

Original Article

Detection and Molecular Typing of Human Adenoviruses Associated with Respiratory Illnesses in Kerala

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SUMMARY: Adenoviruses are responsible for approximately 5–10% of acute respiratory infections globally. However, there are a limited number of reports on the types of circulating respiratory human adenoviruses (HAdV) in India. We detected HAdV in the post-mortem specimens of a young child who died as a result of an acute febrile illness. To retrospectively investigate the circulating adenovirus types in the Alappuzha region, samples ($n = 235$) collected from patients with influenza-like illnesses who participated in the influenza surveillance program were screened for HAdV. Fourteen samples were identified as positive for adenovirus by PCR analysis. Adenovirus was isolated from 3 of the 14 PCR-positive samples cultured using HEK-293 cell lines. The viral strains isolated in the study were from children between 6 and 10 years of age. The isolates were identified as adenovirus species C and E. Sequencing analysis of the fiber gene and a BLAST search revealed that 2 of the isolates were type HAdV-C2, and the third isolate was a HAdV-E4. A fiber gene sequence-based phylogenetic tree showed that the HAdV-E4 isolate was similar to the Japanese HAdV-E4 strain, whereas the HAdV-C2 isolates formed a distinct cluster. Respiratory infections due to HAdV-E4 are generally observed in adults; this study is the first to demonstrate the involvement of the HAdV-E4 strain in respiratory illnesses in children.

INTRODUCTION

Adenovirus is a non-enveloped, double-stranded DNA virus belonging to the *Adenoviridae* family. It was first isolated and characterized in 1953 during the search for the etiologic agents underlying acute respiratory infections (1,2). There are 7 species (A–G) of human adenovirus (HAdV), and 70 types have been classified on the basis of their serological and molecular characteristics to date (3,4). Adenovirus infects different systems of the body, producing clinical symptoms such as those for respiratory conditions, conjunctivitis, cystitis, gastrointestinal conditions, myocarditis, and very rarely, meningoencephalitis.

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

The adenovirus capsid is composed of 3 major proteins: fiber, penton base, and hexon. The hexon gene

consists of conserved and hypervariable regions. Owing to its hypervariable regions, the hexon protein is the most important part of the adenovirus proteome with respect to the classification of adenovirus types (3). The adenovirus and its types were identified using immunological assays, such as the hemagglutination inhibition and cell culture-based type-specific neutralization assays. Adenovirus type-specific sera are employed in both of these assays to identify the adenovirus type (8–10). Currently, the typing can be done through PCR amplification and sequencing of the hexon, penton, and fiber genes. The 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).

In the current study, we received postmortem specimens from a 3-year-old boy who died at our hospital after 3 consecutive days with a fever. The autopsy report did not indicate a specific cause of death. Adenovirus was detected in the trachea, lungs, serum, and nasal swab specimens, and it was identified as HAdV type 2 (14). Therefore, we decided to investigate the circulating HAdV types in the Alappuzha region. The samples were collected from patients with influenza-like illnesses (ILI) as part of an influenza surveillance program and used for HAdV screening. Adenovirus was detected in some of the samples, and the virus was isolated from the positive samples. The isolated virus samples were typed using molecular methods.

MATERIALS AND METHODS

Study area and setting: Kerala lies at the southern end of the Indian peninsula with a population of 33.38

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million (2011 census). The population studied lives in the Alappuzha district of the state of Kerala. Owing to its proximity to the sea, the climate of Alappuzha is humid and hot during the summer. Alappuzha has a Government Medical College Hospital and many Primary Health Centers. There are 6 sentinel sites in different parts of the district, and samples were collected from the outpatient ward of the sentinel hospitals.

This study was approved by Institute Human Ethical Committee of the National Institute of Virology in Pune. The reference number is No. 110 (01)/EC-1/1141. An ILI patient was defined as a person presenting with a sudden onset of fever ($>38^{\circ}\text{C}$) or a history of a sudden onset of fever in the recent past (≤ 3 days) with a cough or sore throat and/or rhinorrhea in the absence of another 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, gently agitated, extracted, and kept at -80°C until testing. A total of 235 samples (including 11 influenza type A and 10 influenza type B positive samples) obtained from June 2013 to October 2013 were tested in this study.

Qualitative real-time PCR: DNA extractions were performed using 140 μL swab specimens using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. 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 the 2 \times Platinum Quantitative PCR Supermix-UDG (Invitrogen, Carlsbad, CA, 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 an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). The adenovirus vector (pAdEasy-1, HAdV type 5) from the AdEasy adenoviral vector system (Agilent Technologies, Santa Clara, CA, USA) was used as a positive control.

Virus isolation: Adenovirus PCR-positive swab specimens were inoculated into a confluent monolayer of human embryo kidney-293 (HEK-293) cells in 25 cm^2 tissue culture flasks. The flasks were kept at 37°C under 5% CO_2 atmosphere up to 7 days. The cells were observed for cytopathic effects, following which the tissue culture fluid was harvested. All culture supernatants were screened using a conventional adenovirus hexon gene PCR assay to confirm the viral presence as well as with gene sequencing using published protocols (17).

Species identification: Multiplex fiber gene-based PCR was carried out for adenovirus species identification (17) with 2 \times 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 settings: 94°C for 5 min of initial denaturation followed by 30 cycles of 94°C for 1 min, 54°C for 45 sec, and 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 genes: The hexon and fiber genes of the virus isolates were amplified for sequencing as described above. The amplified PCR products were separated on 1% agarose gel and purified using the QIAquick Gel Extraction Kit (QIAGEN) as per the manufacturer's protocol. The purified PCR products were outsourced to SciGenomic Technology Pvt. Ltd. (Kerala, India) for sequencing. The fiber and hexon gene sequences of the adenovirus isolates in this study have been deposited in the National Center for Biotechnology Information 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 the fiber gene. Available HAdV sequences from GenBank were used as the reference genome. MEGA version 5 was used for phylogenetic tree construction based on the neighbor-joining method using the Kimura's 2-parameter distance model with 1,000 bootstrap replicates.

RESULTS

The distributions of the adenovirus and influenza virus-positive samples based on patient age are shown in Table 1. Of the 235 samples tested for HAdV, 14 samples were positive by real-time PCR. All 14 PCR-positive samples were from patients 6–29 years of age. Among the positive samples, 9 and 5 were from female and male patients, respectively. As the cycle threshold values were more than 35 in most of the samples, we decided to isolate the virus from the samples for further downstream processing. Virus isolation was successful from 3 samples that were obtained from children who were 6, 7, and 10 years of age.

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 through the analysis of a phylogenetic tree constructed using the hexon gene sequence (432 bp), as shown in Fig. 1. For the construction of the phylogenetic tree, 31 known sequences of hexon genotypes from GenBank were used: 4 of HAdV-A (HAdV-12, 18, 31, and 61), 8 of HAdV-B (HAdV-3, 7, 14, 16, 35, 52, 55, and 66), 4 of HAdV-C (2 of HAdV-2, 1, and 57), 7 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-

Table 1. Age wise analysis of samples of influenza like illness cases ($n = 235$) selected for this study to detect the adenovirus

Age interval (yr)	Total number of samples ($n = 235$) ¹⁾	Real time PCR positive		
		Adenovirus	Influenza Type A	Influenza Type B
0–14	59	6	3	3
15–44	132	8	7	6
45–64	40	0	1	1
≥ 65	4	0	0	0

¹⁾ Samples from the period of June–October, 2013.

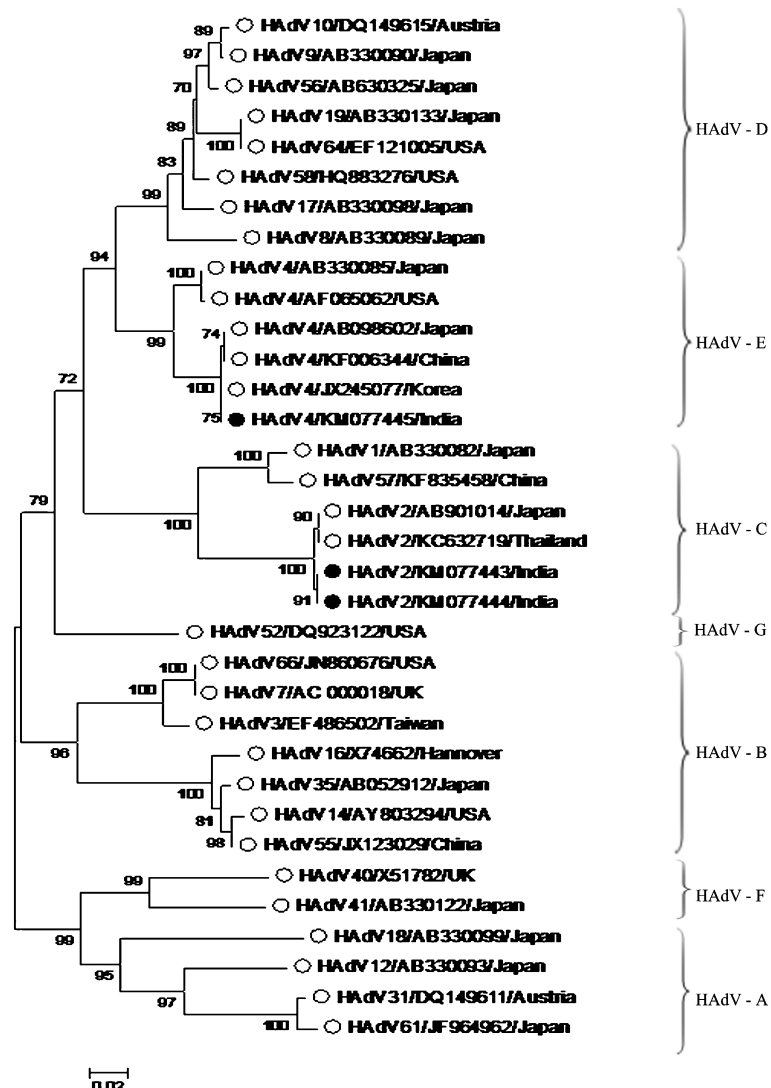


Fig. 1. Phylogenetic analysis of human adenovirus clinical isolates (dark circles) and the other reference strains (white circles) based on the hexon gene (432 bp). Numbers at nodes are indicated as the percentage of 1,000 bootstrap values.

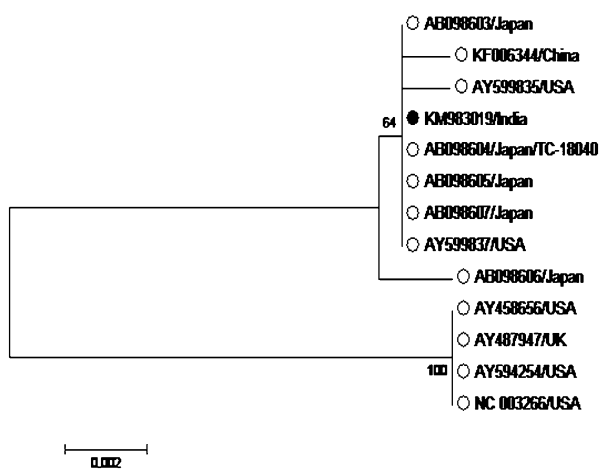


Fig. 2. Phylogenetic analysis of human adenovirus type 4 clinical isolates (dark circles) and the other reference strains (white circles) based on the fiber gene (854 bp). Numbers at nodes are indicated as the percentage of 1,000 bootstrap values.

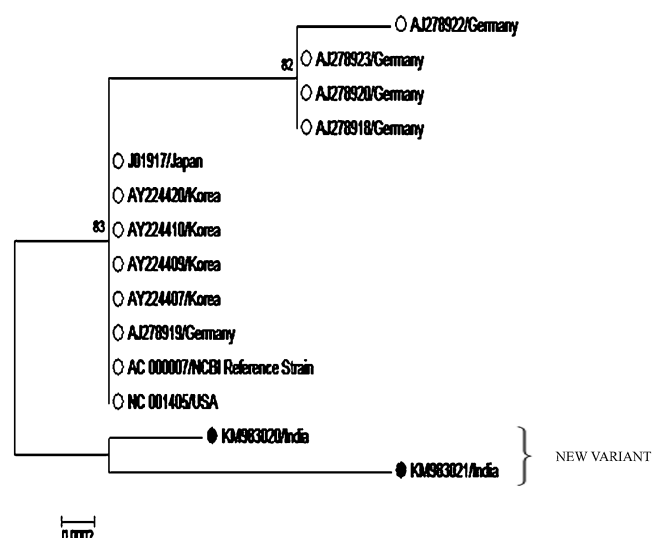


Fig. 3. Phylogenetic analysis of human adenovirus type 2 clinical isolates (dark circles) and the other reference strains (white circles) based on the fiber gene (1,749 bp). Numbers at nodes are indicated as the percentage of 1,000 bootstrap values.

52).

The results of the sequence analysis with the basic local alignment search tool (BLAST) showed that the sequences were identical to those of 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 the phylogenetic analysis of the fiber gene indicated that the HAdV-E4 isolate clustered with the Japanese strain (Fig. 2), and the HAdV-C2 isolate formed a distinct cluster (Fig. 3).

DISCUSSION

Adenoviruses are pathogenic to humans, but they often cause mild and self-limited disease. Some adenovirus types are reported to cause severe diseases, especially types 3, 4, 7, and 21 (18–20). The adenovirus types that are most commonly associated 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, types 2 and 4 of adenovirus species C and E were identified. In the hexon gene sequence-based phylogenetic tree, the adenoviruses formed 7 major clusters of species A–G. One of our isolates was grouped together with species E, whereas the other 2 isolates clustered with species C. However, in the clusters of HAdV-C and HAdV-E, 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 isolate aligned with type 2. Further, to more precisely analyze the genetic relationship of the isolates with other available HAdV isolates, the fiber gene was sequenced. The fiber gene phylogenetic tree of the HAdV-E4 isolate showed 100% similarity with the Japanese TC-18040 strain isolated in 2002 (25), as shown in Fig. 2. However, the fiber gene phylogenetic tree of the HAdV-C2 isolates revealed that the isolates from this study were segregated from the available HAdV-C2 strains and formed a distinct cluster, as shown in Fig. 3.

Although the classification of adenovirus types mostly relies on the sequence analysis of the hypervariable region of the hexon gene together with the fiber gene, this study could not evaluate the hypervariable region of the hexon gene due to limited funding resources. Despite this 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 primarily 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 types 1 and 2 occur significantly more frequently in patients with respiratory diseases (28,29). In India, the

most common adenovirus types associated with respiratory illnesses are HAdV-3, 2, and 7. Adenovirus type 4 was reported in India during an outbreak of epidemic conjunctivitis that occurred in Chennai in 1991 (30,31). The association of adenovirus type 4 with respiratory illnesses was reported elsewhere in military recruits, but not a single case has been reported in India to date (32). In addition, the 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 the fiber gene-based phylogenetic tree, the HAdV-C2 formed a distinct cluster; further molecular studies are needed to confirm this new variant. The HAdV-E4 isolate is closely related to the HAdV-4 Japanese strain isolated during the conjunctivitis epidemic in Japan. Until now, HAdV-E4 was thought to be associated with respiratory infections in adults (17,33). However, this study reveals an association of HAdV-E4 with respiratory illness in children for the first time in India. These results suggest that the continuous surveillance of adenovirus infections in this region is warranted in order to provide more epidemiological information for the improvement of therapeutic strategies.

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Conflict of interest None to declare.

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