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Molecular analysis of human adenoviral keratoconjunctivitis cases: Results of a 2-year survey

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ABSTRACT

Objective: This study aimed to determine the adenovirus genotypes and their epidemiological features between January 2018 and November 2019, in Istanbul, Turkey.

Material and Methods: Conjunctival swab samples were obtained from patients who were clinically diagnosed with keratoconjunctivitis. Samples were screened with an Adeno Detector kit (Rapid Pathogen Screening, RPS Inc., South Williamsport, PA). Nucleic acid extraction and amplification were performed with the ADENOVIRUS ELITe MGB® kit in the ELITe In Genius instrument (Elitech Group, Torino, Italy). For subtyping of the strains, sequencing primers targeted the 'Hypervariable Region 7' (HVR-7) of the hexon gene were used. DNA sequence analysis (n:72) was performed with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA), and subtyping was done by BLAST analysis.

Results: The median viral load in the samples (n: 77) was 7 log10 copies/mL (IQR: 4.5-7.4 log10 copies/mL). The clinical finding score was found to be significantly higher in the high viral load group (Adenovirus DNA≥6 Log 10 copies/mL) than in the low viral load group (Adenovirus DNA<6 Log 10 copies/mL) (p = 0.031).

Conclusion: Our study analyzing hAdV strains collected in 2018 and 2019 revealed that genotype 8 is the dominant type (94.0%) in our region. Molecular methods are very useful for future epidemiological studies and the selection of a vaccine strain.

Keywords: Adenovirus, Genotyping, Viral conjunctivitis, Epidemiological analyzes, Epidemic keratoconjunctivitis

1. INTRODUCTION

Human adenoviruses (hAdVs) are icosahedral, non-enveloped, double-stranded deoxyribonucleic acid (DNA) viruses that can cause an array of diseases including conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia [1]. There are 51 serotypes of hAdVs based on neutralization assays which are classified into seven species HAdV-A to –G [2]. Over 60 types of the genotypes of hAdV have been identified based on sequence homologies as reported by Robinson et al. [3] or 88 as reported by Dhingra et al. [4].

Adenoviruses are the etiologic agents of the most commonly occurring ocular viral infections worldwide. Adenoviral infection in the eye can be in the form of epidemic keratoconjunctivitis (EKC), pharyngoconjunctival fever, and non-specific conjunctivitis [5]. Subgenus D consists of 32 serotypes including Ad8, Ad19, and Ad37, the main agents of EKC, and Ad9 and Ad15, which cause acute follicular conjunctivitis [6]. HAdV-associated follicular conjunctivitis or pharyngoconjunctival fever is relatively mild and short-term. In contrast, EKC is a highly contagious and more serious disease involving the cornea and conjunctiva, with potential long-term consequences on visual acuity leading to decreased quality of life and possible economic consequences. The modes of transmission are mainly through hand-to-eye contact, ocular secretions, respiratory droplets, and contact with medical instruments. Adenovirus is extremely hardy when deposited on environmental surfaces and may be detected on plastic and metal surfaces for more than 30 days [7]. Thus, the elimination of adenovirus from inanimate surfaces and ophthalmic instruments is essential in preventing outbreaks of EKC.

Various methods such as viral culture, antigen detection, serological tests, and nucleic acid detection can be used in the

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laboratory diagnosis of adenovirus infections. In recent years, the development and application of molecular methods using DNA amplification by polymerase chain reaction (PCR) have increased the sensitivity and enabled rapid diagnosis.

PCR primers for the hexon gene, fiber gene, or virally associated ribonucleic acid (RNA) I and II regions are usually preferred because they have some areas that are highly conserved among serotypes.

Identification of the adenovirus genotype is required to understand the geographical distribution of the virus and to improve the knowledge of the relation between a specific genotype and clinical presentation. Epidemiological studies determining genotypes can help to understand the nature of the epidemics and take effective infection control measures [8, 9]. Type-based hAdV surveillance has three objectives: 1) to monitor patterns of circulation for hAdV subtypes over time; 2) to assist with recognition and confirmation of outbreaks associated with circulating types; and 3) to inform the development or use of diagnostics tests, therapeutics, and vaccines [10]. Since, there is limited data about the subtyping of adenoviruses in Turkey, we analyzed hAdV strains obtained from conjunctival swab samples sent from the ophthalmology clinic.

2. PATIENTS and METHODS

Patients

Adult patients whose clinical signs and symptoms were compatible with acute adenoviral keratoconjunctivitis who tested positive for adenoviral antigen at the Marmara University Hospital Ophthalmology Clinic between January 2018 and November 2019 were included in the study. Clinical findings score for each patient was given by evaluation of the presence of eyelid edema, conjunctival injection, and chemosis by an ophthalmologist within the first 72 hours after the onset of symptoms. Rapid pathogen screening (RPS) adeno detector plus (RPS INC., Sarasota, Florida, USA) kit as a rapid immunoassay test for in vitro qualitative detection of adenoviral antigens (hexon protein) was used to detect antigens directly from human tears [11]. The test was performed by an ophthalmologist during a clinical examination.

Conjunctival swab samples (n: 77) obtained from patients clinically diagnosed as having keratoconjunctivitis and confirmed by a rapid antigen test were sent to the clinical microbiology laboratory for molecular analysis.

DNA extraction and real-time PCR Assay

DNA extraction was performed with the extraction cartridges Elite InGenius® SP 200 (ELITech Group, Torino, Italy) and adenovirus DNA was detected and quantified with ADENOVIRUS ELITe MGB® kit, in the fully automated ELITe In Genius TM instrument (ELITech Group, Torino, Italy) by using quantitative real-time PCR method according to the manufacturer's instructions. In each well, two amplification reactions are performed for a specific reaction of the Hexon protein gene and a specific reaction of the human beta Globin gene (Internal Control of inhibition). Measurement range of the assay is 250 to 25,000,000 copies of DNA. All specimens and viral DNA extracts were aliquoted and stored at – 80°C until further testing. One specimen per patient was selected.

Sequencing PCR

Primers targeted 605-629 base pairs including the conserved segments of "Hypervariable Region 7" (HVR-7) that differ according to different genotypes used [12]. The sequences of the sense and antisense primers were 5' – CTG ATG TAC TAC AAC AGC ACT GGC AAC ATG GG-3' and 5' – GCG TTG CGG TGG TGG TTA AAT GGG TTT ACG TTG TCC AT-3', respectively. The total volume of the reaction was 25 µl. Each reaction contained 2.5 µl 10X PCR buffer, 1.5 µl 25mM MgCl2, 0.2 µl of 25mM dNTP mix (Thermo Fisher Scientific, USA), 0.7 µl of each primer, 0,25 µl hot start Taq DNA polymerase (Thermo Fisher Scientific, USA), 16.15 µl distilled water and 3 µl DNA. PCR was performed using a T100™ Thermal Cycler (Bio-Rad, USA). The cycling parameters were as follows: an initial denaturing step of 15 min at 95˚C, 40 cycles consisting of denaturation at 95˚C for 1 min, annealing at 52˚C for 1 min, and elongation at 72˚C for 1min, with a final incubation at 72˚C for 10 min. After this, 5 µl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide. The bands were visualized with a UV transilluminator and then evaluated.

Sequence Analysis

ExoSAP-IT® mixture was used for the enzymatic removal of primers and dNTPs that were not bound in PCR products. The PCR purification reaction cycle was carried out at 37° C for 15 minutes and at 80° C for 15 minutes and the products were ready for the sequence stage. The sequencing reaction was performed by using the primers of HVR-7 sequencing PCR with T100™ Thermal Cycler (Bio-Rad, USA). Each 20 µl reaction contained 4 µl of Big Dye Terminator v 3.1 reaction mix (Thermo Fisher Scientific, USA), 2 µl of 5X sequence buffer (Thermo Fisher Scientific, USA), 0.7 µl of 5 pmol primers, 11.3 µl of distilled water and 2 µl of PCR product. The electrophoresis process of sequence products was performed in the automated DNA sequence analysis instrument of the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained hAdV DNA sequences were typed by BLAST analysis and the genotypes were identified by using the reference hAdV sequences of the NCBI [13]. The phylogenetic tree was drawn with the "neighbor-joining" method using the "MEGA-X" program including reference adenovirus sequences of genotypes 1 to 54 obtained from GenBank [14, 15]. The reliability of the phylogenetic tree was tested using the bootstrap test with 1000 replicates.

Statistical analyses

Statistical analysis was performed using SPSS v 22.0 (Statistical Package for Social Sciences, IBM, USA) software package. The distribution in SPSS according to Shapiro-Wilk was not normal. Therefore, non-parametric tests were used. The Fisher's Exact test was used in the analysis of categorized data. Data is

categorized according to the Adenovirus viral load detected in conjunctival swab samples. Adenovirus viral load was categorized as low (Adenovirus DNA<6 Log 10 copies/mL) and high (Adenovirus DNA≥6 Log 10 copies/mL). Total clinical finding score was compared between high and low viral load groups. The correlation between Adenovirus viral loads and total clinical finding scores was calculated using Spearman's rho test.

3. RESULTS

Demographic data and clinical finding scores of the patients were given in the Table. The median age (IQR) of the patients was 38 years (18-76 years) and 34 (45.3%) of them were male. Detection and quantification of hAdV DNA were performed in the conjunctival swab (n: 77) samples by quantitative real-time PCR. The median viral load in the samples was 7 log10 copies/mL (IQR: 4.5-7.4 log10 copies/mL) (Table). When the data was categorized according to the Adenovirus viral load, the clinical finding score was found to be significantly higher in the high viral load group than in the low viral load group (p = 0.031). The correlation between Adenovirus viral loads and total clinical finding scores was found to be statistically significant (r: 0.348, p = 0.002).

Table. *Characteristics of the patients (n=77)*

Age (years), median (IQR)	38 (18-76)
Sex, male (%)	33 (42.9)
Clinical finding score, median (IQR)	3 (1-7)
Adenovirus viral load in conjunctival swab samples (Log10 copies/mL), median (IQR)	7 (4.5-7.4)

Examination at the diagnosis
Slit lamp inspection
Eyelid edema (0 = absent, 1 = mild, 2 = medium, 3 = severe)
Conjunctival injection (0 = absent, 1 = mild, 2 = moderate, 3 = severe)
Chemosis (0 = absent, 1 = mild, 2 = medium, 3 = severe)
Clinical finding score was determined by evaluation of the presence of eyelid edema, conjunctival injection and chemosis.

After sequencing PCR, amplicons showing a band present in the gel electrophoresis (n: 72) were included in the DNA sequence analysis. Three samples could not be genotyped. Samples were numbered as day/month/year according to the date of admission to the hospital. A phylogenetic analysis based on the obtained sequences classified three genotypes (shown in Figs. 1-2). Three different genotypes, hADV-8 (97.1%, n=67), hADV-4 (1.4%, n=1) (sample no: 2.16_12.07.2019) and hADV-19 (1.4%, n=1) (sample no: 2.9_18.07.2019) were detected (Fig. 1). There were 4 clusters in genotype 8 (Fig. 2)

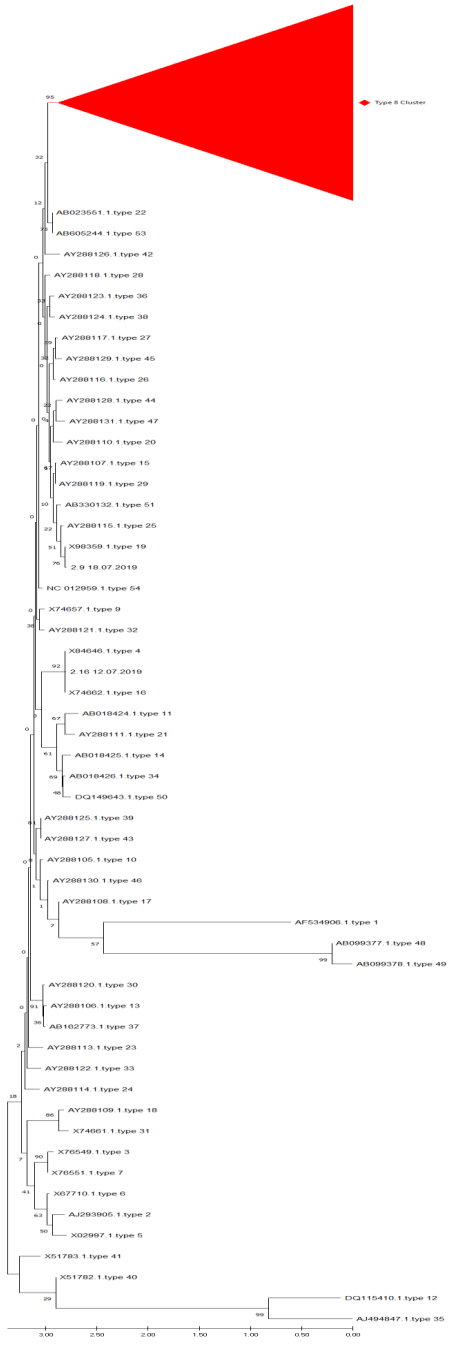

Figure 1. *Phylogenetic tree of hAdV strains*

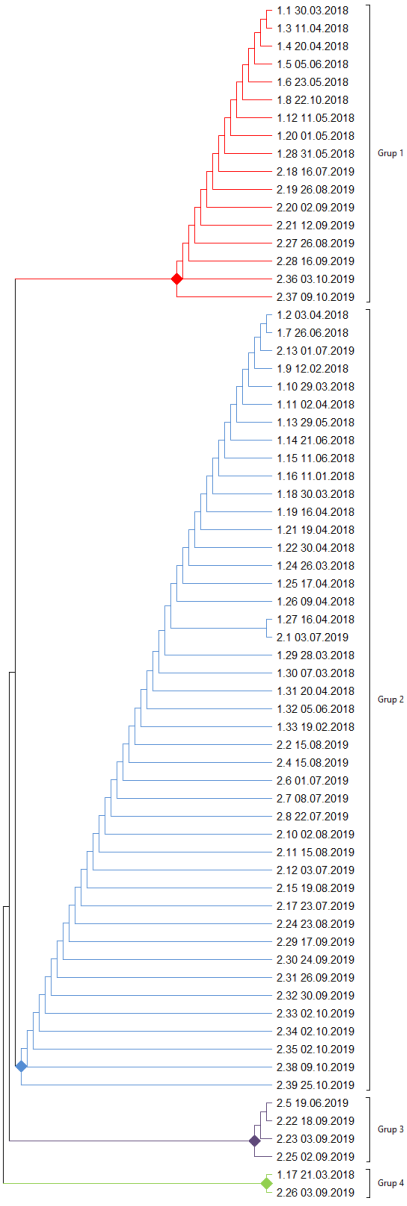

Figure 2. *Adenovirus type 8 cluster groups*

4. DISCUSSION

In our study, HAdV-8 was the most common genotype (97.1%) followed by hAdV-4 (1.4%) and hADV-19 (1.4%). The median viral load in the samples (n: 77) was 7 log10 copies/mL (IQR: 4.5-7.4 log10 copies/mL). The clinical finding score was found to be significantly higher in the high viral load group (Adenovirus DNA≥6 Log 10 copies/mL) than in the low viral load group (Adenovirus DNA<6 Log 10 copies/mL) (p = 0.031). HAdV-8 was the most common genotype (97.1%) followed by hAdV-4 (1.4%) and hADV-19 (1.4%).

The incidence of adenovirus eye infections varies worldwide. In the USA 20 million cases per year were reported whereas the incidence of adenovirus conjunctivitis cases is 0.2 to 0.8 per 100,000 population in Germany [16]. In Russia, more than 300,000 people are annually diagnosed with epidemic keratoconjunctivitis [17]. National Infectious Diseases Surveillance Center of Japan reports hAdV serotypes 3, 8, and 37 as the most common serotypes [18]. In Tunisia, North Africa between 2012 and 2013, hADV-8 (87.6%) was identified as the dominant genotype and this is followed by hADV-4 (6.8%), hADV-3 (3.5%) [19]. In the study conducted in patients with acute conjunctivitis in West India between 2013-2014, hAdV-8 (78.6%), hADV-37 (7.2%), hAdV – 3 (7.2%) and hAdV – 4 (7.2%) serotypes were detected [20].

There are very limited data on the ocular adenovirus infection and genotype distribution in Turkey. The first study published in 2010 reported hAdV genotypes 3, 4, and 8 from conjunctival swab samples (n: 9) collected in 2003 and 2004 [21]. An outbreak of adenovirus conjunctivitis in a neonatal intensive care unit was related to genotype 8 only that were obtained from 14 patients [22]. During the 5-year study period between 2006 to 2010, in adenovirus-positive patients with conjunctivitis (n: 101) type 8 was the dominant genotype (66.3%) followed by genotype 4 (24.7%) [23]. Tezcan et al. [24] included conjunctival swab samples from patients with acute conjunctivitis (n: 100) and from healthy individuals (n: 50) between September 2014-July 2017. A total of 5 genotypes were identified and the most common genotypes were hAdV-8 (n: 17, 63%) and followed by hAdV-53 (n: 4, 14.8%), hAdV-4 (n: 4, 14.8%).

Our study analyzing hAdV strains collected in 2018 and 2019 revealed that genotype 8 is the dominant type (94.0%) in our region. HAdV-8 has been the commonest genotype both in sporadic infections and during epidemics possibility related to high tropism for conjunctival cells produces severe clinical manifestations and pathologic alterations [25]. HAdV-8 is also an important cause of healthcare-associated outbreaks and has been associated with contaminated ocular instruments and ophthalmologic solutions [26]. We could not detect an epidemiologic relation among our isolates. The incidence of hAdV-4 in ocular infection is rare [23, 26]. HAdV – 4 outbreak was demonstrated in a group of fifty patients who had used the same swimming pool [27].

In a multicenter US study, hAdV was detected in 390 (78%) conjunctival swab samples of 500 participants with a 6.52 mean viral load in log10 copies/mL, and high viral load at presentation was associated with poorer clinical outcomes [28]. The mean viral load in our samples was 7 log10 copies/ mL. Measuring viral load in repetitive samples is important to detect the efficacy of treatment in viral blood-borne pathogens like HIV, CMV, and HBV. Latent adenovirus reactivation or transmission during transplantation can be responsible for disseminated infection and graft loss and viral load monitoring is essential to quickly set up an appropriate therapy [29]. We found a significant relationship between the severity of clinical findings and Adenovirus viral load (p=0.03).

Quantification of hAdV in ocular infections by measuring viral load in clinical samples could be investigated to evaluate the efficacy of the treatment, especially for patients having recurrent conjunctivitis.

Conclusion

To our knowledge, this is the first study from Turkey that detects hAdV viral load in clinical samples and analyses epidemiological relations between strains. Our study analyzing hAdV strains collected in 2018 and 2019 revealed that genotype 8 is the dominant type (94.0%) followed by hAdV-4 (1.4%) and hADV-19 (1.4%) in our region. We presented only a small amount of strains since the high cost was a limiting factor. Molecular methods are very useful for future epidemiological studies and the selection of a vaccine strain.

Compliance with the Ethical Standards

Ethical Approval: The study protocol was approved by the Institutional Review Board and the Ethics Committee of Marmara University School of Medicine (Protocol number: 4.01.2019-09).

Human and animal rights

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. No animals were used in this research. All humans research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20 activities/10ethics/10helsinki/). General written consent including the laboratory tests to be made was obtained from patients who admitted to our hospital as a routine application.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

AG: Doing PCR and sequencing tests, analyse results, manuscript preparation, RCS: Method determination, doing real time PCR tests, analyse real time PCR results, manuscript preparation, AKY: Method determination, analyse PCR and sequencing results, manuscript preparation, FOA: Conception and design, data collection, analysis and interpretation of data, critical revision of the manuscript, SAT: Conception and design, data collection, analysis and interpretation of data, critical revision of the manuscript, MAK: Analyse PCR and sequencing results, doing phylogenetic analyses, manuscript preparation, AET: Conception and design, data collection, analysis and interpretation of data, critical revision of the manuscript,

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