Detection and molecular typing of human adenovirus associated with respiratory illness in Kerala

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**KEYWORDS:** Human adenovirus; respiratory illness; Kerala; HAdV-E4; HAdV-C2

Running title: Human adenovirus in respiratory infection in India
SUMMARY: Adenoviruses are responsible for approximately 5-10% of acute respiratory infections globally. However, there are limited reports on circulating respiratory human adenovirus (HAdV) types in India. We detected HAdV in the post mortem specimens of a young child died due to acute febrile illness. In order to know the circulating adenovirus types in the Alappuzha region, retrospectively, the samples (n=235) collected from influenza-like illness patients for influenza surveillance program were screened for HAdV. Fourteen samples were positive for adenovirus by PCR. Adenovirus was isolated from three out of fourteen PCR positive samples in HEK 293 cell lines. The viral strains isolated in the study were from children between ages 6 and 10 years. The isolates were identified as adenovirus species C and E. Further, the sequencing of fiber gene and BLAST search revealed that the two of the isolates were type HAdV-C2 and the third isolate was a HAdV-E4. Fiber gene sequence based phylogenetic tree showed that the isolate HAdV-E4 closed to the Japanese HAdV-E4 strain, whereas the isolates HAdV-C2 formed a distinct cluster. Although respiratory infections due to HAdV-E4 are generally seen in adults, this is the first instance showing the involvement of HAdV-E4 strain with respiratory illness in children.

INTRODUCTION
Adenovirus is a non-enveloped, double stranded DNA virus belonging to the *Adenoviridae* family. It was first isolated and characterized during the search for the etiologic agents of acute respiratory infection in the year 1953 (1, 2). There are seven species (A-G) of human adenovirus (HAdV) and so far 70 types have been classified on the basis of their serological and molecular characteristics (3, 4). Adenovirus infects different systems of the body and produces clinical symptoms like respiratory, conjunctivitis, cystitis, gastrointestinal, myocarditis, and very rarely meningo-encephalitis.

Acute respiratory tract illness is a globally recognized major health problem in children. About 5 to 10% of the lower respiratory tract infections (LRTIs) in infants and children are caused by human adenovirus (5). Adenovirus species B, C, and E generally cause mild respiratory infection. However, fatal infection occurs particularly in infants, immunocompromised individuals and in individual with neurological disorders and pulmonary problems (6, 7).

Adenovirus capsid is composed of three major proteins: fiber protein, penton base protein and hexon protein. Hexon gene consists of conserved and hyper variable regions. Owing to its hyper-variable regions, the hexon protein is the most important part of the adenovirus proteome for the classification of adenovirus types (3). Adenovirus and its types were identified by immunological assays such as hemagglutination inhibition and cell culture-based type-specific neutralization assays. Adenovirus type specific sera are employed in both of the assays to identify the adenovirus types (8-10). Now a days, the typing can be done by PCR amplification and sequencing of hexon, penton and fiber genes. Hexon gene sequence analysis has widely been used for the classification of adenovirus types; however sequence analysis of the fiber gene has also been incorporated to determine the recombination events in the adenovirus genotype (11-13).
We have received the postmortem specimens from a three and half year old boy who died at hospital after consecutive 3 days fever. The autopsy report did not indicate a specific cause of death. Adenovirus was detected from trachea, lungs, serum, and nasal swab specimens and it was human adenovirus type 2 (14). Therefore, it was decided to find out the circulating human adenovirus type in Alappuzha region. The samples collected from influenza like illness (ILI) patients for influenza surveillance purpose were used for HAdV screening. Adenovirus has been detected in some samples and the virus was isolated from the positive samples. The isolated virus was typed by molecular methods.

**MATERIALS AND METHODS**

**Study Area and Setting:** Kerala lies at the southern end of Indian peninsula with a population of 33.38 million (2011 census). The population studied lives in Alappuzha district of the state of Kerala. Owing to its proximity to the sea, the climate of Alappuzha is humid and hot during summer. Alappuzha has a Government Medical College Hospital and many Primary Health Centers. There are six sentinel sites in different parts of the district and samples were collected from the outpatient ward of the sentinel hospitals.

The study was approved by Institute Human Ethical Committee, National Institute of Virology, Pune. The reference number is No.110 (01)/EC-I/1141. An ILI patient was defined as a person presenting with sudden onset of fever (>38°C) or history of sudden onset of fever in the recent past (≤ 3 days) with cough or sore throat and/or rhinorrhea in the absence of other diagnosis (15).

**Specimen Collection and Processing:** The respiratory specimens (nasopharyngeal swabs) were collected from patients who fit into the ILI case definition. Collected swabs were then placed in sterile screw-capped containers with Viral Transport Media (VTM) and transported under refrigeration to the laboratory. Swabs were gently agitated and extracted for further processing. The extracts were kept at -80°C until used. A total of 235 samples (including 11 influenza type A
and 10 influenza type B positive samples) from the period June—October, 2013 were tested for adenovirus in this study.

**Qualitative real time PCR:** DNA extractions were performed from 140 µL of swab specimens using QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to manufacturer’s instruction. The viral DNA was eluted in 50 µL elution buffer. The extracted DNA was tested for adenovirus by Real Time PCR using the published hexon gene based primers and probe (16) with 2x Platinum Quantitative PCR Supermix –UDG (Invitrogen, Carlsbad, USA) in 20 µL reaction volume. Amplification was carried out at 95 °C for 10 min of initial denaturation followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min in ABI 7500 instrument (Applied Biosystems, Foster City, CA). Adenovirus vector (pAdEasy-1, human adenovirus type 5) from AdEasy Adenoviral vector system (Agilent Technologies, USA) was used as a positive control.

**Virus Isolation:** Adenovirus PCR positive swab specimens were inoculated into confluent monolayer of human embryo kidney-293 (HEK-293) cells in 25 cm² tissue culture flasks. The flasks were kept at 37 °C under 5% CO₂ atmosphere up to 7 days. The cells were observed for cytopathic effects (CPE) following which the tissue culture fluid was harvested. All culture supernatants were screened by conventional adenovirus hexon gene PCR to confirm the viral presence as well as gene sequencing using published protocol (17).

**Species identification:** Multiplex fiber gene based PCR was carried out for adenovirus species identification (17) with 2x Emerald Amp GT PCR Master Mix (TaKaRa, Otsu, Japan) and multiple sets of adenovirus species-specific primers in 25 µL reaction volumes. Amplification was carried out at the following setting: 94 °C for 5 min of initial denaturation followed by 30 cycles of 94 °C for 1 min, 54 °C for 45 sec, 72 °C for 2 min, with a final extension of 72 °C for 5 min. The amplified PCR products were resolved in 1% agarose gel and stained with SYBR green for visualization.
Sequencing of fiber and hexon gene: Hexon and fiber gene of the virus isolates were amplified for sequencing as described above. The amplified PCR products were separated on 1% agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) as per the manufacturer’s protocol. The purified PCR products were outsourced to SciGenomic Technology Pvt. Ltd, India for sequencing. The fiber and hexon gene sequences of adenovirus isolates determined in this study have been deposited in the National Center for Biotechnology Information (NCBI) GenBank under the accession numbers KM077443- KM077445 (Hexon gene sequences) and KM983019-KM983021(Fiber gene sequences).

Phylogenetic analysis: Phylogenetic trees were constructed from both the partial hexon nucleotide sequences and the entire open reading frame of fiber gene. Available HAdV sequences from GenBank were used as the reference genome. MEGA version 5 was used for phylogenetic tree construction based on neighbor-joining (NJ) method using the Kimura’s two-parameter distance model, with 1000 bootstrap replicates.

RESULTS

Age-wise break-up of adenovirus and influenza virus positive samples were given in table 1. Of the 235 samples tested for HAdV, fourteen samples were found positive by Real Time PCR. All 14 PCR positive samples were from patients with age between 6 to 29 years old. Among the positive samples, 9 were females and 5 were males. As the cycle threshold (Ct) values were more than 35 in most of the samples, it was decided to isolate the virus from the samples for further downstream process. Virus isolation was successful from three samples which were obtained from children of ages 6, 7 and 10.
The fiber gene based multiplex PCR revealed that the isolates were adenovirus species C (n=2) and E (n=1). The results were further confirmed by phylogenetic tree constructed from the hexon gene (432bp) as shown in Fig.1. For construction of phylogenetic tree, 31 known hexon sequences of genotypes including 4 of HAdV-A (HAdV-12, 18, 31 and 61), 7 of HAdV-B (HAdV-3, 7, 14, 16, 35, 52, 55 and 66), 4 of HAdV-C (2 of HAdV-2, 1 and 57), 8 of HAdV-D (HAdV-8, 9, 10, 17, 56, 58 and 64), 5 of HAdV-E (HAdV-4), 2 of HAdV-F (HAdV-40 and 41) and 1 of HAdV-G (HAdV-52) from GenBank were used.

The basic local alignment search tool (BLAST) sequence analysis results showed that the sequences were identical to HAdV-C2 and HAdV-E4. A phylogenetic tree of HAdV-E isolate (854 bp) was constructed with the available 12 HAdV-E4 partial fiber nucleotide sequences. For HAdV-C, the available 12 known full length fiber nucleotide sequences of HAdV-C2 from GenBank were used for the nucleotide sequence alignment and the phylogenetic tree construction. The result of phylogenetic analysis of the fiber gene indicated that the isolate HAdV-E4 was clustered with the Japanese strain (Figure 2) and the isolate HAdV-C2 formed a distinct cluster (Figure 3).

DISCUSSION

Adenoviruses are pathogenic to human but often cause mild and self-limited disease. Some adenovirus types are reported to cause severe diseases, especially type 3, 4, 7, and 21 (18-20). Most commonly associated adenovirus types with respiratory infections are HAdV-3, 7, and 21 of species B; HAdV-1, 2, 5, and 6 of species C and HAdV-4 of species E (21, 22). HAdV-E4, the only member of species E often causes conjunctivitis and respiratory infections (2, 23, 24).

In this study 235 samples were tested for HAdV, of which, type 2 and 4 of adenovirus species C and E were identified. In hexon gene sequence based phylogenetic tree, the
adenoviruses formed seven major clusters of species A-G. One of our isolate was grouped together with species E whereas other two isolates were with species C. In the clusters HAdV-C and HAdV-E, however, the isolates had formed a different sub-cluster from the known adenovirus strains. In BLAST nucleotide analysis, HAdV-E isolates aligned with sequences of type 4 and the HAdV-C aligned with type 2. Further, to analyze the genetic relationship of the isolates with other available HAdV more precisely, the fiber gene was sequenced. The fiber gene phylogenetic tree of the HAdV-E4 isolate showed 100% similarity with Japanese TC-18040 strain isolated in 2002 (25) as shown in the Fig. 2. However, the fiber gene phylogenetic tree of the HAdV-C2 isolates revealed that the Kerala isolates were segregated from the available HAdV-C2 strains and formed a distinct cluster as shown in the Fig. 3.

Although the classification of adenovirus types were mostly relied on the sequence analysis of hyper variable region of the hexon gene together with the fiber gene, this study could not target the hyper variable region of the hexon gene due to the shortage of project fund. Despite the limitation, to our knowledge the information provided by this study equally contributes the importance of adenovirus type prevalence in Kerala, India.

Adenovirus types 1, 2, 3, 5, 6 and 7 mainly cause respiratory diseases (26). Among these adenovirus types, HAdV-1, 2, 3 and 7 are the major types associated with acute respiratory illness (27). Adenovirus type 1 and 2 are occurred significantly more often in respiratory diseases (28, 29). In India, the most common adenovirus types associated with respiratory illnesses are HAdV-3, 2 and 7. Adevovirus type 4 was reported in India during an outbreak of epidemic conjunctivitis occurred in Chennai, in the year 1991 (30, 31). Association of adenovirus type 4 with respiratory illnesses was reported elsewhere in the military recruits but not a single case has been observed in India so far (32). In addition, molecular characterization of respiratory adenovirus types in India is very limited. This study describes the association of
adenovirus type 4 with respiratory illness and the molecular characterization of the isolated HAdV-E4 for the first time in India.

In fiber gene based phylogenetic tree, the HAdV-C2 formed a distinct cluster, which indicates the requirement of further molecular study to confirm the new variant. The isolate HAdV-E4, which is closely related to HAdV-4 Japanese strain isolated from the epidemic conjunctivitis in Japan. Until now, HAdV-E4 was thought to be associated with respiratory infection in adults (17, 33). However, this study showed the association of HAdV-E4 with respiratory illness in children for the first time in India. This study warrants continuous surveillance of adenovirus infection in this region in order to provide more epidemiological information for therapeutic approaches.

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Conflict of interest: None to declare.

REFERENCES


**Figure Legends**

Figure 1: Phylogenetic analysis of human adenovirus clinical isolates (dark circles) and the other reference strains (white circles) based on the hexon gene (432bp). Numbers at nodes are indicated as the percentage of 1,000 bootstrap values.

Figure 2: Phylogenetic analysis of human adenovirus type 4 clinical isolates (dark circles) and the other reference strains (white circles) based on the fiber gene (854 bp). Numbers at nodes are indicated as the percentage of 1,000 bootstrap values.
Figure 3: Phylogenetic analysis of human adenovirus type 2 clinical isolates (dark circles) and the other reference strains (white circles) based on the fiber gene (1749 bp). Numbers at nodes are indicated as the percentage of 1,000 bootstrap values.

Table 1 Age wise analysis of samples of influenza like illness cases (n=235) selected for this study to detect the adenovirus
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<table>
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<th>Age interval (years)</th>
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<tr>
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<tr>
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<td>0</td>
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<td>≥65</td>
<td>4</td>
<td>0</td>
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\(^1\)Samples from the period of June—October, 2013