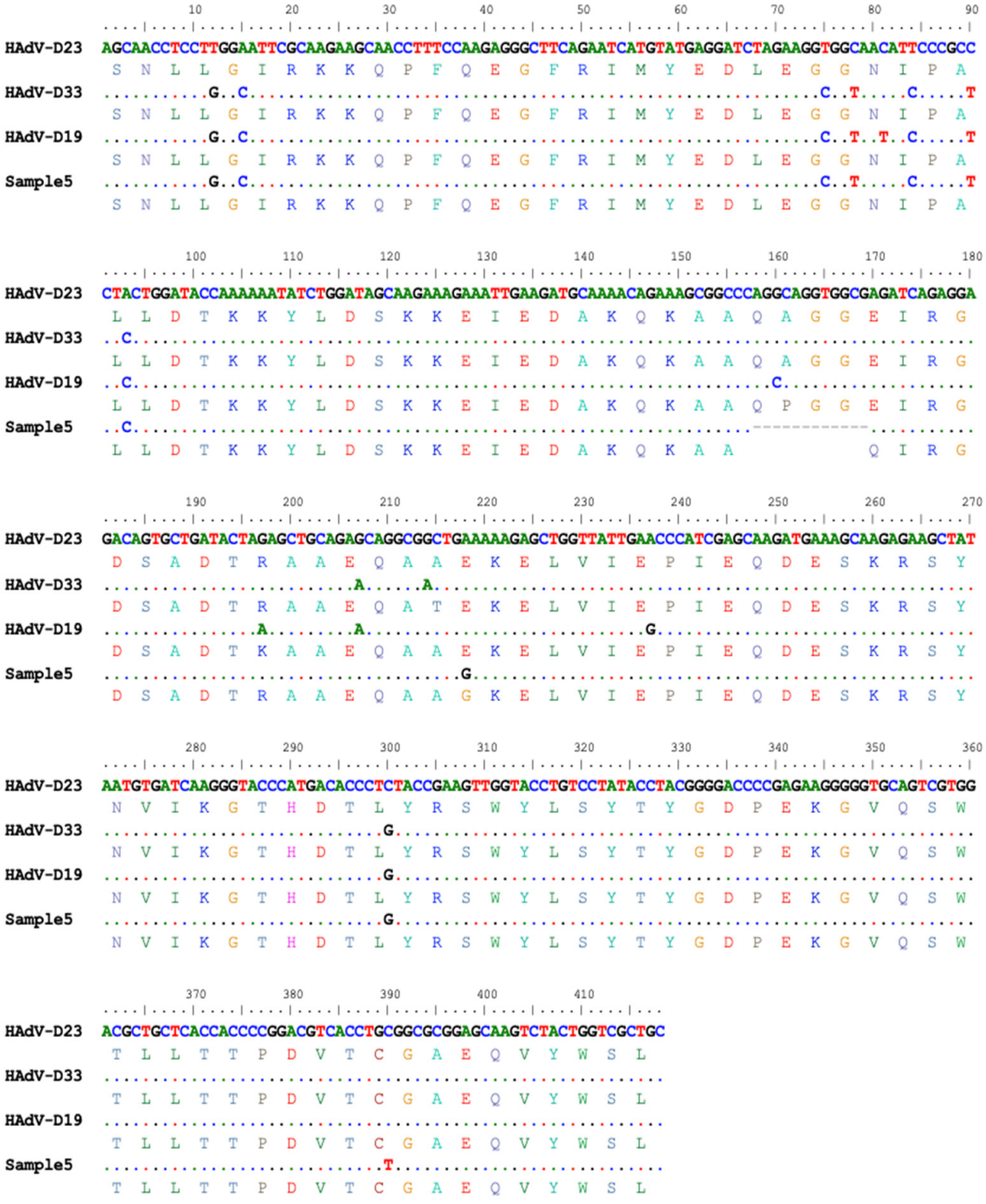

Figure 5. **Nucleotide and amino acid alignment of the RGD region of the penton of HAdV-D 13, 32, 44, 51, and 59 with samples 5.**

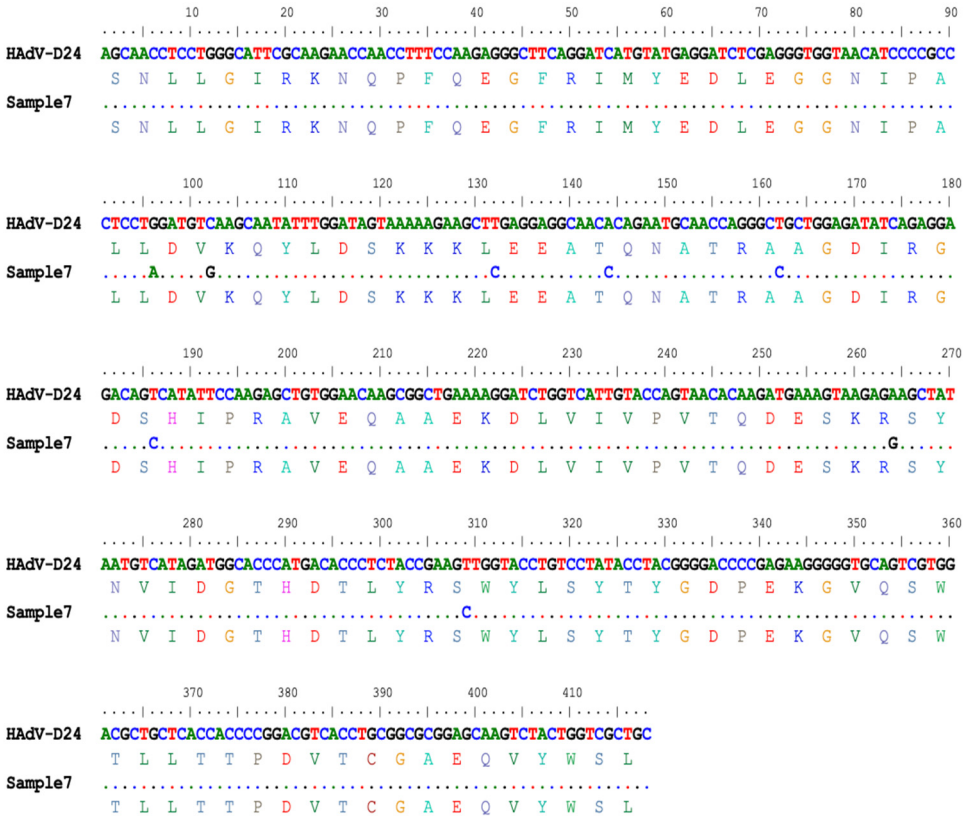

Figure 6. Nucleotide and amino acid alignment of the RGD region of the penton of HAdV-D 24 with sample 7.

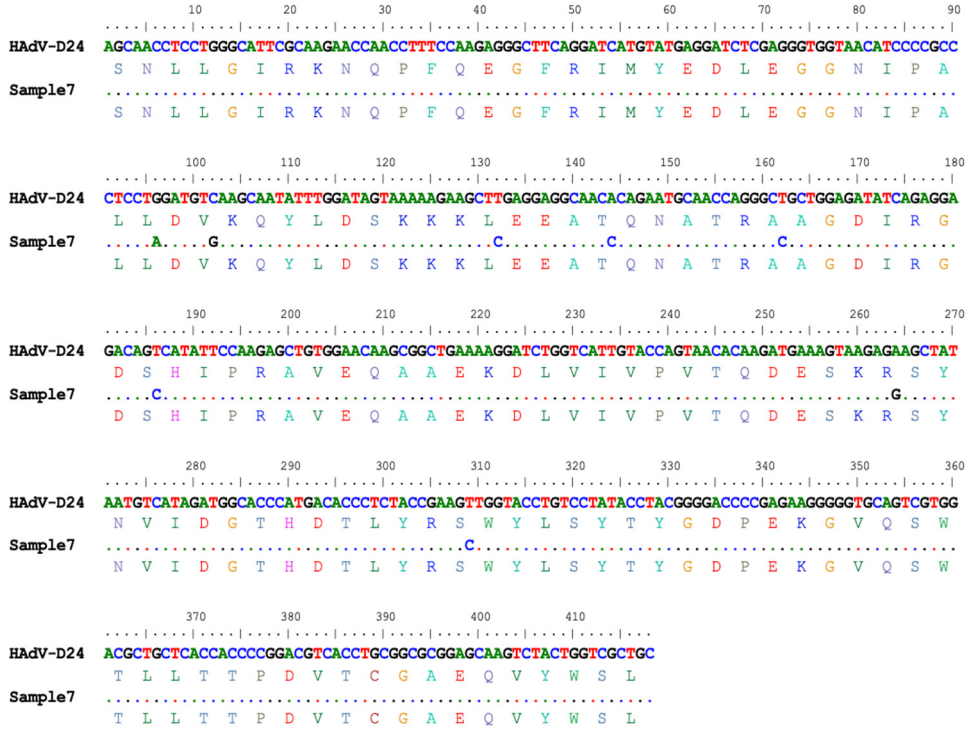

Figure 7. Nucleotide and amino acid alignment of the RGD region of the penton of HAdV-D 9, 10, 47, and 56 with samples 9.

DISCUSSION

The novelty of this study lies in its comprehensive analysis of the penton gene across multiple adenovirus isolates, revealing new insights into adenovirus genetic diversity, especially within species HAdV-D, in immunocompromised patients. Unlike traditional typing that focuses only on the hexon and fiber genes, this study includes an investigation into the penton gene's genetic diversity, identifying recombination events and distinct clades. Such findings suggest that limited-region typing approaches may overlook critical genomic diversity and misclassify adenovirus strains. This study not only detects isolates that do not align with known HAdV types, suggesting novel or recombinant strains, but also underscores the divergence within the penton gene's RGD loop, a functionally significant region that interacts with host cells. The discovery of conserved and variable regions within the RGD loop highlights both evolutionary pressures and possible host-specific adaptations among clinical isolates, insights particularly relevant in immunocompromised individuals. Furthermore, the alignment of nucleotide and amino acid sequences identifies potential new adaptations and recombination events in clinical samples, underscoring the study's importance for understanding adenovirus evolution, pathogenicity, and epidemiology. These findings collectively emphasize the need for whole-genome sequencing and advanced molecular techniques to better characterize adenovirus diversity and evolution, contributing valuable knowledge to the field of adenovirus research and its application in developing adenovirus-based vectors. This study's approach and novel insights represent a meaningful advancement in comprehending adenovirus pathogenesis, especially in vulnerable populations.

The antigenic properties of human adenoviruses are primarily determined by the hexon, fiber, and penton proteins. These capsid proteins are hypervariable regions, which are prone to amino acid substitutions, driving antigenic diversity[18]. However, species D adenoviruses exhibit a unique evolutionary dynamic, characterized by a high rate of homologous recombination[19]. This genetic exchange can lead to the emergence of novel adenovirus types with altered biological properties, including virulence, tropism, and transmissibility. For example, HAdV-D53 and HAdV-D70 are notable examples of recombinant viruses that have arisen through complex recombination events involving multiple parental strains, some of which were previously unknown[20,21]. These findings highlight the significant impact of recombination on the evolution and diversity of human adenoviruses.

In this study, we conducted a comprehensive analysis of the penton gene in nine species of D adenovirus isolates obtained from individuals with AIDS. These isolates had previously been characterized based on their hexon and fiber gene sequences, displaying identical typing results. To delve deeper into the potential for recombination events, we sequenced and analyzed the penton gene. By comparing these sequences to a reference dataset of 70 human adenovirus types obtained from GenBank, we constructed phylogenetic trees to elucidate evolutionary relationships.

Our analysis of the partial penton region, which encompasses the critical RGD loop, revealed a striking pattern of genetic diversity. Only three of the nine isolates (22, 23, and 24 exhibited consistent typing results across all three structural genes: hexon, fiber, and penton. The remaining six isolates displayed discordant penton types, suggesting the occurrence of recombination events involving different parental strains. Furthermore, we observed identical sequences within the analyzed region for samples 3 and 4, as well as samples 6 and 7, indicating potential clonal relationships or recent transmission events.

These findings highlight the complex evolutionary dynamics of species D adenoviruses and emphasize the importance of considering multiple genetic markers for accurate typing and characterization. Further investigation is warranted to elucidate the mechanisms underlying the observed genetic diversity and its potential impact on viral pathogenesis and disease severity.

The study revealed frequent recombination events within species D adenoviruses, including exchanges between penton and hexon/fiber genes. This genomic instability, particularly in immunocompromised individuals, likely arises from prolonged viral shedding in the gastrointestinal tract[25]. As species D adenoviruses are considered for gene therapy[26], understanding their evolutionary dynamics is crucial to prevent unintended consequences from recombination with viral vectors.

Our findings underscore the significant limitations inherent in traditional adenovirus typing methods, which often rely solely on analyzing specific regions

like the hexon and fiber genes. These conventional techniques, while useful to a degree, fail to capture the full genomic complexity and potential for recombination within the adenovirus genome. As our study has shown, adenoviruses can undergo recombination events not only within hexon and fiber genes but also involving other critical genes like the penton. These recombinations can lead to substantial genetic variability, making traditional, limited-region typing methods unreliable for comprehensive adenovirus classification. The insights from this research highlight the importance of adopting full genome sequencing in adenovirus studies. By sequencing the entire genome, researchers can gain a more complete understanding of the viral genetic structure, identify novel recombinant strains, and reveal hidden diversity that would otherwise be overlooked. This approach is particularly essential for accurately characterizing and monitoring adenoviruses, especially in immunocompromised populations where diverse adenovirus strains can lead to serious health outcomes. In addition, full genome sequencing enables a deeper understanding of the virus's evolutionary patterns, pathogenic potential, and ability to adapt to different host environments. Our findings advocate for a shift toward full genome sequencing as a standard practice in adenovirus research, emphasizing that a broader, more detailed genomic perspective is necessary for accurate characterization, epidemiological tracking, and the development of targeted therapies or vaccines.

Based on the findings of this study, future research could further enhance our understanding of adenovirus genetic diversity and recombination patterns by expanding sequencing efforts beyond specific regions and focusing on full-genome sequencing across a larger sample of adenovirus strains. This would allow for a more comprehensive analysis of recombination hotspots across the genome and may reveal novel regions involved in recombination events.

Additionally, exploring the functional implications of genetic variability, especially within the hypervariable regions and RGD loop of the penton gene, could offer insights into adenovirus-host interactions and pathogenesis. In-depth studies investigating how these genetic changes affect virus fitness, infectivity, and immune evasion could provide critical data for the design of more effective adenovirus-based vectors.

Another promising area for future work would be a comparative analysis of adenovirus recombination patterns across different species, including non-human adenoviruses, to understand how host-specific adaptations influence viral evolution. Finally, research on developing more sophisticated bioinformatic tools to predict recombination events and genetic stability within adenoviruses would be invaluable. These advancements in both methodology and analytical tools would contribute to more accurate adenovirus classification, ultimately informing the development of safer, more effective adenovirus-based therapeutics and vaccines.

In conclusion, this study highlights the limitations of traditional adenovirus typing methods, which focus on partial sequencing of hexon and fiber genes, and underscores the prevalence of recombination events throughout the adenovirus genome. Our findings demonstrate that recombination within the penton gene, particularly in the hypervariable and RGD loop regions, contributes to significant genetic diversity among species D adenoviruses. These insights emphasize the importance of full-genome sequencing for precise adenovirus classification and understanding evolutionary dynamics. This comprehensive approach is essential for advancing adenovirus-based therapies and ensuring the stability and efficacy of adenovirus vectors in clinical applications.

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 an 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.

REFERENCES CITED

[1] Kovács E. 2011. Siadenoviruses: a comparative olecular and phylogenetic study. Budapest: Eotvos Lorand University.

[2] Syyam A, Nawaz A, Ijaz A, Sajjad U, Fazil A, Irfan S, Muzaffar A, Shahid M, Idrees M, and Malik K. (2022). Adenovirus vector system: construction, history and therapeutic applications. Biotechniques, 73(6), 297-305.

[3] Lynch III JP, and Kajon AE. (2021, December). Adenovirus: epidemiology, global spread of novel types, and approach to treatment. In Seminars in respiratory and critical care medicine (Vol. 42, No. 06, pp. 800-821). Thieme Medical Publishers, Inc..

[4] Takuissu G, Kenmoe S, Ebogo-Belobo J, Kengne-Ndé C, Mbaga D, Bowo-Ngandji A, Ondigui Ndzie J, Kenfack-Momo R, Tchatchouang S, and Kenfack-Zanguim J. (2024). Exploring adenovirus in water environments: A systematic review and meta-analysis. International Journal of Environmental Health Research, 34(6), 2504-2516.

[5] Kulanayake S, and Tikoo SK. (2021). Adenovirus core proteins: structure and function. Viruses, 13(3), 388.

[6] Al-Heeti OM, Cathro HP, and Ison MG. (2022). Adenovirus infection and transplantation. Transplantation, 106(5), 920-927.

[7] Gu J, Su Q-q, Zuo T-t, and Chen Y-b. (2021). Adenovirus diseases: a systematic review and meta-analysis of 228 case reports. Infection, 49, 1-13.

[8] Grebe, UF, and Suomalainen M. (2022). Adenovirus entry: Stability, uncoating, and nuclear import. Molecular Microbiology, 118(4), 309-320.

[9] Karabulut OC, Karpuzcu BA, Türk E, Ibrahim AH, and Süzek BE. (2021). ML-AdVInfect: a machine-learning based adenoviral infection predictor. Frontiers in Molecular Biosciences, 8, 647424.

[10] Ricobaraza A, Gonzalez-Aparicio M, Mora-Jimenez L, Lumbreras S, and Hernandez-Alcoceba R. (2020). High-capacity adenoviral vectors: expanding the scope of gene therapy. International Journal of Molecular Sciences, 21(10), 3643.

[11] Nestić D, Božinović K, Pehar I, Wallace R, Parker AL, and Majhen D. (2021). The revolving door of adenovirus cell entry: not all pathways are equal. Pharmaceutics, 13(10), 1585.

[12] Zubieta C, Blanchoin L, and Cusack S. (2006). Structural and biochemical characterization of a human adenovirus 2/12 penton base chimera. The FEBS journal, 273(18), 4336-4345.

[13] Liu L, Qian Y, Jia L, Dong H, Deng L, Huang H, Zhao L, and Zhu R. (2021). Genetic diversity and molecular evolution of human adenovirus serotype 41 strains circulating in Beijing, China, during 2010–2019. Infection, Genetics, and Evolution, 95, 105056.

[14] Cai R, Mao N, Dai J, Xiang X, Xu J, Ma Y, Li Z, Han G, Yu D, and Yin J. (2020). Genetic variability of human adenovirus type 7 circulating in mainland China. PLoS One, 15(4), e0232092.

[15] Hang J, Kajon AE, Graf PC, Berry IM., Yang Y, Sanborn MA, Fung CK, Adhikari A, Balansay-Ames MS., and Myers CA. (2020). Human adenovirus type 55 distribution, regional persistence, and genetic variability. Emerging Infectious Diseases, 26(7), 1497.

[16] Robinson CM., Seto D, Jones MS, Dyer DW, and Chodosh J. (2011). Molecular evolution of human species D adenoviruses. Infect Genet Evol, 11(6), 1208-1217. https://doi.org/10.1016/j.meegid.2011.04.031

[17] Wu X, Zhang J, Lan W, Quan L, Ou J, Zhao W, Wu J, Woo PC, Seto D, and Zhang Q. (2022). Molecular typing and rapid identification of human adenoviruses associated with respiratory diseases using universal PCR and sequencing primers for the three major capsid genes: penton base, hexon, and fiber. Frontiers in Microbiology, 13, 911694.

[18] Madisch I, Hofmayer S, Moritz C, Grintzalis A, Hainmueller J, Pring-Akerblom P, and Heim A. (2007) Phylogenetic analysis and structural predictions of human adenovirus penton proteins as a basis for tissue-specific adenovirus vector design. Journal of Virology 81, 8270-8281.

[19] Robinson CM., Singh G, Lee JY, Dehghan S, Rajaiya J, Liu EB, Yousuf MA, Betensky RA, Jones MS, Dyer DW, and Seto D. (2013). Molecular evolution of human adenoviruses. Scientific reports, 3.

[20] Walsh MP, Chintakuntlawar A, Robinson CM, Madisch I, Harrach B, Hudson NR, Schnurr D, Heim A, Chodosh J, Seto D, and Jones MS. (2009). Evidence of molecular evolution driven by recombination events influencing tropism in a novel human adenovirus that causes epidemic keratoconjunctivitis. PloS one, 4(6), p.e5635.

[21] Hage E, Liebert UG, Bergs S, Ganzenmueller T, and Heim A. (2015). Human mastadenovirus type 70: a novel, multiple recombinant species D mastadenovirus isolated from diarrhoeal faeces of a haematopoietic stem cell transplantation recipient. Journal of General Virology, 96(9), pp.2734-2742.

[22] Rowe WP, Huebner RJ, Hartley JW, Ward TG, and Parrott RH. (1955). Studies of the Adenoidal-Pharyngeal-Conjunctival (APC) Group of Viruses. American Journal of Hygiene, 61(2), pp.197-218.

[23] Davison AJ, Benkő M, and Harrach B. (2003). Genetic content and evolution of adenoviruses. Journal of General Virology, 84(11), 2895-2908.

[24] Davison AJ, Benkő M, and Harrach B. (2003). Genetic content and evolution of adenoviruses. Journal of General Virology, 84(11), 2895-2908.

[25] Curlin ME, Huang ML, Lu X, Celum CL, Sanchez J, Selke S, Baeten JM, Zuckerman RA, Erdman DD, and Corey L. (2010). Frequent detection of human adenovirus from the lower gastrointestinal tract in men who have sex with men. PloS one, 5(6), p.e11321.

[26] Robinson CM, Seto D, Jones MS, Dyer DW, and Chodosh J. (2011). Molecular evolution of human species D adenoviruses. Infection, Genetics and Evolution, 11(6), pp.1208-1217.