Molecular characterization of human adenoviruses isolated in Italy

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There is little information on the epidemiology of Human Adenoviruses (HAdVs) in Italy. In this study, 103 HAdV isolates, collected by the A. Gemelli Hospital (Catholic University Medical School of Rome, Italy) between 1987 and 2005, were genotyped by sequencing and phylogenetic analysis on a partial hexon gene region. Nine different serotypes belonging to all six HAdV species were identified. Serotype 2 was the most frequent (53.4%), followed by serotype 1 (15.53%) and serotype 41 (9.7%). Partial-hexon-based identification was confirmed as an effective tool for studying the molecular epidemiology of HAdVs.

KEY WORDS: Adenovirus, Epidemiology, Hexon gene, PCR, Serotyping, Sequencing.

INTRODUCTION

Human Adenoviruses (HAdVs) belong to the genus Mastadenovirus, family Adenoviridae, and are grouped into six species, HAdV-A to HAdV-F, according to their biological properties, DNA homology and fiber protein characteristics. 51 different serotypes have so far been identified (De Jong, Wermenbol et al., 1999); (Kidd, Jonsson et al., 1996); These viruses cause a broad spectrum of diseases including pharyngitis, acute respiratory disease, pneumonia, pharingoconjunctival fever, epidemic keratoconjunctivitis, genitourinary infections and gastroenteritis. Although most infections are self-limited, severe or lethal infections can occur; especially in infants (Cusi, Valensin et al., 1986); (Cevenini, Varoli et al., 1985); young children and immunocompromised patients (Munoz, Piedra et al., 1998); (Pichler, Reichenbach et al., 2000). Specific serotypes are associated with manifestation and severity of the disease (Adhikary, Numaga et al., 2001; Allard, Girones et al., 1990); (Elnifro, Cooper et al., 2000), and rapid identification of particular virulent serotypes can help prevention and disease control. There is therefore a growing clinical interest in determination of the serotype of clinical isolates.

Diagnosis of adenoviral infections and determination of adenovirus type is usually based on virus isolation in cell culture followed by antibody studies or antigen detection by immunofluorescence, and visualization by electron microscopy (Wadell 1984; Hierholzer, Stone et al., 1991; Wigand 1987). However, these procedures are laborious and time-consuming. The introduction of Polymerase Chain Reaction (PCR)-based assays has opened a new pathway...
to rapid, sensitive and specific identification of adenovirus infections at the species or type level. Most of the PCR assays used rely on the detection of sequences in the hexon gene region, the most highly conserved gene in the Adenovirus genome (Allard, Albinsson, 2001; Raty, Klemola, 1999; Banik, Adhikary, 2005; Frantzidou, Pavlitou, 2005; Sarantis, Johnson, 2004; Shimada, Ariga, 2004; Heim, Ebnet, 2003). Others use sequences in the VA RNA gene (Kidd and Tiemessen 1993), the pIX gene (Akalu, Seidel, 1998) or in the fiber gene (Kidd and Tiemessen 1993; Xu, McDonough, 2000).

Molecular and epidemiological studies on Adenovirus have been carried out in many countries (Cusi, Valensin, Barberi, Guiglielmi, and Rossolini 1986); (Cevenini, Varoli, Rum pianesi, Mazzaracchio, Nanetti, and La Placa 1985), (Li, Zheng, 1996; Lin, Lin, 2004; Aoki and Tagawa 2002; Noda, Yoshida, 2002; Azar, Varsano, 1998; Kajon, Mistchenko, 1996); in Italy, as far as we know, no recent epidemiological studies on human adenovirus infections have been conducted and no serotype distribution information is available. This study reports the genetic characterization by PCR and sequencing of adenoviruses collected by the Agostino Gemelli Hospital, Rome, from 1987 to 2005. The PCR assay was carried out using a previously published degenerate primer pair (Allard, Albinsson, and Wadell 2001), targeting a region of the hexon gene able to detect all known human adenovirus serotypes and to establish phylogenetic relationships on the bases of sequence analysis.

**MATERIAL AND METHODS**

**Field isolates, Reference strains, RNA Extraction and PCR**

This study analyzed 103 viral strains isolated in clinical specimens from patients who had received medical care at the A. Gemelli Hospital (Catholic University Medical School of Rome) from 1987 to 2005. The specimens included faeces (70.87%), pharyngeal swabs (25.24%), urine (1.94%), liquor (0.97%) and intestinal biopsy (0.97%). Two reference strains from ATCC (American Type Culture Collection) were included in the study: Adenovirus 40 (strain Dugan, 931-VR) and Adenovirus 41 (strain TAK, 930-VR).

Viruses were grown in green monkey kidney (Vero) and human epidermoid carcinoma (HEp-2) cells. Infected cell cultures were tested with a genus specific monoclonal antibody conjugated to fluorescein isothiocyanate (IMAGEN Adenovirus, DakoCytomation) to confirm the presence of adenoviruses. Nucleic acid extracts were obtained from 600 µl of positive culture cells. Samples were subjected to enzymatic proteolysis in a mixture containing 200 µg of proteinase K, 0.1M Tris-HCl (pH 7.5), 5mM EDTA, and 1% sodium dodecyl sulfate (SDS) and incubated for 60 min at 37°C. Subsequent extractions were performed, first with an equal volume of phenol, and then with an equal volume of chloroform/isoamyl-alcohol (24:1). Nucleic acids were precipitated with sodium acetate and cold isopropyl alcohol and dried pellet was dissolved in 50 µl of sterile water.

A partial hexon sequence of 301 bp was amplified from 2 µl of extracted DNA, with 22 pmol of a previously described pair of degenerate primers (Allard, Albinsson, and Wadell 2001), ID-991 (5'-GCCSCARTGGKCWTACATGCACATC-3', position 18858 to 18883 based on the complete nucleotide sequence of AdV-2 strain GenBank accession no. J01917) and ID-992 (5'-CAGCACA-SCCIICGRATGTCAAA-3', position 19138 to 19158).

PCR was carried out in a GeneAmp PCR System 9700 thermo-cycler (Applied Biosystems) under the following conditions: 1 cycle of template denaturation at 95°C for 4 min, 35 cycles at 95°C for 1 min, 54°C for 1 min, 72°C for 1 min, followed by one cycle of elongation for 10 min at 72°C.

After amplification, 10 µl of PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide, together with a marker of known molecular weight (1Kb DNA Ladder, Invitrogen). The gel was visualized under UV light and photographed. The PCR products were purified prior to sequencing by Microcon-100 microconcentrator columns (Amicon, Beverly, Mass.). The ID reported in this work for primers and samples came from a PostgreSQL open source database (www.postgresql.org) which keeps track of all primers, PCR and samples used.
Gene Sequencing and Data Analysis
Both strands of the amplicons were sequenced by automated sequencing using the PCR primers as sequencing primers, with Big-Dye Terminator labelling and PCR cycle sequencing (Applied Biosystems, Foster City, CA). After cycle sequencing, unincorporated dyes were removed with CEN-TRI-SEP spin columns (Princeton Separations, Adelphia, N.J.). Sequencing analysis was performed in a capillary automatic sequencer (ABI PRISM™ 310 Genetic Analyser, Applied Biosystems). The consensus sequences were constructed by comparing forward and reverse electropherograms using the AutoAssembler sequence assembly software, version 2.1.1 (Applied Biosystems, Foster City, CA, USA) and exported in GCG format to a Sun Blade 2000 workstation (Sun Microsystems, Palo Alto, CA, USA) implemented with Wisconsin GCG v. 10.3 (University of Wisconsin Genetic Computer Group, Madison, WI, USA). Database searches were run using the Fasta program of GCG and the Blast (Basic Local Alignment Search Tool) service provided by the National Centre for Biotechnology Information (NCBI, Bethesda, MD, USA) web server. The consensus sequences were submitted to the EMBL Nucleotide Sequence Database using the Sequin software tool, designed for submitting sequences to public databases, available at www.ncbi.nlm.nih.gov/projects/Sequin/. Multiple alignment, with a final manual adjustment, was carried out using the gcg program PILE-UP and the subsequent phylogenetic analyses were performed using the ClustalW program, version 1.8, with the Neighbor-Joining method. The robustness of the grouping was assessed using bootstrap resampling of 1000 replicates of the data sets. Bootstrap values of ≥60% were considered statistically significant for the grouping. The tree was displayed with the NJplot program and the post-script file was imported into CorelDraw (version 10) for adjustments.

The sequences described in this report have been submitted to the EMBL database.

RESULTS

An amplicon of 301 bp was obtained for each sample by using the degenerate hexon primers targeting a conserved region within the hexon gene. Amplicon identity was verified by sequencing analysis. Sequences were submitted to the Blast program to find the most related sequences available in GenBank. Among the 103 isolates, nine different serotypes were identified, belonging to all six AdV species. The predominant serotype was serotype 2 (55 samples, 53.4%), followed by serotype 1 (16 samples, 15.5%), serotype 41 (10 samples, 9.7%), serotype 6 (9 samples, 8.7%) and serotype 7 (5 samples, 4.9%). Serotypes 3, 4, 31 and 37 were scarcely represented (2 samples each, 1.9%). With regard to species, the most frequently detected (77.7%) was species C (including serotypes 1, 2, and 6), followed by species F (9.7%). Percentages for serotypes and species are listed in Table 1.

To assess the genetic relationships among samples, a phylogenetic tree, shown in Figure 1, was constructed based on a multialignment of 211 nucleotides (using the shortest sequence) of the hexon gene, from the 103 samples and two reference strains, Adenovirus 40 (Dugan, ATCC, 931-VR) and Adenovirus 41 (TAK, ATCC, 930-VR). Reference strains obtained from GeneBank were included from each serotype identified: af161559 for serotype 1, j01917 for serotype 2, x76549 for serotype 3, ay458656 for serotype 4, af161560 for serotype 6, ay495969 for serotype 7, af161576 for serotype 31 and af161567 for serotype 37. The list of reference sequences used for comparison is shown in Table 2.

As shown in Figure 1, the HAdVs were grouped into nine different clusters (corresponding to nine different serotypes), along with their respective prototype strains. The nine clusters segregated into
six major groups corresponding to the six HAdV species, A to F. Within clusters, the isolates were divided into different groups showing strain variability, except for serotype 3 and 7 in which strains showed 100% hexon sequence identities.

Adenovirus A species (from the bottom of Figure 1) included two identical serotype 31 strains with a 98.4% identity with prototype (accession AF161576).

The Adenovirus B species included serotype 7, with five identical members having 100% identity with the Adenovirus 7 reference strain (Accession AY495969), and serotype 3 with two identical members having 100% identity with the reference strain G.B. (accession X76549).

Adenovirus E species included two Adenovirus serotype 4 strains, of which one has 100% identity with the reference strain CL68578 (Accession AY458656), and the other has 98.7% identity with the same reference strain.

Adenovirus F species included serotype 41 with ten identical members showing 98.1% identity with the reference strain TAK (ATCC-930-VR, from this study), and serotype 40 with the only reference strain Dugan (ATCC 931-VR from this study).

Adenovirus D species included serotype 37, with two strains of which one has 100% identity with the reference strain (accession AF161567), and the other has 99.6% identity with the same reference strain.

Adenovirus species C included serotypes 2, 6 and 1. The Adenovirus 2 group included 55 members accounting for 53.4% of isolates. Two main subgroups within the group were found, one with 13 isolates (212, 508...516) showing 100% identity with the prototype strain (accession J01917) and isolates 158 and 151 (99.6% and 99.3% identity, respectively), and the other with 38 identical isolates (500, 503, ...441) with 98.2% identity with the reference strain and samples 164, 146 and 449 (97.7%, 97.5%, and 97.6% identity, respectively).

The Adenovirus 6 group was almost heterogeneous, including nine isolates of which one (574) had 100% identity with the reference strain (Accession AF161560), while the others averaged 98.4% similarity with the same reference. The Adenovirus 1 group is the second most frequently detected serotype, with 14 out of 16 isolates having 100% identity to the reference strain (Accession AF161559) and two isolates with 98.8% similarity to the same sequence.

DISCUSSION

Adenoviruses are associated with a wide variety of clinical syndromes, the majority of which concern the respiratory tract. However, depending on the infecting serotype, they may also cause other illnesses, such as gastroenteritis, con-
FIGURE 1 - Phylogenetic tree of the partial hexon region from the 103 adenovirus genomes. The phylogenetic tree was constructed using the ClustalW program, version 1.8, with the Neighbor-Joining method, based on a multialignment of 211 nucleotides (using the shortest sequence) of the hexon gene, from the 103 samples and two reference strains: Adenovirus 40 (Dugan, ATCC, 931-VR) and Adenovirus 41 (TAK, ATCC, 930-VR). Reference strains obtained from GenBank were included from each serotype identified: af161559 for serotype 1, j01917 for serotype 2, x76549 for serotype 3, ay458656 for serotype 4, af161560 for serotype 6, ay495969 for serotype 7, af161576 for serotype 31, and af161567 for serotype 37. The HAdVs were grouped into 9 different clusters (corresponding to 9 different serotypes), along with their respective prototype strains. The 9 clusters segregated into 6 major groups corresponding to the six HAdV species, A to F.
junctivitis and genitourinary infections. People exposed to adenoviruses, which are often found in a crowded, stressful environment, may fully recover without ill effects or develop flu-like symptoms. Some people with weakened immune systems or other health problems may develop more serious illnesses; infants and young children are especially susceptible to severe complications of adenovirus infections. Because specific serotypes are associated with severity of disease presentation, there is a growing clinical interest in determining the serotype of clinical isolates. There are few epidemiological studies of adenovirus infections in Italy (Cusi et al., 1986; Cevenini et al., 1985) and no information is available on the distribution of serotypes. The present work, although limited to a small number of clinical specimens, gives an idea of the Italian adenovirus strains responsible for diseases requiring hospitalization, as well as serotype distribution. We found that serotype 2 is the most common (53.4%), followed by serotype 1 (15.53%) and serotype 41 (9.7%). Serotypes 1 and 2 have been shown to be endemic in other parts of the world, where they have been studied. Infection has usually occurred during childhood, and they are responsible for upper respiratory tract infections, especially in children under four. Serotypes 41 and 40, on the other hand, are mainly associated with enteric infections (Shinozaki, Araki et al., 1991) (De Jong, et al., 1993). Other serotypes detected were type 6 (8.2%), type 7 (4.9%), and, less frequently, serotypes 3, 4, 31 and 37 (1.9% each). Among these, adenovirus type 3, 4 and 7 are known to be associated with acute respiratory disease (ARD) in soldiers and, less often, in the civilian population (Rose, et al., 1970; Gray, et al., 1999; Top, et al., 1971; Gray, et al., 2000; Ryan, et al., 2002). In the USA, the serotype 4 and 7 was reported to account for 60% of all acute respiratory diseases among soldiers requiring hospitalisation (USAMMDA Information paper 2004, http://seattletimes.nwsource.com/news/nation-world/links/virus/warning.pdf). In the past, live adenovirus vaccines orally administered to soldiers up to 1999 were proven safe and highly effective in decreasing hospitalization rates for adenoviral acute respiratory diseases. Newly emerged strains have made acute respiratory disease due to adenoviruses a re-emerging disease among soldiers, (Ryan et al., 2002; Kolavic et al., 2002) making the development of a new adenovirus vaccine almost a “top priority” in the USA. (Gerry J. Gilmore, American Forces Press Service, http://www.af.mil/news/story.asp?storyID=123008879). Adenovirus type 3 (and occasionally serotypes 4 and 7) is also known to be a causative agent of a characteristic syndrome of acute pharyngoconjunctival fever (APC) in older children and adults, especially in summer camps and swimming pools. The same serotype may be an infrequent cause of encephalitis/encephalopathy in young children (Okamoto, Fukuda et al., 2004). Adenovirus type 31 is an important causative agent of gastroenteritis in infants (after type 40 and 41) (Shinozaki et al., 1991; Hammond et al., 1985; Adrian et al., 1989) while adenovirus 37 causes epidemic keratoconjunctivitis (Kemp et al., 1983; Ariga et al., 2005).

Although this study used a limited number of clinical specimens, it gives an idea of the types of adenovirus circulating in Italy. The circulation of especially virulent serotypes such as type 7 and 4, which have been associated with severe illness, is of special interest. In particular, subtypes 7b and 7h have been sometimes associated with severe morbidity and mortality. It is therefore our opinion that stricter surveillance is needed to evaluate risks for new genetic variants of these viruses.

This work confirms the effectiveness of the partial hexon nucleotide assay for detecting human adenoviruses belonging to all six species, and the usefulness of phylogeny-based clustering for the identification of HAdV isolates at the serotype level, in a broad range of patient specimens, including pharyngeal swab, faeces, liquor, intestinal biopsies and urine specimens. Moreover, this method can be a useful tool for detecting adenoviruses not only in clinical samples for clinical diagnosis but also in environmental samples, to determine, for example, the degree of faecal contamination in wells, sewage or seawater. It is well known that water sources, including surface water, groundwater and treated drinking water may be polluted by various microbial pathogens including adenoviruses (Abbaszadegan et al., 1999; Szewzyk et al., 2000) and that the survival of adenoviruses is high in
external environments and during water treatment processes such as chlorination and UV irradiation. Enteric Ad40 and Ad41, especially, are potentially important waterborne viruses, relatively resistant to sewage treatment and identified as the second most common agents of gastroenteritis in children after rotavirus in many studies (Ko et al., 2003). The U.S. Environmental Protection Agency drinking water lists them as a candidate for contamination. The 6th Framework Programme of the European Community Research is currently supporting a research program aimed at evaluating the possibility of including adenoviruses among the indicators of human viral pollution in water quality standards for swimming areas.

In conclusion, the partial hexon nucleotide assay and phylogeny-based clustering should be of use not only for the rapid clinical diagnosis and typing of HAdVs but also for global epidemiological study of these viruses.

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REFERENCES


