RESEARCH PAPER

Molecular epidemiology of human Adenovirus among patients with Keratoconjunctivitis

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A B S T R A C T:
Various Human Adenoviruses (HAdV) serotypes are reported to be accompanied with keratoconjunctivitis infections. Seventy-three conjunctival swabs were obtained from (39 male and 34 female) who presented to the ophthalmic unite in the hospital of Erbil province with clinical features of a acute keratoconjunctivitis. Immunochromatography (IC) was performed 51(69.86%) samples were positive out of 73 eyes swab. In the present study, nested PCR was employed to amplify the partial hexon gene, and the sequence of the 37 isolates were specified and compared to sequences deposited in NCBI. The results illustrated that all isolates showed 96.7 to 100% nucleotide identity and all sequences were submitted to Genbank-NCBI which documented and received accessions number start from (MK615151 to MK615187) respectively. Phylogenetic analysis classified these isolates into different genotype-related groups A, B, D, E, and F and human adenovirus HAdV-D8 were the most frequent type. The results of nucleotide Blast of HAdV-D8 with the deposited sequence in NCBI were revealed that similar to genotype sequence documented in Germany in (99%).

KEY WORDS: Molecular epidemiology Adenovirus keratoconjunctivitis partial hexon gene
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INTRODUCTION:
As a highly contagious eye infection with probable global outbreaks, epidemic keratoconjunctivitis (EKC) can cause a severe type of conjunctivitis on the surface of the eye (Wold and Horwitz, 2007). Adenoviruses are regarded as the main cause of viral conjunctivitis, and they contain double-stranded non-enveloped DNA viruses that belong to the family Adenoviridae, genus Mastadenovirus which are highly resistant to environmental influence and can transmit from person to person through infectious secretions and particularly as tear fluids (Aldloch et al., 2010). Ocular adenoviral infections are associated with different keratoconjunctivitis (EKC), chronic papillary conjunctivitis, and non-specific follicular conjunctivitis. One of the specifications of EKC is severe bilateral conjunctivitis with involvement of corneal. Period of incubation is 8 to 10 days, and corneal affection is likely to continue for months (O’Brien et al., 2009). Sixty-eight types of HAdVs were identified and assigned into 7 groups (A–G) based on immunological distinctiveness, genome sequencing, phylogenetic, biological characteristics and sequence (Walsh et al., 2009). Of all conjunctivitis cases, the global prevalence of adenoviral conjunctivitis has been reported to be between 15 and 70% in both sporadic and epidemic forms. Moreover, epidemic keratoconjunctivitis may break out in large scales in schools, hospitals, and military establishments (Weiss et al., 1993; Shamsi-Shahrabadi et al., 2009).

Adenoviruses have been typed differently in various parts of the world. According to different
studies, the major causes of adenoviral conjunctivitis are HAdV-4 (in HAdV-E), HAdV-3 (in HAdV-B), and HAdV-8, HAdV-19 and HAdV-37 (in HAdV-D) with three HAdV-D serotypes that are known to cause EKC with severe symptoms including severe discharge, membrane formation, multiple subepithelial corneal infiltrates, and lacrimation (Aoki and Tagawa, 2002; Jin et al., 2006; Kaneko et al., 2005; Janani et al., 2012). Different HAdV types are recombined, and there are cases of co-infection with two or more types of HAdV, as a result of which intermediate types emerge and more virulent strain may be resulted. Moreover, new types like HAdV-53 or changes in tropism as in HAdV-56 may be observed (Kaneko et al., 2009; Robinson et al., 2011). According to DNA sequence analysis, hexon, penton, and fiber genes were the most frequent variables among various adenovirus serotypes (Madisch et al., 2005; Miura-Ochiai et al., 2007). Currently, no vaccine exists for the civilians; however, a vaccine against type 4 and 7 are available on the license, which is only provided to U.S. military recruits (Tate et al., 2009).

Few studies in Iraq have focused on the molecular epidemiology of adenoviruses, particularly adenoviral keratoconjunctivitis. In this regard, the present study was conducted in order to identify the molecular epidemiology of HAdV infections so as to achieve a clear picture of the prevalent types.

2. MATERIALS AND METHODS

2.1. Patients and clinical specimens

Seventy three conjunctival swabs (39 men, 34 women) were collected from patients with age groups ranging from nine to seventy six year with suspected viral keratoconjunctivitis based on clinical features (with at least one of the findings and complaints below: sudden pinkness or redness, discomfort, pain, tearing, burning, conjunctival hemorrhages, and eyelid swelling) which attending the clinical ophthalmic unite in Rizgary hospital in the period 2016 to 2017. An ophthalmologist examined the patients, and two specimens were retrieved from each patient with two sterile Dacron swabs by ophthalmologists. One of the swabs was employed for IC adenovirus test, and the other one was gathered in 3 ml M5 viral transport medium (Remel Microbiology product, USA), immediately placed in ice and transported to the laboratory. The samples were first vortexed in transport medium for 30 seconds, then the excess fluid was removed from the swabs, and finally the swabs were discarded. The processed specimens were kept at -70°C.

2.2. Immunochromatography test (IC)

The immunochromatography test was carried out based on the manufacturer’s instruction (Aden test, SA Scientific™, USA). Directly after sample collection using sterile swab this test performed in which 4 drops (about 150μl) of the specimen were put into the kit specimen well. Sufficient time was devoted for the specimen to filter through the kit to the specimen and control positions, and the results were read within a period of 15 minutes. Depending on the concentration of antigen, some positive results can be observed in as short as 30 seconds. When two colored lines appeared at specimen and control position, the test was regarded positive, when only one colored line appeared in the C (control) position, the test result was interpreted as negative, and when there was no line, it was considered invalid and the same specimen was retested. Within 30 minutes from application of specimen to the kit, the readings were finalized.

2.3. Molecular detection of HAdV

2.3.1. Human Adenovirus DNA extraction

DNA was extracted from 37 isolates which gave positive IC adenovirus test through QIAGEN DNA extraction kit (Hilden, Germany). According to the protocol of the manufacturers’ instructions, the kit reagents were reconstituted before use. The molecular detection of virus was done in Genome center/ Koya University.

2.3.2. Partial hexon gene amplification using nested PCR

Nested PCR was utilized to amplify a partial hexon sequence of HAdV from all of the 37 isolates in two steps (Shimada et al., 2004; Miura-Ochiai et al., 2007). Using 50pmol of a pair of primers AdTU7 and AdTU4 during the first step, the 1004bp fragment of the hexon gene was amplified (Table 1). With a pair of primers, AdnU-S, and AdnU-A using 10μl of the first step
PCR product, the 956bp DNA fragment was amplified using nested PCR. PCR was conducted for 36 cycles in a PCR Thermal Cycler (Eppendorf, Germany). Each cycle included annealing at 50°C for 1 minute, denaturation at 94°C for 1 minute, and primer extension at 72°C for 2 minutes. Following the last cycle, the extension was continued at 72°C for 7 minutes. The products of the PCR were separated on 1% agarose gels, and a QIAquick gel extraction kit was used to purify them (Qiagen, Germany). PCR amplifications were conducted in 50µl volumes containing 25 µl GoTaq green master mix (Promega, USA), 2 µl of amplicon and 1 µl of each primer needed for the specific reaction and reaction completed using dH₂O. Using a cycle of denaturation at 94°C for 1 minute, primer extension at 72°C for 2 minutes, and annealing at 50°C for 1 minute, PCR was performed in a PCR thermal cycler (fermentas, Germany) for 36 cycles. Following the final cycle, the samples were extended at 72°C for 7 minutes. This PCR amplification was carried out by utilizing the same protocol as used for the first reaction. Following the nested-PCR amplification, 5 µl of the reaction mixture was subjected to electrophoresis on a 1% agarose gel that contained ethidium bromide.

Table (1): Primer used for partial hexon gene amplification and sequencing

| Primer   | Sequence                  | Product size/bp | Reference                  |
|----------|---------------------------|-----------------|----------------------------|
| AdTU7    | 5'-GCCACCTTCTTCCCCATGCGC-3' | 1004            | Shimada et al., (2004)     |
| AdTU4    | 5'-GTAGCGTTGC CGCCCGAGAAA-3' |                | Miura-Ochiai et al., (2007) |
| AdnU-S   | 5'-TTCCCCATGG CGCACAACAC-3' | 956             |                            |
| AdnU-A   | 5'-GCCTCGATGAGCGGCACCTGGT-3' |                |                            |

2.3.3. Partial hexon gene amplification using nested PCR

An Applied Biosystem 3130 Genetic analyzer was used to determine the nucleotide sequence of the PCR products, and sequencing reaction was done by employing an ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Genome Centre, Koya University). To analyze the nucleotide sequences of the partial hexons, they were compared to those of prototype strains of all HAdV serotypes obtained from GenBank using ClustalX for Windows version 1.81, with the default parameters. MEGA7 version (7.0.26) was used to construct phylogenetic trees. In brief, Kimura’s two-parameter method was utilized to compute the evolutionary distances (Kimura, 1980), and the neighbor-joining (N-J) method was employed to construct unrooted phylogenetic trees (Saitou and Nei, 1987). Bootstrap analyses were performed by 1,000 replicates of the data sets (Felsenstein, 1985). The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Kimura two-parameter method (Kimura, 1980) with a transition/transversion ratio of 2.0 was utilized to calculate the similarity of the hexon gene between the HAdV and the prototype strains in each window of 200 nucleotides. The nucleotide sequences of the partial hexon genes (956bp) of all 37 strains of HAdV were determined in Genome center/ Koya University.

3. RESULTS AND DISCUSSION

3.1. Circulating types and associated diseases

Adenoviruses are a main common cause of conjunctivitis all over the world. Recently 68 human adenovirus genotypes were identified and grouped into 7 species A to G. Distinguishing between serotypes associated with simple conjunctivitis and those accompanied with more severe epidemic keratoconjunctivitis was desirable in ocular infections (Sarantis et al., 2004; Walsh et al., 2009; Hogan and Crawford, 2018). In 2016, some cases of nosocomial EKC were observed at a Rizgary hospital located in Erbil, the Kurdistan Region of Iraq. The seventy-three samples (39 male, 34 female) with age groups ranging from (9-76) years were tested using IC test in which 51(69.86%) samples (27 male, 24 female) were positive HAdV results and 22 (30.13%) negative to HAdV IC test (See Table 2). This method was developed for rapid diagnosis of human adenovirus and commercially available kit. The frequency of HAdV infections in males and
females was normal and same distribution, as well as age. Genotype and seasonal differences were observed all over the study and the peak season was mostly during the spring months because in the spring season there is a lot of subject travel, pool swimming, and different other activities, as in figure (1).

Table (2): Immunochromatography test (IC) and nested PCR for detection of HAdV in the patient with adenoviral keratoconjunctivitis

| IC test | Nested PCR |
|---------|------------|
| Positive (%) | Negative (%) | Positive (%) | Negative (%) |
| 51(69.86%)     | 22 (30.13%)  | 37(72.54%)   | 14(27.45%)   |

Furthermore, genotypes of the virus vary based on population and geographical distribution for this the DNA genomes from positive IC test were extracted, and two steps nested PCR was applied. For confirmation and genotyping, a set of primers was employed to amplify a partial hexon gene, which was found to be effective in amplifying all of the HAdVs strains that it selected based on conserved sequences of the hexon gene as in table (1). In this study thirty-seven (72.54%) Adenoviral DNA was detected using nested PCR and 14 (27.45%) were negative, The PCR products of all clinical specimens were 956bp when applied on 1% agarose gel electrophoresis as in figure (2). The targets band excised and purified from agarose gel using DNA purification kit (Fermentas, Germany). Amplification using nested PCR has become more applicable to clinical specimens and popular alternative methods, sensitive, fast, and accurate. Approximately the two used methods gave the same results which indicated high specificity, and these findings are in agreement with those reported by Jin et al. (2006), Levent et al. (2009), Al-Kasaby et al. (2011), and Demian et al. (2014).

Figure (1): Seasonal genotypes distribution of human Adenovirus in keratoconjunctivitis patients

Figure (2): Agarose gel electrophoresis analysis 1% (stained with ethidium bromide) of PCR products showing partial hexon gene amplified from the seventy three Human Adenovirus with specific primer that indicated the expected size 956 bp. Lane L: is 1 kb DNA Ladder; Lanes 1-12,13,15: HAdV Hexon gene product amplified from HAdV genome run on 75V for 45min.

3.2. Phylogenetic analysis and sequencing of partial hexon region

An ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Genome Centre, Koya
University) was used to sequence and type 37 purified partial hexon region genes successfully. After the DNA sequences were analyzed, the BLAST search engine National Center for Biotechnology Information (NCBI) was used. Through a BLAST search of all partial hexon gene (956bp), it was showed that the highest homology was found with the hexon proteins sequences of human adenovirus deposited in NCBI and it was found that samples belonged to different groups of HAdV-A, -B, -D, -E, and -F (Figure 3) and all 37 sequences were submitted and received Genbank-NCBI accessions number start with (MK615173 to MK615187) respectively. Moreover, the sequences obtained in this study and HAdV reference sequences retrieved from GenBank(http://www.ncbi.nlm.nih.gov/Genbank) were utilized to construct a phylogenetic tree. Based on the phylogenetic analysis, there was similarity between most of the isolates and the selected reference strains; however, in some cases a degree of divergence from the reference strains was observed but the analysis gave acceptable bootstrap values. Furthermore, Serotypes belonging to the same species made cluster together in which serotypes 8, 9, 19, and 37 were encompassed the species D. Genotype 7, 11, 16, and 50 encompassed together within the species B. Also serotype 40 and 41 within the species F. Genotypes 18 and 4 within species A and E respectively as in figure (3). Results showed that 19 (51.3%) isolates were HAdV-D, 9 (24.3%) isolates were HAdV-B, 4 (10.8%) were HAdV-F, 3 (8.1%) were HAdV-E and 2 (5.4%) were HAdV-A with different genotypes (Table 3).

The constructed phylogenetic trees demonstrated that the amplicon of each serotype possessed a readily identifiable sequence and belonged to different human adenovirus groups A, B, D, E, and F with a different genotype beside that it showed that HAdV-8 was most prevalent causing adenoviral keratoconjunctivitis and it showed serotypes are quite distinct and the bootstrap values are overall quite high particularly at the nodes between species; however, they were less good for some nodes within species. This finding agrees with other studies reported in Turkey and India (Erdin et al., 2015; Akhil et al., 2016). It should also be noted that group B was second predominates genotypes. According to the results of most previous studies, EKC is typically resulted from one of the three HAdV-D serotypes -8, -19, and -37, and these strains lead to frequent nosocomial outbreaks. These viruses originally were isolated from patients that had EKC in 1951, 1955, and 1976. So far, several variants of these viruses have been reported as the causes of keratoconjunctivitis (Tabery, 1995; Tanaka-Yokogui et al., 2001; Ariga et al., 2005; Zhou et al., 2012). Many subjects and travellers from various regions around the world and have moved to Erbil, and they may bring viruses such as adenovirus with them, and this may have contributed to the spread of epidemics of adenoviral keratoconjunctivitis. Beside that the virus may obtain pathogenesis through some unknown mechanisms, leading to large nosocomial EKC infections because mutations of the hexon gene might change the antigenicity of the adenovirus, these changes of antigenicity may allow the virus to escape from acquired immunity and may result in an outbreak of EKC. Another reason simultaneous infection of two or more HAdV types results in recombination, and a large number of these intermediate strains are accompanied with different diseases of varying intensity, which has been well documented between 1973 and 1992 and typed by De Jong et al. (1999) and Janani et al. (2012).

Genotypes of the virus are diverse and differ according to population and geographical distribution. The HAdV-D8 was most predominant genotypes were compared with the other nucleotide sequences of HAdV-D8 which isolated and sequenced from other countries and deposited in NCBI using Blast engine. Results were showed that 33 samples from Germany have 99% identity rate to detected HAdV-D8 of study, 20 samples from Denmark in 97.2%, 18 samples from the USA in 93.2% identity (Table 4).
Table (3): Adenovirus serotypes and frequency associated with keratoconjunctivitis

| HAdV subgroup | Serotype   | Frequency of serotype (percentage) |
|---------------|-----------|-----------------------------------|
| A             | 18        | 2 (5.4%)                          |
| B             | 7, 11, 16, 50 | 9 (24.3%)                        |
| D             | 8, 9, 19, 37 | 19 (51.3%)                       |
| E             | 4         | 3 (8.1%)                          |
| F             | 40, 41    | 4 (10.8%)                         |

Table (4): HAdV-D8 identity with the other sequences isolated from others country.

| No. of Sample | Country   | Identity % |
|---------------|-----------|------------|
| 33            | Germany   | 99%        |
| 20            | Denmark   | 97.2%      |
| 18            | USA       | 93.2%      |
| 14            | Tunisia   | 89.1%      |
| 8             | Japan     | 89%        |

Few epidemiological and limited data exist on ocular adenoviral infections and genotype distribution in Iraq and to the best of the author’s knowledge, this study has been the first report on the molecular epidemiology of HAdV infections from Iraq but several outbreaks of keratoconjunctivitis caused by adenovirus have been reported within other countries supported our study in Saudi Arabia, Egypt, Iran, and Turkey (Casas et al., 2005; Tabbara et al., 2010; Ayoub et al., 2013; Sohrabi et al., 2014; Lee et al., 2015; Akçay et al., 2017).

Figure (2): The phylogenetic analysis included 37 nucleotide sequences of HAdV inferred using the Neighbor-Joining method [1]. The percentage of the replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated next to the branches [2]. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 version (7.0.26).

4. CONCLUSIONS

In the present study the circulating HAdV strains and their epidemiology in Erbil, the Kurdistan region of Iraq between 2016 and 2017 were documented that causes keratoconjunctivitis
using direct sequencing techniques is an accurate, efficient, and rapid means of diagnosing and the most common cause of adenoviral keratoconjunctivitis was human adenovirus types 8 these data will be useful in predicting future outbreaks of adenovirus infection. However, due to the limited sample size, the seasonal peak of adenovirus infection was in spring. It is recommended that a well-designed epidemiological study be conducted in order to obtain generalizable findings on the relationships among the serotypes, the sequences of their hexon gene, and prevalent serotypes and their associated keratoconjunctivitis diseases in Erbil province. Epidemiological surveillance of HAdV serotypes will improve understanding of the global infection of HAdV.

Conflict of Interest
There is no conflict of interest.

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