VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 213-219

Method Development for Detection and Classification of Conjunctivitis-Causing Adenoviruses in Human

Nguyen Viet Ha[1], Nguyen Thi Thu Huyen[1], Do Thi Thanh Huyen[2], Nguyen Quang Hung[1], Tran Thuy Anh[1], Hoang Anh Tuan[3], Nguyen Van Sang[1,*]

[1]*Falcuty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam*

[2]*High School for Gifted students, VNU University of Science, 182 Luong The Vinh, Thanh Xuan, Hanoi, Vietnam, PhD candicate from Project 911, VNU University of Science*

[3]*Vietnam National Institute of Ophthalmology, 85 Ba Trieu, Hai Ba Trung, Hanoi, Vietnam*

Received 02 June 2016
Revised 02 August 2016; Accepted 09 Septeber 2016

Abstract: Conjunctivitis or "pink eye" disease caused by human adenoviruses (HAdVs) is highly contagious and persistent morbidity without any effective treatments. Types of human adenoviruses (HAdV) are also very diverse. Therefore, precise determination of HAdV-types causing conjunctivitis is important for epidemiological studies. In this study, we aimed to develop a method for detection and classification of human adenoviruses causing conjunctivitis in Vietnam. The HAdV genome was extracted from clinical samples of conjunctivitis patients in Vietnam. The hypervariable region 7 (HVR-7) in hexon gene of adenoviruses was amplified with the redesigned primers. HVR-7 region was then sequenced to investigate and determine the HAdV-types. The HAdV types were identified by analyzing HVR-7 sequence of the hexon gene. In this research, HAdV-3, -4, -7, -8, -37 were identified as the cause of conjunctivitis, in which serotype 8 was the predominant type, detected in 19/23 samples.

Keywords: Human adenovirus, hypervariable region-7, type, conjunctivitis, PCR.

1. Introduction

Conjunctivitis, also known as "pink eye" is an inflammation of conjunctiva caused by the infection of various pathogens including viruses, bacteria, and fungi. The most prevalent cause of conjunctivitis is the infection of human adenovirus (HAdV) [1, 2]. Conjunctivitis caused by HAdVs is highly contagious and persistent morbidity without any effective treatment. HAdV conjunctivitis can be self-cured after 7 to 14 days of infection, but occasionally it may develop complication and lead to unanticipated long-term impact to the patients [3]. In Vietnam as well as in many countries, HAdV conjunctivitis is quite popular and frequently leads to the disease outbreak [2, 4].

HAdVs belong to the genus Mastadenovirus. HAdVs are divided into 7 species (A-G) based on different oncogenic, hemagglutinating, morphological and DNA sequence properties. To date, 52 serotypes have been identified. There are many HAdV serotypes associated with conjunctivitis, in

* Corresponding author. Tel.: 84-967776046
Email: nvsangvnu@yahoo.com

which type 8, 19, 37 are known to have the greatest epidemic potential and are the leading cause of severe keratoconjunctivitis [5]. HAdV-3, HAdV-4 and HAdV-7 also commonly cause mild conjunctivitis as a part of pharyngoconjunctival fever [5]. There is limited data on the prevalence of HAdV types in Vietnam [4]. Determination of HAdV-types has been one of principal and necessary directions for an effective epidemiological surveillance and qualified diagnosis.

The adenovirus genome is non-segmented, linear double-stranded DNA [6]. The hexon gene belonging to Late gene 3 (L3) with average size about 2.7-2.9 kb long encodes hexon proteins. Hexon is the most abundant and largest of the structural proteins and plays a crucial role of the major antigen [7, 8]. Particularly, loop 1 and loop 2 of the hexon protein contain type-specific epitopes, as known as neutralization ε determinant classified into seven hypervariable regions, HVR1−6 in loop 1 and HVR7 in loop 2 [9].

Currently, PCR, sequencing analysis of HAdVs genome regions have provided a rapid and sensitive alternative for adenovirus detection and typing in clinical samples [9-11]. The diagnosis by PCR and sequencing was mainly based on the type-specific determinants on hexon, penton and fiber gene [8]. HVR-7 of hexon gene is a potential candidate for the type-specific epitope with the low mean maximum homology among serotypes of HVR-7 as 58% [12]. Therefore, in this study, the HVR-7 was used for PCR amplification and sequencing to detect and classify HAdV-types in HAdV conjunctivitis case in Vietnam.

2. Materials and Methods

Clinical sample collection: The HAdV source was isolated from clinical samples of conjunctivitis patients in Hanoi, Vietnam. 36 samples of ocular washing solution of different conjunctivitis patients supplied by National Institute Ophthalmology, Hanoi were kept in 1.5mL eppendorf tubes separately and stored at -20^{o}C until testing. The total volume of each sample was in a range of 90-180 µl. The patients who volunteered to give these samples were not from the same family and they live and work in different locations in Hanoi, Vietnam.

DNA extraction: Viral DNA was extracted by using The Viral Gene-Spin Virus RNA/DNA Isolation Kit (iNtRon, Korea). The extracted DNA was stored at -20^{o}C for further experiments.

PCR amplification of HVR-7 of hexon gene: The sequence of the primer pair targeting conserved segments that bracketed the HVR-7 of the hexon gene used in this study was 5'-GTA CTA CAA CAG CAC TGG CAA CAT GGG -3' (forward primer) and 5'- GCR TTG CGG TGG TGG TT-3' (reverse primer). This primer pair was modified based on a previous research [11]. The primers targeted the conserved segments, but they encompassed the highly variable region. There existed some different nucleotides in these segments among HAdV-types, especially at both ends of the original primers which could form mismatches between the original primers and templates from certain HAdV types. The redesigned primers were changed by reducing those different nucleotides at ends of original primers, which possibly improve the PCR efficiency as well as sensitivity. The desired size of PCR product is also about 600 bp. Each *PCR* reaction with 20 µl of total volume contained 10 µl of 2X PCR Master mix solution (i-Taq) (iNtRon), 1.25 µl of 3.2 µM for each primer, 8.9 µl of double-distilled water and 0.3 µl of DNA template. PCR was performed using the PCR machine Kyratec with an initial denaturation at 95^{o}C for 2 min; followed by 35 cycles composing of denaturation at 95^{o}C for 20 sec, annealing at 58^{o}C for 10 seconds, and elongation at 72^{o}C for 35 seconds; and a final extension at 72^{o}C for 5 min. After PCR, a 3µl of each reaction was examined by gel electrophoresis on 2% agarose gels containing

Redsafe. The bands were visualized under UV and acquired by Alphaimager MINI System.

Sequencing: The sense strands of the amplicons were sequenced with the PCR forward primer by 1st BASE sequencing service using the BigDye® Terminator v3.1 cycle sequencing kit chemistry.

Sequencing analysis: Sequencing editing and analysis were done by using nucleotide BLAST tool on NCBI and BioEdit software, version 7.2.5. Sequence alignments were also performed by using ClustalW Multiple Alignment method in BioEdit software. The phylogenetic analysis based on the nucleotide sequences of HVR-7 including the sequences from 23 samples and the hexon gene of HAdV-1 to HAdV-46 was carried out by using the Mega6 software. The tree was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. The accession numbers of the used sequences will be listed in the below appendix section.

3. Results and discussion

Amplifying HVR-7 of hexon gene by PCR: Of the 36 samples studied, 23 were PCR positive for HAdVs with amplified DNA fragment of approximately 600 bp as the same length as expected HVR-7 of the hexon gene. The other 13 remaining samples might be negative with HAdVs because of failing in PCR without any amplified product. The amplified products were fractionated on a 2% agarose gel, which is shown in figure 1. The desired PCR products were then purified by using purification kit and sent for DNA sequencing.

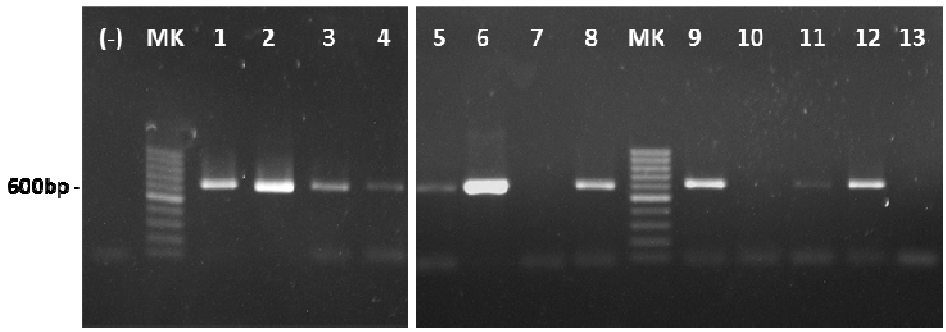

Figure 1. PCR-amplified products of HVR-7 of hexon gene from clinical samples.

A total of 3 µl for each of the PCR products was examined on a 2% agarose gel. Lanes (-) are the negative control, lanes MK are marker 100 bp (Norgen; the lowest band is 100bp, the distance between bands is 100 bp), Lane 1-13 are PCR products from clinical samples.

Table 1. Summary of sequencing analysis by Nucleotide Blast tool on NCBI

Number of samples	Obtained sizes	HAdV-(Type)	Query cover	Identity	Accession
1	542 bp	Type 3	100%	100%	KM458623.1
1	440 bp	Type 7	100%	98%	KM458626.1
1	524 bp	Type 4	100%	100%	KF006344.1
1	558 bp	Type 37	100%	100%	AB448778.1
19	494-550 bp	Type 8	100%	100%	AB500121.1

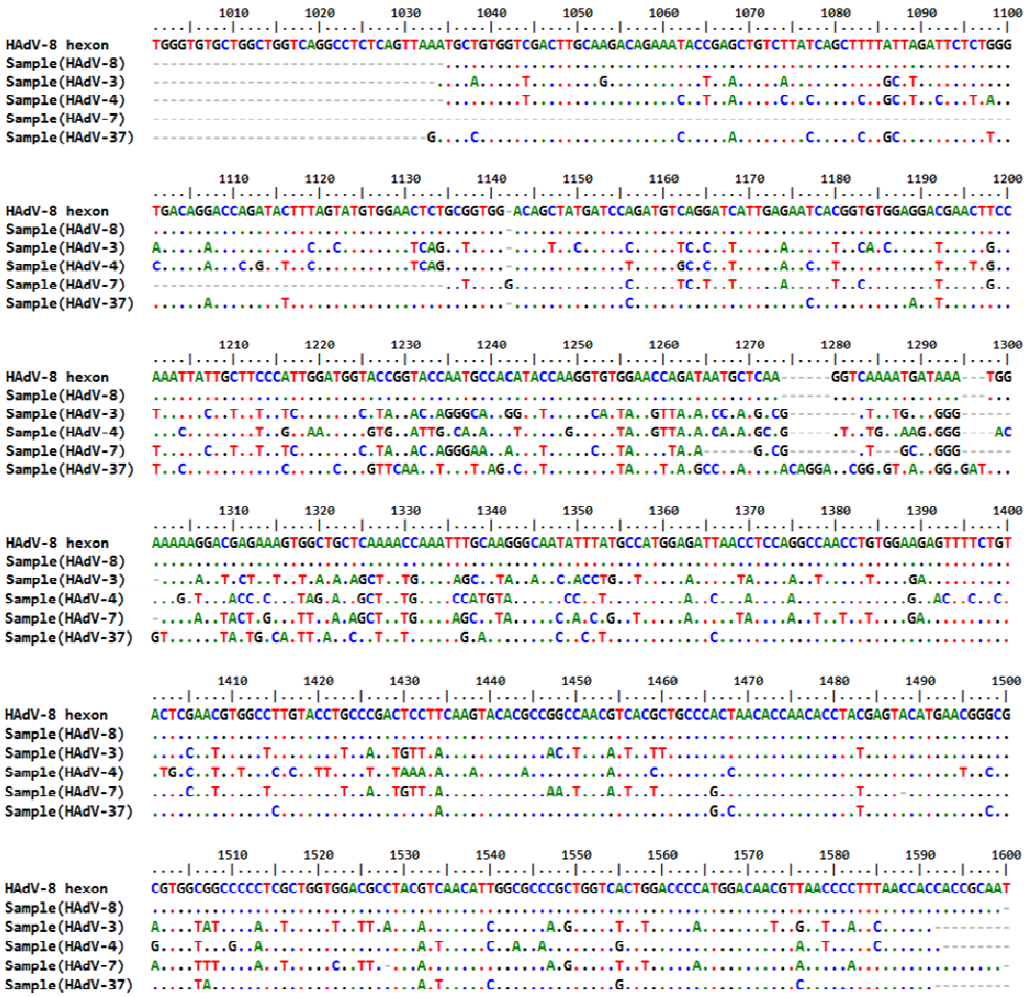

Figure 2. Alignment of DNA sequence from samples and HAdV-8 from GeneBank by BioEdit software.

4. DNA sequencing analysis

The identity of amplicons with expected size was confirmed by single pass DNA sequencing with PCR forward primer. The sequencing results had good sequence trace with clear peak and the noise only appeared at the two ends of the sequence. The sequences and traces were interpreted by BioEdit software.

The sequences (440-545 bp) obtained by eliminating the noisy nucleotides were then analyzed online by nucleotide BLAST tool on NCBI to find out the most homology sequences in GeneBank with studying sequences. The BLAST results have shown that all 23 samples were successfully determined the HAdV-serotypes, which was illustrated in Table 1. The homology rate of these sequences to those from Genebank was reached nearly 100%, excluded 1 sample containing similarity of DNA sequence to serotype 7 by 98%. There was no existence of different HAdV-types in the same sample. The HAdV-8 was the most common type found in this study with 19/23 samples.

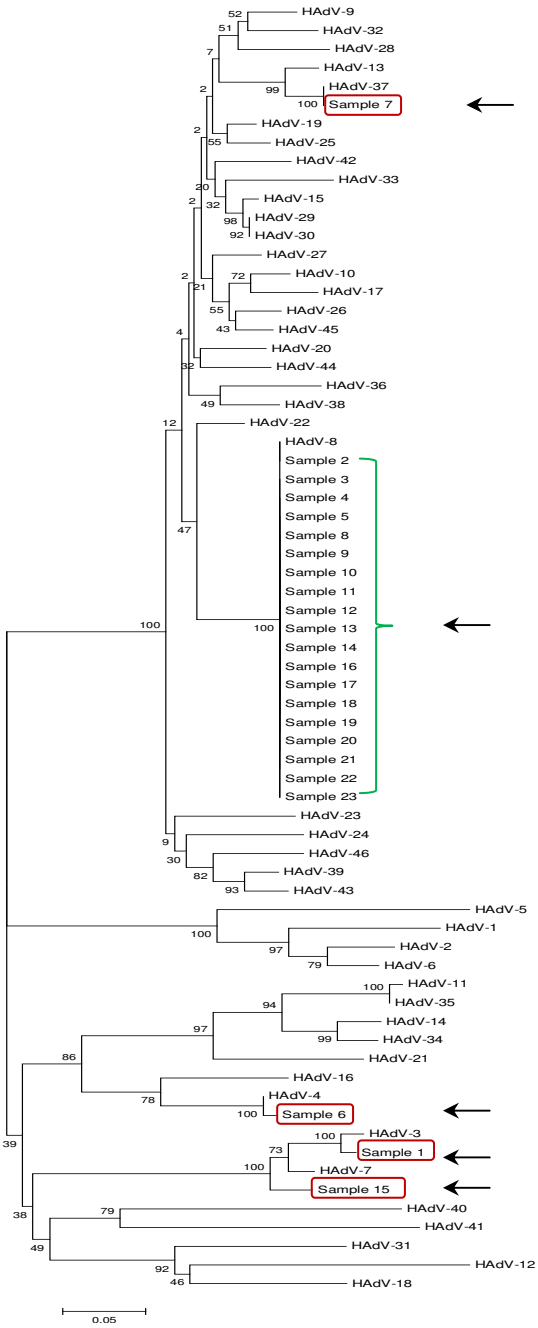

Figure 3. The phylogenetic tree of HAdVs based on the HVR-7 nucleotide sequence.

In addition, the sequence of HAdV-8 hexon gene which was a known sequence from GeneBank database (AB330089.1) and the sequences of samples identified above as type -8, -3, -4, -7, -37 were aligned together by using ClustalW Multiple Alignment method in BioEdit software. The noisy nucleotides from sequencing results had already been erased

before alignment. The sequences from samples were fit to the anticipated range comprising HVR-7 (approximately from 1000th nucleotide to 1600th nucleotide within hexon gene length of ~2900 bp), which indicates that the region containing HVR-7 was precisely amplified and sequenced by the edited primer pair. The alignment result was shown in Figure 2. The figure 2 also displayed that the sequences of the HAdV-8, -3, -4, -7, -37 samples (as defined above) contained many dissimilar nucleotides to each other. These different nucleotides could be adequate to distinguish HAdV-types.

Besides, phylogenetic analysis based on the nucleotide sequences of HVR-7 was conducted by using the neighbor-joining method within the Mega6 software. As shown in all Figure 3, each type was clearly separated in the phylogenetic analysis based on the HVR-7 sequence of hexon gene. The samples which have high homology to type 3, -4, -7, -8, and -37 were localized within the same branch and at the same internode with HAdV-3, -4, -7, -8, -37 respectively, as expected. This phylogenetic tree was not aimed at reflecting the phylogeny of HAdVs, and for more precise calculation of genetic distance among types, the much larger sequences, ideally whole genomes are required. In summary, the phylogenetic tree herein supported to display the distinct categories of HAdV-type based on HVR-7 of the hexon gene.

However, it should be considered that although this typing scheme based on the HVR-7 of hexon gene was proposed as one of the HAdV-typing systems, this method could fail to recognize the recombinant strain, especially if the new recombinant strains owning the similar regions at HVR-7 of the hexon gene. Nevertheless, it was suggested that these arising recombinant strains may occur relatively infrequently [11].

5. Conclusion

HVR-7 of the hexon gene was successfully amplified from clinical samples by PCR with using the edited primer pair. Human adenovirus DNA was detected in 23/36 clinical samples. The HAdV-types from clinical samples were recognized by sequencing the HVR-7 of the hexon gene. HAdV-3, -4, -7, -8, -37 were identified as the cause of conjunctivitis in this study, in which type 8 was the dominant type, detected in 19/23 samples.

Acknowledgements

We would like to thank the BIOFUND II grant 2016 (Faculty of Biology, HUS-VNU) for supporting this study.

References

[1] Pihos, A.M., Epidemic keratoconjunctivitis: A review of current concepts in management. Journal of optometry, 2013. 6(2): p. 69-74.

[2] Ishii, K., et al., Comparative studies on aetiology and epidemiology of viral conjunctivitis in three countries of East Asia—Japan, Taiwan and South Korea. International journal of epidemiology, 1987. 16(1): p. 98-103.

[3] Jawetz, E., The story of shipyard eye. British medical journal, 1959. 1(5126): p. 873.

[4] Jin, X.-H., et al., Molecular epidemiology of adenoviral conjunctivitis in Hanoi, Vietnam. American journal of ophthalmology, 2006. 142(6): p. 1064-1066.

[5] Ghebremedhin, B., Human adenovirus: Viral pathogen with increasing importance. European Journal of Microbiology and Immunology, 2014. 4(1): p. 26-33.

[6] Acheson., N.H., Fundamentals of molecular virology. 2nd ed. 2011. 274-275.

[7] Norrby, E., The structural and functional diversity of adenovirus capsid components. Journal of General Virology, 1969. 5(2): p. 221-236.

[8] Pichla-Gollon, S.L., et al., Structure-based identification of a major neutralizing site in an adenovirus hexon. Journal of virology, 2007. 81(4): p. 1680-1689.

[9] Madisch, I., et al., Phylogenetic analysis of the main neutralization and hemagglutination determinants of all human adenovirus prototypes as a basis for molecular classification and

taxonomy. Journal of virology, 2005. 79(24): p. 15265-15276.

[10] Avellón, A., et al., Rapid and sensitive diagnosis of human adenovirus infections by a generic polymerase chain reaction. Journal of virological methods, 2001. 92(2): p. 113-120.

[11] Sarantis, H., et al., Comprehensive detection and serotyping of human adenoviruses by PCR and sequencing. Journal of Clinical Microbiology, 2004. 42(9): p. 3963-3969.

[12] Takeuchi, S., et al., Serotyping of adenoviruses on conjunctival scrapings by PCR and sequence analysis. Journal of Clinical Microbiology, 1999. 37(6): p. 1839-1845.

Xây dựng quy trình phát hiện và phân loại adenovirus gây bệnh đau mắt đỏ ở người

Nguyễn Việt Hà[1], Nguyễn Thị Thu Huyền[1], Đỗ Thị Thanh Huyền[2], Nguyễn Quang Hưng[1], Trần Thùy Anh[1], Hoàng Anh Tuấn[3], Nguyễn Văn Sáng[1]

[1]*Khoa Sinh học, Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam*

[2]*Trường THPT Chuyên Khoa học Tự nhiên, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 182 Lương Thế Vinh, Thanh Xuân, Hà Nội, Việt Nam*

[3]*Bệnh viện Mắt Trung ương, 85 Bà Triệu, Hai Bà Trưng, Hà Nội, Việt Nam*

Tóm tắt: Bệnh viêm kết mạc hay đau mắt đỏ do virut adeno gây ra ở người có độ lây nhiễm cao và thường sinh bệnh kéo dài mà chưa có thuốc điều trị hiệu quả. Các chủng adenovirus ở người cũng rất đa dạng. Vì thế, việc xác định chính xác chủng virut adeno gây bệnh đau mắt đỏ cho người là vô cùng ý nghĩa trong các nghiên cứu dịch tễ. Trong nghiên cứu này, mục đích được đưa ra là phát triển phương pháp phát hiện và phân loại các chủng virut adeno gây bệnh đau mắt đỏ ở Việt Nam. Hệ gen của adenovirus được tách chiết từ các mẫu lấy từ người bệnh đau mắt đỏ ở Việt Nam. Vùng siêu biến đổi số 7 (HVR-7) nằm trên gen hexon của adenovirus được nhân lên với một cặp mồi cải biến. Vùng HVR-7 trên gen hexon sau đó được giải trình tự và phân tích để dựa vào đó xác định chủng adenovirus. Kết quả đạt được là các chủng 3, 4, 7, 8, 37 được tìm thấy trong các mẫu bệnh đau mắt đỏ, trong đó đa số các mẫu phát hiện được là chủng 8, với số lượng là 19 mẫu trên tổng số 23 mẫu dương tính với virut adeno.

Từ khóa: Adenovirus ở người, vùng biến đổi cao số 7 (HVR-7), chủng, bệnh đau mắt đỏ, PCR.