Genotyping of human adenoviruses circulating in Southwest India

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Abstract Adenoviruses are found to be associated with a wide range of diseases in children and adults. There is little data available on the circulating serotypes of Human Adenoviruses (HAdVs) in the southwest region of India. In this study, we explore the molecular epidemiology of HAdVs circulating in southwest India. Twenty-three samples (Adenovirus PCR positive), collected between January 2011 and March 2013, have been typed based on the partial hexon gene sequence and phylogenetic analysis. The commonest serotypes were HAdV-3 and HAdV-2. The other serotypes were HAdV-7, HAdV-1, HAdV-8 and HAdV-40. Respiratory illness was the most common clinical manifestation of HAdV-3, HAdV-2 and HAdV-7 serotypes. HAdV-3, HAdV-7 and HAdV-8 were found to cause conjunctivitis, whereas HAdV-1, HAdV-2 and HAdV-3 caused encephalitis. In conclusion, this study documents the circulating HAdV strains and the epidemiology in southwest India. To the best of our knowledge, this is the first study on the molecular epidemiology of HAdVs in India.

Keywords Human adenovirus · HAdV · Molecular epidemiology · Southwest India · Phylogenetic 17 analysis · Hyper variable region

Introduction

The human adenoviruses (HAdVs) belong to the genus *Mastadenovirus*. A total of 68 types of HAdVs have been identified and classified into seven species or subgroups (A–G). Adenoviruses are found to be associated with a wide range of diseases including respiratory diseases, conjunctivitis, encephalitis, gastroenteritis, and hemorrhagic cystitis [5]. The clinical manifestations may vary according to the age and immunocompetence of the host. These infections are usually mild in immunocompetent patients but could be severe in immunocompromised patients [5]. The clinical manifestations, the pattern of infection, and disease severity vary in species and serotype. Serotypes 1, 2, 3, 5, 6 and 7 mainly causes respiratory tract infections, while types 40 and 41 are responsible for gastroenteritis. A severe form of conjunctivitis named as epidemic keratoconjunctivitis (EKC) is associated with Adenovirus Types 8, 19 and 37. Other diseases such as acute hemorrhagic cystitis and myocarditis are also caused by these viruses.

The typing of adenoviruses has been carried out in different parts of the world. HAdV-3, 2, 1, and 7 are the major types associated with acute respiratory illness (ARI), whereas Type 40 and 41 are the major etiologic agents of gastroenteritis [7]. Adenoviruses can infect people of all ages, but they are most common in children under 5 years. Several outbreaks of severe lower respiratory tract infections (LRTI) caused by HAdV-14 in closed communities such as military camps and day care centres have been reported. There are cases on recombination between different types of HAdV and co-infection with two or more HAdV types, being the major reason for the emergence of intermediate types [6]. This recombination may result in a more virulent strain. Also, the emergence of new types

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such as HAdV-53 or a change in tropism as in HAdV-56 have been witnessed [12]. Currently, there is no vaccine available for the civilians. However, there is a vaccine against type 4 and 7, available on the license, only for U.S. military recruits [14].

A limited number of studies have been conducted on the molecular epidemiology of adenoviruses in India, especially of respiratory adenoviruses. The studies which have been conducted in India mainly focused on molecular epidemiology of adenovirus associated with gastroenteritis as well as keratoconjunctivitis. In this context, we attempted to identify the molecular epidemiology of HAdV infections in South West part of India to obtain a clear picture of the prevalent types, associated diseases and the seasonality of infections caused by these viruses.

Materials and methods

Study population

The study population included patients from different parts of Kerala and Karnataka States, India, during April 2011 to March 2013. From 51 archived samples of patients (with adenovirus infection), 30 samples, presenting clinical symptoms of respiratory illnesses, gastroenteritis, conjunctivitis and encephalitis, were selected and used for the study at MCVR. Samples included throat swabs, blood samples and stool samples. A total of 16 respiratory cases and 6 gastro enteritis cases (including 3 cases of severe diarrhoea) were selected. Remaining 5 cases were of encephalitis and 3 cases were of conjunctivitis.

DNA extraction and PCR

Viral DNA was extracted from the clinical samples using MagMAXTM Total Nucleic Acid Isolation Kit (Life Technologies, USA, Cat. No. AM1840) according to manufacturer's instructions. A nested PCR was used to amplify the hexon L1 region containing hyper variable region 1 (HVR 1–6) to HVR 6 of the genome [11].

The external primers used were **AdhexF1**: 5'TIC TTT GAC ATICGI GGI GTI CTI GA3' (position 19135–19160) and **AdhexR1**: 5'CTG TCI ACI GCC TGR TTC CAC A3' (20009–20030). A second round of PCR was performed using internal primers **AdhexF2**: 5'GGY CCY AGY TTY AAR CCC TAY TC3' (19165–19187) and **AdhexR2**: 5'GGT TCT GTC ICC CAG AGA RTC IAG CA3' (19960–19985). Reference for the nucleotide binding position is Genbank accession number AC_000007.1 [11].

Amplification was carried out on Veriti[®] Thermal Cycler (Life Technologies, USA) with the reaction condition as described by Lu and Erdman [11]. In a 50 µL

reaction mixture, 0.2 µM of each primer and 2.5 U of Superscript III RT/Platinum Taq Enzyme Mix (Life Technologies, USA) was used. For the first round amplification, 10 µL of extracted nucleic acid was added.

In brief, amplification was performed at 94 °C for 2 min denaturation followed by 35 cycles of 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min, with a final extension of 72 °C for 5 min. For the nested reaction, 1 µL of the primary PCR product amplified using the same conditions mentioned above. A known positive control and negative control included in each reaction.

The PCR products expected to be ranging in size from 764 to 896 bp (primary amplification) and 688–821 bp (secondary amplification) which containing HVR 1 to HVR 6. Along with this region, a non-specific region of size approximately 1250 bp also amplified by the PCR reaction.

Sequencing

For the extraction of specific product of approx. 750–800 bp size, GenEluteTM Gel Extraction Kit (Sigma-Aldrich, USA) was used. Extraction was carried out according to manufacturer's protocol. Sequencing was performed in a single direction using the internal forward primer (AdHex F2) and the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) on an ABI 3500 sequencer (Life Technologies, USA).

Nucleotide sequence analysis

Serotypes were identified using Genbank BLAST tool from NCBI, with identity score $\geq 97\%$. The hexon gene sequences of another 33 Adenoviruses (different types obtained from GenBank) were included as reference strains for phylogenetic analysis. These strains were isolated from different parts of the world during different time periods.

Phylogenetic analysis was done by neighbor-joining method with 1000 repetitions (bootstrap value: 1000) by Mega 5.2 software. Analysis of recombination in hyper variable region was performed using the software Simplot 3.5.1.

Results

Circulating types and associated diseases

Of the 30 samples tested, 23 samples were successfully sequenced and typed.

On BLAST analysis with the sequences from GenBank, the most predominant serotypes identified were HAdV-B3

and HAdV-C2. Less common types identified were HAdV-B7, HAdV-C1, HAdV-D8 and HAdV-F40.

HAdV-2 and 3 were associated with almost similar clinical syndromes and mean age; whereas all type 7 cases were found in adults with a mean age of 31. Distribution of HAdV infections in males and females were found to be almost similar (Table 1).

Most of these cases were mild but some severe cases such as LRTI and diarrhoea were also observed.

HAdV-2 and 3 were found to be majorly associated with respiratory tract infections. Lymphadenopathy, rashes, conjunctivitis, seizures and gastroenteritis were also presented by these two HAdV types. The two adenoviral LRTI cases during the study period were found to be caused by HAdV-3 and were characterized by breathlessness. Typing revealed that HAdV-3 was associated with suspected myocarditis in a child presenting with LRTI. Type 2 and 3 were also associated with pharyngoconjunctival fever in the study population.

Adenovirus 1 was identified from a child with severe diarrhoea and the stool was found to be containing blood. Adenovirus from another encephalitis case was also typed as 1. Both ARI and conjunctivitis were caused by Type 7. The conjunctivitis cases by type 7 were presented with or without fever and lymphadenopathy. Type 8 was associated with one case of conjunctivitis and one gastroenteritis case was found to be caused by enteric adenovirus type 40.

Month wise distribution of adenovirus infection

The majority of the infections occurred during the months ranging from January to March. More number of respiratory illnesses and gastroenteritis cases were observed in

Fig. 1 Seasonality of typed and untyped Human adenovirus (HAdV) infections in southwest India, March-2011 to March-2013

the months of January and February (2012 and 2013) (Fig. 1).

Phylogenetic analysis of hyper variable region 1–6 (HVR 1–6)

Phylogenetic analysis revealed that most of the isolates were similar to the selected reference strains, however, some strains exhibited varying degree of divergence from the reference strains (Fig. 2). Most of the HAdV-3 strains were similar to the reference strains, whereas type 1, 2 and 7 were divergent. The isolates HAdV2/MCVR/26640/2013 and HAdV3/MCVR/26712/2013 diverged from other isolates (of the same type) as well as from the reference strains. HAdV40/MCVR/26617/2013 and HAdV8/MCVR/23219/2012, single representatives in the study from the genera D and F respectively, were found to be distant from reference strains. Adenovirus isolates of type 7 and 8 and 40 also showed differences from reference strains. The

Table 1 Description of demographic characteristics and clinical syndromes of HAdV positive patients and identified HAdV types (N = 23)

Description	HAdV-1 (n = 2)	HAdV-2 (n = 7)	HAdV-3 (n = 9)	HAdV-7 (n = 3)	HAdV-8 (n = 1)	HAdV-40 (n = 1)
Age						
Mean (SD)	2 (1.4)	3.4 (2.9)	3.8 (3.2)	31 (14.4)	34	5
Median (IQR)	2 (1–3)	3 (2–3)	3 (1–5)	25 (20–47)	34	5
Mode	–	3	–	–	–	–
Sex						
Male	1 (50 %)	3 (43 %)	5 (55.6 %)	2 (66.7 %)	1	–
Female	1 (50 %)	4 (57 %)	4 (44.4 %)	1 (33.3 %)	–	1
Clinical syndromes						
ARI	–	5 (71.4 %)	7 (78 %)	2 (66.7 %)	–	–
Gastroenteritis	1 (50 %)	–	–	–	–	1
Conjunctivitis	–	–	1 (11 %)	1 (33.3 %)	1	–
Encephalitis	1 (50 %)	2 (28.6 %)	1 (11 %)	–	–	–

HAdV human adenovirus, SD standard deviation, IQR intra quartile range, ARI acute respiratory illness

Fig. 2 Neighbor-joining tree constructed from amino acid sequences of Hexon gene hyper variable region 1–6 (HVR 1–6) from study HAdV types and reference strains (using MEGA6.06). The study HAdV sequences are marked by a red square. Bootstrap value (1000 replicates) showed at internal branch nodes. Nucleotide sequences of partial hexon gene containing HVR 1–6 of study isolates were deposited in Genbank under Accession No. KM274903–923

analysis gave acceptable bootstrap values. There was no evidence of recombination in the hyper variable region of these isolates.

Discussion

Human adenoviruses are one of the major pathogen associated with ARI and gastroenteritis in children worldwide. These viruses are also associated with severe LRTI, CNS infections, conjunctivitis and urinary tract infections. Pediatric population is most commonly affected by HAdV infections. In the current study, children below 5 years of age were majorly infected by HAdV-1, 2, 3, and 40, whereas type 7 was identified in adults.

The epidemiologic study conducted in Thailand reported that HAdV infection occurs throughout the year, with a peak observed from January to March. HAdV genotype distribution can change on a yearly basis [13]. The probable seasonality that was obtained from this study also reflected the same result. HAdV-3 was predominant in the year of 2012 whereas, in 2013, type 2 was more common. The conjunctivitis cases were observed in July and August, which matched with the previous report on seasonality occurrence in India [10].

HAdV-2 and 3 are mostly associated with respiratory tract infections worldwide. Serotype 1–3 have shown predominance in pediatric adenovirus lower respiratory tract infection in Canada [2] and in our study, 2 pediatric cases of LRTI were found to be associated HAdV-3.

Several outbreaks of conjunctivitis caused by adenovirus type 7 and 8 have been reported in adults and neonate [3, 4]. Isolates of type 7 and 8 in this study were also found to be associated with conjunctivitis in adults. From an outbreak of HAdV-7 ARI in Malaysia, which cost 5 lives [17], the presence of circulating HAdV-7 type in the current study area was considered a significant issue. Adenovirus type 2 has been isolated from an adult man presenting with encephalitis and Huang et al. [9] have reported 3.3 % of CNS involvement in the pediatric population with adenovirus infections, with seizures being the most commonly noticed symptom (64 %). In the current study, type 2 was associated with two pediatric encephalitis cases.

Type 1 was found to be the etiologic agent of encephalitis in a child presenting with fever, rashes and seizures. No reports were available linking HAdV type 1 with encephalitis and gastroenteritis. Type 1 and 40 were detected from two cases of gastroenteritis. Type 1 was associated with a case of severe diarrhoea. However, coinfection with other viruses and asymptomatic shedding in faeces have to be considered in this case. Quantification of viral load may help to resolve this problem. Several studies showed that types 1, 2, 3, 5 and 21 are associated with myocarditis in children [16]. Fatal cases of HAdV-3 myocarditis have been reported by Treacy et al. [15]. Adenovirus type-3 was identified as the causative agent of LRTI and suspected myocarditis in the current study.

Recombination between serotypes, within the HVR 1-6 also have been reported [11], however on analysis, no such evidence was obtained in this study. Sequencing of only partial hexon gene may fail to identify novel HAdV types, for example, Ad4, which shows high sequence identity to Ad16 hexon and a species C fiber, could have resulted from recombination between a species B and a species C virus [8]. An HAdV strain, M86, contains recombinant HVR 4 and entire fiber gene from Ad11. Recombination has been also noticed among the highly conserved Ad3 type [1]. Coinfection of two or more HAdV types allows the recombination and many of these intermediate strains are associated with different diseases of varying severity.

In conclusion, this study documents the circulating HAdV strains and their epidemiology in southwest India. To the best of our knowledge, this is the first report on the molecular epidemiology of HAdV infections from India. However, due to limited sample size, a well-designed epidemiological study is required to obtain generalizable data on the prevalent serotypes circulating in India and associated diseases.

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References

- Adhikary AK, Inada T, Numaga J, Suzuki E, Ushijima H, Banik U, et al. Characterisation of hexon and fibre genes of a novel strain of adenovirus involved in epidemic keratoconjunctivitis. *J Clin Pathol.* 2004;57:95–7.
- Alharbi S, Caesele PV, Consunji-Araneta R, Zoubeydi T, Fanella S, Souid A-K, et al. Epidemiology of severe pediatric adenovirus lower respiratory tract infections in Manitoba, Canada, 1991–2005. *BMC Infect Dis.* 2012;12:55.
- Birenbaum E, Linder N, Varsano N, Azar R, Kuint J, Spierer A, et al. Adenovirus type 8 conjunctivitis outbreak in a neonatal intensive care unit. *Arch Dis Child.* 1993;68:610–1.
- Caldwell GG, Lindsey NJ, Wulff H, Donnelly DD, Bohl FN. Epidemic of adenovirus type 7 acute conjunctivitis in swimmers. *Am J Epidemiol.* 1974;99:230–4.
- Donald R. Adenovirus infections in immunocompromised patients. *Am J Med.* 1997;102:71–4.
- Echavarría M, Maldonado D, Elbert G, Videla C, Rappaport R, Carballal G. Use of PCR to demonstrate presence of adenovirus species B, C, or F as well as coinfection with two adenovirus species in children with flu-like symptoms. *J Clin Microbiol.* 2006;44:625–7.
- Gray GC, McCarthy T, Lebeck MG, Schnurr DP, Russell KL, Kajon AE, et al. Genotype prevalence and risk factors for severe clinical adenovirus infection, United States 2004–2006. *Clin Infect Dis.* 2007;45:1120–31.
- Hierholzer JC, Gamble WC, Dowdle WR. Reference equine antisera to 33 human adenovirus types: homologous and heterologous titers. *J Clin Microbiol.* 1975;1:65–74.
- Huang Y-C, Huang S-L, Chen S-P, Huang Y-L, Huang C-G, Tsao K-C, et al. Adenovirus infection associated with central nervous system dysfunction in children. *J Clin Virol.* 2013;57:300–4.
- Janani MK, Malathi J, Madhavan HN. Isolation of a variant human adenovirus identified based on phylogenetic analysis during an outbreak of acute keratoconjunctivitis in Chennai. *Indian J Med Res.* 2012;136:260.
- Lu X, Erdman DD. Molecular typing of human adenoviruses by PCR and sequencing of a partial region of the hexon gene. *Arch Virol.* 2006;151:1587–602.
- Robinson CM, Seto D, Jones MS, Dyer DW, Chodosh J. Molecular evolution of human species D adenoviruses. *Infect Genet Evol.* 2011;11:1208–17.
- Sriwanna P, Chieochansin T, Vuthitanachot C, Vuthitanachot V, Theamboonlers A, Poovorawan Y. Molecular characterization of human adenovirus infection in Thailand, 2009–2012. *Virol J.* 2013;10:193.
- Tate JE, Bunning ML, Lott L, Lu X, Su J, Metzgar D, et al. Outbreak of severe respiratory disease associated with emergent human adenovirus serotype 14 at a US air force training facility in 2007. *J Infect Dis.* 2009;199:1419–26.
- Treacy A, Carr MJ, Dunford L, Palacios G, Cannon GA, O'Grady A, et al. First report of sudden death due to myocarditis caused by adenovirus serotype 3. *J Clin Microbiol.* 2010;48:642–5.
- Valdés O, Acosta B, Piñón A, Savón C, Goyenechea A, Gonzalez G, et al. First report on fatal myocarditis associated with adenovirus infection in Cuba. *J Med Virol.* 2008;80:1756–61.
- Yusof MA, Rashid TRTA, Thayan R, Othman KA, Hasan NA, Adnan N, et al. Human adenovirus type 7 outbreak in police training center, Malaysia, 2011. *Emerg Infect Dis.* 2012;18:852.