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ORIGINAL ARTICLE

Evolution and diversity of human adenoviruses isolated from patients with keratoconjunctivitis

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ABSTRACT

Background and Objectives: Human adenovirus type 8 is a highly contagious eye disease and is considered as the most common epidemic keratoconjunctivitis worldwide. The virus may alter the course of detection as mutations and recombination in surface antigens are associated with binding and pathogenesis in human adenovirus. The recognition of new recombinant human adenovirus has been based on sequencing of three genes, penton base, hexon and fiber.
Materials and Methods:50 suspected samples of ocular keratoconjunctivitis were selected over 6 months. Following DNA extraction from isolates positive for cytopathic effect in each well, the complete sequences of hexon, fiber, and penton regions were performed on the genome of human adenovirus isolates using PCR. The sequences of capsid genes, including hexon, fiber, and penton were assessed to observe the evidence of recombination at the molecular level using genetic tools.
Results: The results of nucleotide and amino acid sequence of 5/ 50 patients with epidemic keratoconjunctivitis positive for hypervariable region of hexon (132 -449), hypervariable of knob fiber (183 -362) and hypervariable penton (106 -466) isolates showed nucleotide and amino acid identity of 98% and 99.41%, 99% and 100%, 95% and 99.72% with hexon, fiber and penton of human adenovirus 8 subtypes. The results of phylogenetic tree and Simplot of the entire sequences and hypervariable regions of isolated hexon, fiber and penton showed all the isolates of human adenovirus from Ahvaz, Iran, were clustered with human adenovirus 8A, B, E, P and J, subtypes isolated strains from different regions of the world.

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Conclusion: The results of this study revealed that the human adenovirus isolates from patients with epidemic keratoconjunctivitis were closed to human adenovirus 8A, B, E, P and J subtypes. To determine the emergence of new human adenovirus D8 subtypes strain, analysis of complete genome sequence of human adenovirus was required.

Keywords: Human adenovirus; Keratoconjunctivitis; Infections; Evolution; Recombination

INTRODUCTION

Epidemic keratoconjunctivitis, characterized by inflammation of the conjunctiva and cornea, can lead to a sudden onset of acute follicular conjunctivitis and stromal keratitis which is a significant worldwide problem sometimes causing long-term morbidity (1). Human adenovirus are non-enveloped viruses with an icosahedral capsid, containing a dsDNA genome of 26-36 kb, which are classified into seven species (HAdV-A to HAdV-G) (2). Recent classification of adenovirus is based on evaluation and the evidence of recombination in hexon, fiber, and penton base regions of adenovirus genome (3). So far, 90 HAdV genotypes have been recognized according to the complete genome sequencing and characterized by HAdV working group (http://hadvwg.gmu.edu/) (4). Evidently, HAdV serotype D including HAdV-D8, D19, D37, D53, and D54 are identified as the most common causes of EKC worldwide (5). The hexon region comprises of 240 trimers which build up the icosahedron protein shell; 12 trimetric fibers protrude from the capsids and are linked to the hexons via a pentameric penton base.

As an important process in virus evolution, recombination is considered as a definite feature of HAdV genetics and pathogenesis. HAdV recombination continuously happens between strains of the same species (6). Mutations in hexon, fiber, and penton base proteins result in new strains of the adenovirus (7). The regions of loop1 and 2, and hypervariable regions 1-7 of hexon gene have shown to be highly susceptible to homologous recombination among HAdV-D (8). When a cell is infected simultaneously with two different adenovirus types, due to the exchange of the genetic material, a new adenovirus genotype may emerge (9).

Hence, the recombination between species D types may generate novel neutralization epitopes ('immune escape') which contributes to the huge diversity of HAdV-D types. In present study, the sequences of capsid genes, including hexon, fiber, and Penton, were assessed to observe the evidence of recombination and evolution at the molecular level using different genetic tools. Due to the vital functions of each of the hexon, fiber, and penton base proteins, any mutations or recombination in their genes cause a change in the binding proteins as a result of the deception of the immune system and the severity of the virus pathogenesis. In this study, we sought to examine these types of changes and the evolution of the virus. Over such investigations, mutant species can be quickly identified and emerging pathogens can be prevented.

MATERIALS AND METHODS

Ethical statement. This project was approved with registration number No:OG-9713 and the ethical code of IRAJUMS1397.219 by ethic committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The informed consent was signed by each patient with Keratoconjunctivitis.

Specimen collection. To collect specimens, this cross-sectional study was conducted in the Virology Department of Ahvaz Jundishapur University of Medical Sciences from April to June 2018. In this study, 50 eye swab samples of suspected ocular keratoconjunctivitis were selected at Imam Khomeini hospital and subsequently isolated in A549 cell culture. Specimens that produced cytopathic effect (CPE) in cell culture were used in PCR testing for adenovirus diagnosis.

EKC symptoms usually manifest with fever, muscle aches, malaise, and swollen lymph nodes. When eye symptoms develop, they typically occur in different phases. The acute phase, which may last between 7 to 10 days, is characterized by inflammation of the conjunctiva with or without corneal involvement. The chronic phase is defined by subepithelial corneal infiltrates, a condition in which the immune system inadvertently draws white blood cells into the cornea that can lead to corneal opacity. Patients with fungal, bacterial, and other infections were excluded; patients with symptoms of adenoviral Keratoconjunctivitis (inclusion) were only studied based on the symptoms

in consultation with an ophthalmologist.

Preparation of cell line A549. The A549 cell lines were obtained from Razi Vaccine and Serum Research Institute Karaj, Iran. *Mycoplasma* contamination was detected with specific primers. A549 cells were supplemented in DMEM (Gibco- Germany) with 5% FBS (Gibco- Germany), penicillin 100 unit/ml, streptomycin 100 µg/ml, amphotericin B, using 24 wells, tissue culture micro-plates (Nunc, Denmark). The plates were incubated at 37°C in an atmosphere containing 5% CO_2. Each specimen was inoculated into a well containing confluent monolayers cells, incubated at 37°C for 1 hour. Finally, the cells supplemented with maintenance DMEM media containing 2% FBS were incubated at 37°C for 4 days to reveal the cytopathic effect. Five wells with 100% CPE were selected for molecular characterization of complete ORF of hexon, fiber, and penton base regions of the isolated HAdV.

DNA extraction. After transferring to the laboratory, the samples were placed in a freezer at -20°C until the conditions were ready for extraction. On the day of extraction, the samples were taken out of the freezer and thawed to be ready for extraction. DNA was extracted from supernatant of infected cell culture with significant CPE using high pure viral nucleic acid kit (Roche) according to the manufacturer's instructions. Initially, the PCR assay was carried out to detect partial hexon region, using outer primer pair, forward primer (5-GCC SCA RTG GKC WTA CAT GCA CAT C-3) and reverse primer (5-CAG CAC SCC ICG RAT GTC AAA-3), which produced a 301-bp product and was used for both sequencing and diagnosis. The results of partial sequencing of PCR product and blasting revealed that all the samples belonged to HAdV-D8. Five samples were selected and PCR assay was fulfilled particularly to amplify entire hexon, fiber and penton base genes, using designed primers set scheduled in Table 1.

Primer design and PCR. Based on the complete sequence of HAdV retrieved from Gene bank, including AB448768, AB448769, AB746853, KY002684, KY002685 DQ900900, AB448778, AY599834, LC215442, AB605241, AB605240, AB605242, AB605243, AB605244, AB605245, AB605246, LC215446, LC215427, AB448770, LC215445, and the results of performing a blast on NCBI, the primers were designed to cover the complete ORF of hexon, fiber, and penton genes for isolated HAdV from Ahvaz city using Oligo7 software (Table 1). All the primers exhibited 100% similarity with the hexon, fiber, and penton genes recorded in the Gene Bank.

The following thermal condition steps were performed for the detection of different regions of hexon, penton, and fiber genes by PCR using thermal cycler (PEQLAB, Germany). Denaturation at 95°C for 5 min, followed by 35 cycles: denaturation at 95°C for 30 s, annealing (55-59°C) for 45 s, extension at 72°C for 2 min, final extension 72°C for 5 min. subsequently, the PCR products of different regions were sequenced (Applied Biosystems, USA).

Sequencing analysis. Nucleotide sequences of each region of the target genes were aligned using MAFFT online server 24 (https://mafft.cbrc.jp/alignment/server/) for the hexon, fiber, and penton base of the isolated HAdV, trimmed and assembled using SnapGene software. The Phylogenetic tree was constructed with neighbor joining, Tamura-Nei model, and Gamma distributed with Invariant sites (G+I) with 500 bootstrap replicates and then analyzed for each complete gene of hexon, fiber, and penton base using the MEGA X software package. Next, the complete sequence of the hexon, fiber, and penton gene was sent to GeneBank to obtain the accession number. Hexon: MH507071, MH513942, MH513943, MH513944, MH513945 fiber: MH545936, MH545937, MH545938, MH545939, MH545940 and penton MH545941, MH545942, MH545943, MH545944, MH545945.

Recombination analysis. The evidence of recombination in entire gene of hexon, fiber, and penton of the isolates HAdVs was demonstrated by Simplot 3.5.1 software and RDP4 software (version 4.80). The bootstrap analysis of the genes was completed with window size 200 bp, step size 20bp, 50% consensus and 1000 replicates with kimura 2-parameter model.

RESULTS

The results of the complete genes of the hexon, penton, and fiber of isolated HAdV from patients with EKC were as follows.

Analysis of hexon. The nucleotide sequences of

Table 1. The nucleotide sequences of used primers for detection and analysis

Primer name	Nucleotide	Length	Tm °C
Forward hexon	F1-GAGCTTAACTTGTATGTGCCTT	17733-17754	57
Reverse hexon	R1-CCCTCCTTTTTTATTAGTTGT	18459-18480	57
Forward hexon	F2-GAGCTCTTAAAAAGGAAACCAA	18407-18428	57
Reverse hexon	R2-AATTTGGTT TTGAGCAGCCACT	19080-19101	57
Forward hexon	F3-TGCTCAAGGTCAAAATGATAAA	19041-19062	57
Reverse hexon	R3-AGTCTTGGTTTTTAGGCGG	19788-19709	57
Forward hexon	F4-CCATTTCCATCCCCTCGCGCAA	19736-19757	57
Reverse hexon	R4-CGCCCATAGACATGAAGTTGCT	20374-20395	57
Forward hexon	F5-CCTACCCGCTTA TCGGACAGAC	20285-20306	57
Reverse hexon	R5-GTACTCGAAGCTGTAAATCTGC	20852-20873	57
Forward penton	F1-GGTTAACCCG TTCGCGCATCTG	13419-13440	58
Reverse penton	R1 -ACTTAACTCCAATATCGCTCTC	14106-14127	58
Forward penton	F2-TGGTGGCTAGGAAGCAC CCACA	13924-13945	58
Reverse penton	R2-CATGAGGTCCGGCAGCGACCAG	14684-14705	58
Forward penton	F3-CTGT CCTATACCTACGGGGACC	14592-14613	58
Reverse penton	R3-GGCCGCTCGTCTGCGGTCCTG	15539-15560	58
Forward fiber	F1-TTATTCAATAAAGATCACTTAC	30584-30605	58
Reverse fiber	R1-GCTACCAAGTCTCCATTGTCAT	31260-31281	58
Forward fiber	F2,CCTGGCTTGGTTAATAC TCTTG	31145-31166	58
Reverse fiber	R2-TGGTAAATCCTTTAAGAGCTGG	31469-31490	58
Forward fiber	F3- CAGGAT AAGGACTCTAAGCTAA	31352-31373	58
Reverse fiber	R3- GCTGAGAGAATTGTTTACACTG	31954-31975	58

each 5 complete hexon isolated HAdV-D8 strains comprised of 2829 bp and were recorded in the GenBank database with accession numbers MH507071, MH513942, MH513943, MH513944, and MH513945. The entire aa sequence of each isolated hexon composed of 942 aa and was deposited in the GeneBank database with accession numbers QB171904, QB171905, QB171906, QB171907, and QBH72539. The hypervariable regions of hexon (132_{aa}-449) of isolated HAdV-8 comprised of two loops; loop 1 encompassed 6 HVR including HVR1 (132-168 aa), HVR 2 (174-190 aa), HVR 3 (198-209 aa), HVR 4 (233-253 aa), HVR 5 (261-280 aa), HVR 6 (297-309 aa), and loop 2 contained HVR 7 (403-449 aa). The analysis of aa alignment of hexon revealed that in hypervariable region 1 of hexon isolate QBH72539 at positions 142, 144 and 152, there were aa substitutions alanine → valine, valine → alanine and arginine → glycine compared to consensus isolates HAdV-8A, B, E, P and J sequences. The results of phylogenetic tree of the entire sequences and hypervariable regions of hexon of HAdV isolated from Ahvaz, Iran, were clustered with HAdV-8A, B, E, P, J subtypes isolated strains from different regions of the world (Fig. 1). The results of Simplot revealed high nucleotide similarity >98-100% with HAdV-8A, B, E, P and J subtypes.

Fiber analysis. The nucleotide sequences of each 5 complete fiber isolated HAdV-D8 strains comprised of 1089 bp and was recorded in the GenBank database with accession numbers MH545936, MH545937, MH545938, MH545939, MH545940. The result of entire nucleotide sequence of each fiber of five isolated HAdVs showed nucleotide identity of 98% to 99.41% with isolated fiber HAdV-8A, B, E, P and J subtypes isolated strains from different regions of the world. The entire aa sequence of each isolated HAdV fiber composed of 363 aa and were deposited in the GenBank database with accession numbers QBI59924, QBI59925, QBI59926, QBI59927 and QBI59928. The aa sequence of each five complete fiber isolated HAdV-8 strains comprised of 363 aa (Tail 1-43aa, shaft 44-72 aa and 76-100 aa, and knob 183-362aa). The analysis of fiber knob region showed that at position 165 aa there was an aa substitution glutamic acid with glutamine in comparison with HAdV-8A, B, P

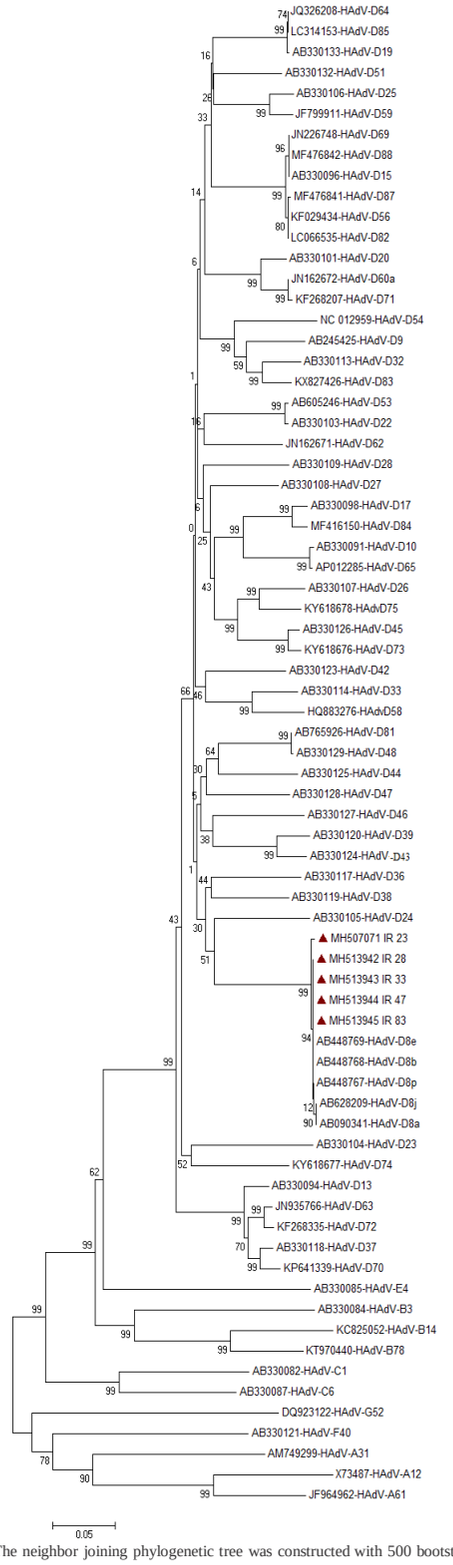

Fig. 1. The neighbor joining phylogenetic tree was constructed with 500 bootstrap replicates for complete hexon region of isolated HAdV-D8 strains in Ahvaz city. The Iranian isolates strains with accession numbers MH507071, MH513942, MH513943, MH513944, MH513945 compared with HAdV-8P AB448767, HAdV-8B AB448768, HAdV-8E AB448769, HAdV-8J AB628209 and HAdV-54 NC-012959 were obtained from GenBank. The Iranian HAdV-D8 isolates were labeled with red triangle and were clustered with HAdV-D8 subtypes isolates strains from different regions of the world.

and J, subtypes. The result of entire nucleotide and aa alignment of hyper variable knob fiber (183_{aa}-362) region of isolated HAdV-D8 revealed nucleotide and protein identity of 99-100% with HAdV-8A, B, E, P and J subtypes. The results of phylogenetic tree of the entire sequences and hypervariable regions of fiber of isolates HAdV from Ahvaz, Iran, were clustered with HAdV-8A, B, P, J subtype strains isolated from different regions of the world (Fig. 2). The results of Simplot showed high nucleotide similarity >98-100% with HAdV-8A, B, E, P and J subtypes.

Analysis of penton base. The nucleotide sequences of each 5 complete penton genes of isolated HAdV-D8 strains comprised of 1563 bp and were recorded in the GenBank database with accession numbers MH545941, MH545942, MH545943, MH545944, MH545945. The result of entire nucleotide blasting of each penton of 5 isolated HAdVs showed nucleotide identity of 99% to 100% with isolated penton HAdV-8A, B, E, P and J subtypes isolated strains from different regions of the world (Fig. 3). The entire aa sequences of each 5 isolated HAdV penton composed of 520 aa and were deposited in the GenBank database with accession numbers QBI59929, QBI59930, QBI5993, QBI59932 and QBI59933. Each 5 complete penton genes of isolated HAdV-8 strains comprised of two hypervariable regions, HVR1(136-152aa) and RGD loop (282-335aa). The results of phylogenetic tree of the entire sequences and hypervariable regions of penton base of HAdV isolates from Ahvaz, Iran, were clustered with HAdV-8A, B, P, J subtype strains isolated from different regions of the world (Fig. 3). The results of Simplot showed high nucleotide similarity >98-100% with HAdV-8A, B, P and J subtypes.

Overall, The analysis of nucleotide and aa identity, multiple sequence alignment phylogenetic tree and Simplot for the hexon, fiber and penton of isolated HAdV-8 showed that all Iranian HAdV-8 isolates were very close to HAdV-8A, B, P, J subtypes. However, to determine the exact HAdV-D8 subtypes, analysis of the complete sequence of HAdV-8 genome is required.

DISCUSSION

HAdV serotype D including HAdV-D8, -D19, D37, D53 and D54 are the most common cause of EKC worldwide. Previously, the HAdVs serotypes were classified based on serological tests such as Hem-

Fig. 2. The neighbor joining phylogenetic tree was constructed with 500 bootstrap replicates for complete fiber region of isolate HAdV-D8 strains in Ahvaz city. The Iranian isolates with accession numbers MH545936 MH545937, MH545938, MH545939, MH545940 were compared with other HAdV groups and HAdV-8PAB448767, HAdV-8B AB448768, HAdV-8E AB448769, AB628208 HAdV-8J subtypes and were obtained from GenBank. The Iranian HAdV-8 isolates were labeled with red triangles and clustered with HAdV-D8 subtypes isolated strains from different regions of the world.

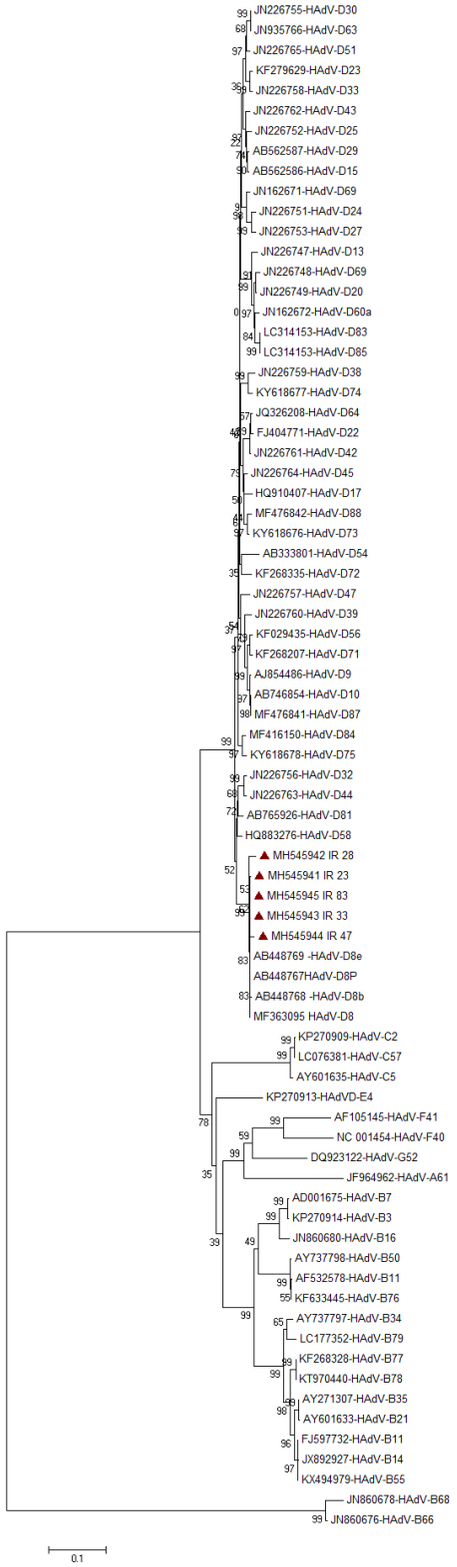

Fig. 3. The neighbor joining phylogenetic tree was constructed with 500 bootstrap replicates for complete penton region of HAdVD8 isolates strains in Ahvaz city. The Iranian isolates with accession numbers MH545941, MH545942, MH545943, MH545944, MH545945 were compared with other HAdV groups and HAdV-D8 subtypes HAdV-8PAB448767, HAdV-8B AB448768, HAdV-8E AB448769 subtypes and HAdV-54 were obtained from GenBank. The Iranian HAdV-8 isolates, labeled with red triangles, were clustered with HAdVD8 subtypes isolated strains with different regions of the world.

agglutination and Neutralization (NT) assay. Using application of advanced molecular techniques, a new serotype of the HAdV has been classified (10). The present study aimed to detect HAdV in patients with EKC and analyze the obtained results using RDP4 and Simplot software. Subsequently, the phylogenetic relationships of HAdV isolates were compared with seven other groups of HAdV reference strains. It was found that the hexon fiber, and penton gene of the isolated HAdV had a close relationship with HAdV-8A, E B, P, J subtypes isolates strains from different regions of the world. The hexon gene of isolated HAdV-D8 comprised 2829 bp and 942 aa that showed that at hypervariable region 1 of hexon isolate (QBH72539) at positions 142, 144 and 152, there are aa substitutions alanine→valine, valine →alanine and arginine glycine as compared to isolates HAdV-8A, B, E, P and J sequences. The analyses of the hexon hypervariable isolated showed discrepancy in the length. Moreover, the beginning of the hypervariable positions (132_{aa}-449) were unlike any other known reported HAdV19, 21, 28, 29, 40, 41, 46 reported by Ebner et al. (11). Crawford-Miksza evaluated HAdV2, 5, 12, 16, 48, 40, 41 B3 hexon genes and found that the length and the beginning of the hypervariable positions was 138 to 460aa (12). Thus, the hexon region and its relevant aa composition played a critical role in diversity and evolution of the new genotype. The results of alignment of isolated HAdV penton showed two hypervariable loops (HVR1 from 136 to 152aa and RGD loop from 282 to 335 aa). While Madisch et al. reported that the penton base of HAdVs D8, A12, B3, C2, E4, F40 had two hypervariable loops (HVR1 from 157 to 177aa and RGD 307-405 aa). This finding supported that our HAdV RGD loop was more conservative than RGD loop HAdVs reported by Madisch et al. (13). Fiber knob domain functions as a primary receptor binding site. In this study, fiber was organized in three regions, tail 1-43aa, shaft 44-72 aa and 76-100 aa, and knob 182-362aa. The shaft/knob domains comprised of highly conserved TLWT motif (14). Hexon gene of all HAdVs contains the major neutralization epitope but the occurrence of mutations in this region can result in immune escape and rapid evolution of the hexon gene.

Recombination is the most important naturally occurring phenomenon among the HAdVs serotype D which contributes to evolution and reclassification of adenoviruses (15). Homologous recombination between types can be facilitated by the ability of adenoviruses to development of persistent infections in humans (16).

In the current study, there was no recombination event in hexon, fiber and penton genes. Thus, the analysis of variable region in the hexon, fiber, and penton base, will provide additional information on the extent of diversity of HAdVs isolates from clinical samples. This description of outbreaks and circulating genotypes will have an impact on improving the detection of HAdVs with enhanced virulent and transmission, tending to a new tissue tropism, epidemiological surveillance, or pathogenic properties. Currently, no FDA approved drug or vaccine is available for adenoviral keratoconjunctivitis and current management of HAdV EKC is palliative (17). In fact, further research is needed to be done on complete genome, pathogeneses, and determination of fiber, penton and hexon epitopes, antiviral vaccine and animal study.

CONCLUSION

The analysis of sequencing and aa alignment, phylogenetic tree and Simplot for the hexon, fiber and penton of isolated HAdV-D8 showed that all Iranian HAdV-D8 isolates were very close to HAdV-8A, E, B, P, J subtypes. However, to determine the exact subtypes of HAdV, analysis of the complete genome sequence of HAdV-D8 isolates is required.

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