Published in final edited form as:

Pediatr Infect Dis J. 2005 January ; 24(1): 29–33.

Quantitation of Adenovirus Genome During Acute Infection in Normal Children

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Abstract

Background—Adenovirus infection causes a wide range of clinical illness in normal children. New molecular techniques allow quantitation of viral genome to study the natural history of adenovirus infection and viral load in normal children.

Methods—Clinical samples were collected from 38 previously healthy, febrile children, and viral cultures were performed. Quantitative polymerase chain reaction (PCR) was used to detect adenovirus genome and to determine viral load. Adenovirus isolates were genotyped with a PCR-based assay.

Results—Adenovirus culture was positive in 6 children who were diagnosed with acute adenovirus infection. Throat swabs contained high copy numbers of adenovirus genome (1.6×10^6 – 6×10^7 copies/swab) from 4 of 4 adenovirus culture-positive children. Only 2 of 32 adenovirus culture-negative children had detectable adenovirus genome from throat swabs, but with a lower copy number (8×10^2 copies/swab). Adenovirus genome was not detected in blood samples from 5 of 6 adenovirus culture-positive children with uncomplicated upper respiratory tract infection and from all adenovirus culture-negative children. High level viremia (1.8×10^8 /ml) was detected in an adenovirus culture-positive 6-month-old infant with fever, pneumonia, conjunctivitis and hepatitis. Subsequent reduction in viral load paralleled her clinical recovery. Adenovirus viruria (1×10^9 copies/ml) with normal urinalysis was detected in another adenovirus culture-positive child. All 6 adenovirus isolates were genotyped as adenovirus type 7h.

Conclusion—Viral load assessment in clinical samples determined by quantitative PCR can be useful in the diagnosis of adenovirus infection in immunocompetent, febrile children.

Keywords

rapid viral diagnosis; TaqMan polymerase chain reaction; quantitative polymerase chain reaction; adenovirus infection; children

Adenovirus accounts for 5–10% of upper and lower respiratory tract infections in infants and children.^{1,2} The clinical course of adenovirus infection among healthy children is usually benign but can be complicated by severe or fatal pneumonia, myocarditis and hepatitis. The 51 currently identified human adenovirus serotypes³ are divided into 6 subgroups, A–F, based on their DNA sequence and their ability to agglutinate erythrocytes.⁴ Adenovirus type 7 (Ad7),

a group B virus, accounts for nearly 20% of reported adenovirus isolates worldwide. Ad7 usually causes mild upper respiratory infection and conjunctivitis² but is the most frequent isolate from patients with severe or fatal respiratory infection.^{5–7} Ad7h, the most virulent of 19 Ad7 genotypes, became the predominant genotype in South America in 1986^{5,8} and has circulated in North America since 1998.⁹

Recent advances in molecular methods have improved our understanding of the relationship between viral replication and clinical outcome. In immunocompromised individuals with disseminated adenovirus infection, viral load reflects disease activity and can be used to monitor the response to antiviral treatment.^{10–12} Although the presence of adenovirus genome has been transiently detected by nested polymerase chain reaction (PCR) in the serum of 25% of immunocompetent children hospitalized with adenovirus infection,¹³ quantitative analysis of adenovirus viral load has not been described in this group.

We studied 38 previously healthy children who presented with fever, 6 with Ad7h infection and 32 diagnosed with other illnesses. We present for the first time data regarding viral load as determined by quantitative TaqMan PCR in previously healthy children.

METHODS

Subjects and Clinical Samples

Patients who presented to the Emergency Department at Children's Hospital and Health Center in San Diego were enrolled from May 2003 to March 2004 in a clinical study of Kawasaki disease patients and febrile controls with other illnesses. Inclusion criteria for children with Kawasaki disease were 4 of 5 standard clinical criteria (rash, conjunctival injection, cervical lymphadenopathy, changes in the extremities, changes in the oropharynx)¹⁴ or 3 of 5 criteria with dilated coronary arteries by echocardiogram. Inclusion criteria for the other febrile children were tympanic temperature of $>38.3^{\circ}\text{C}$ accompanied by any of the following signs: rash; conjunctival injection; cervical lymphadenopathy; oropharyngeal erythema; or peripheral edema. All patients required phlebotomy for routine laboratory studies. As part of the research protocol, all patients had a nasopharyngeal (NP) viral culture and blood collection. Urine samples and throat swabs were obtained from a subset of patients. The protocol for this study was approved by the institutional review board, and informed consent was given by the parents of all subjects.

Laboratory Assays

NP swabs were placed in viral transport medium, which was inoculated onto A549 (lung carcinoma), rhabdomyosarcoma and primary monkey kidney cell monolayers. Samples for rapid screening of respiratory viral antigen in NP epithelial cells were obtained with a Rhinoprobe curette (Arlington Scientific, Springville, UT), washed in phosphate-buffered saline and fixed with acetone on a glass slide for direct fluorescent assay (DFA; Respiratory Screen, Light Diagnostics, Temecula, CA). Pooled monoclonal antibodies were used to detect adenovirus, respiratory syncytial virus, influenza viruses A and B and parainfluenza viruses 1–3. Positive results were confirmed with specific monoclonal antibody staining.

DNA was extracted from a throat swab or 100 μL of serum, plasma, whole blood with ethylenediaminetetraacetic acid (EDTA) or urine, by standard phenol-chloroform extraction. 15 DNA was resuspended in 100 μL of $0.1 \times$ Tris-EDTA, and 2 μL were used for PCR. Quantitative PCR with a TaqMan probe (Applied Biosystems) was performed in a GeneAmp 5700 sequence detection system (PE Biosystem) as previously described,¹² with the following modifications. The primers were designed with a degenerate sequence to promote hybridization to a broader range of adenovirus genomes: DAQ1 (GCC SCA RTG GKC DTA CAT GCA

CAT C) and DAQ2 (GCC ACB GTG GGG TTY CTA AAY TT). Thermal cycling conditions were 50°C for 2 minutes and 95°C for 7 minutes, followed by 45 cycles of 95°C for 5 seconds, 55°C for 10 seconds and 60°C for 50 seconds. These primers bind to a conserved region of adenovirus hexon gene and allow detection of all 51 serotypes. The adenovirus genome copy number was determined by comparison with a standard curve generated with serial dilutions of a positive control plasmid, constructed by TOPO TA cloning (Invitrogen) of the PCR product from an adenovirus NP isolate (case 5). The lower limit of detection of the TaqMan PCR assay was approximately 15–30 copies of positive control plasmid. A linear standard curve was obtained between 15 and 3×10^8 plasmid copies. The number of adenovirus genomes in clinical samples was expressed per mL for blood or urine and per swab for throat samples by extrapolation from the volume and dilution of DNA used in the assay. Presence of PCR inhibitor in samples was tested in a separate PCR tube by spiking positive control plasmid (3×10^4 copies) into the sample DNA.

Adenovirus genotyping was performed using a species-specific multiplex PCR (fiber gene primers) from viral culture supernatant,¹⁶ followed by direct sequencing. Human adenovirus subgroups A through F were determined by the amplicon size analyzed by agarose gel electrophoresis. The sequences of purified PCR products were compared with known adenovirus sequences in GenBank with the use of Blast 2 sequence similarity search BLASTN 2.2.6 by Genome Net WWW Server (<http://www.genome.ad.jp>).¹⁷ To differentiate between Ad3 and Ad7h, we designed primers that amplify the early region E3: F5 (AAG GTG ATG CAT TAC TAA ATT TTG A); and R270 (GAG CCA TAT GGG GAC GAC T). The size of the PCR product was determined by agarose gel electrophoresis. Ad7h contains nucleotide variations and deletions in this region¹⁸ and yields a shorter PCR product (208 bp) compared with the product from Ad3 (266 bp).

RESULTS

Thirty-eight febrile children enrolled during a 10-month period were studied by viral culture and adenovirus PCR. The children were 6–77 months of age, and 14 of them were diagnosed with Kawasaki disease. Demographic, clinical and virologic data for the 6 adenovirus culture-positive children are shown in Tables 1 and 2. Four of the 6 patients required hospitalization, either for brief evaluation of their illnesses (cases 2 and 6), treatment with oxygen and intravenous fluids (case 1) or for treatment of suspected Kawasaki disease (case 5). All patients had fever and some constellation of respiratory symptoms. Clinical samples were collected between the fifth and ninth days after onset of fever. DFA was positive in 2 of 5 patients in whom the test was performed. Adenovirus genome was detected by quantitative PCR in the 4 throat swabs tested, and copy number ranged from 1.6×10^6 to 6×10^7 /swab.

Among 32 adenovirus culture-negative, febrile children, we found 2 children with adenovirus genome in throat swabs. The copy number was $\sim 8 \times 10^2$ copies for both swabs. The diagnoses in these 2 children were Stevens-Johnson syndrome and Kawasaki disease. The remaining 30 adenovirus PCR-negative children had the following diagnoses: Kawasaki disease, 12 patients; culture-negative viral syndrome, 10 patients; Epstein-Barr virus infection, 2 patients; scarlet fever, 2 patients; staphylococcal scalded skin syndrome, 1 patient; enteroviral meningitis, 1 patient; influenza A infection, 1 patient; and appendicitis, 1 patient.

Only a 6-month-old infant (case 1) had documented viremia with adenovirus genome detected in whole blood samples (Fig. 1). No adenovirus genome was detected in blood samples from the other 5 adenovirus culture-positive children with uncomplicated upper respiratory tract infection or from the adenovirus culture-negative febrile control children. Urine was tested in only 1 adenovirus culture-positive patient (case 3) by quantitative PCR on illness day 6, the last day of her fever. Viruria was detected despite a normal urinalysis and the absence of

urinary tract symptoms. Of the urine samples from 21 adenovirus culture-negative patients, none had detectable adenovirus genome.

Analysis of adenovirus fiber gene PCR product by gel electrophoresis established that all 6 patients had group B adenovirus. The sequences of all 6 amplicons (672 bp) matched both Ad7h (GenBank number, MAV7HE3) and Ad3 (GenBank number, AY224416) sequences. Further amplification of the adenovirus E3 region and analysis of PCR product size (208 bp) established that all 6 isolates were Ad7h.

Case 1

A 6-month-old Hispanic female infant presented on illness day 6 with fever, emesis, diarrhea, cough, tachypnea, irritability and dehydration. Physical examination revealed a temperature of 38.9°C, heart rate 160/min, blood pressure 97/54 mm Hg and respiratory rate 33/min. Oxyhemoglobin saturation was 90% in room air. Unilateral exudative conjunctivitis was noted. Breath sounds were diminished on the left. Cardiac and abdominal examinations were unremarkable. The mother was also ill with cough and exudative conjunctivitis.

Laboratory examination yielded the following results: leukocyte count, 14,300/ μ L with 46% band forms and 23% polymorphonuclear leukocytes; aspartate aminotransferase, 326 IU/L; alanine aminotransferase, 133 IU/L; C-reactive protein, 13.1 mg/dL; and erythrocyte sedimentation rate, 41 mm/h. Analysis of cerebrospinal fluid was unremarkable, and the culture was sterile. DFA was negative for adenovirus, but adenovirus grew from NP and rectal viral cultures. Serial chest radiographs revealed worsening diffuse bilateral infiltrates. The patient responded to supportive treatment and was discharged on illness day 14. Serial adenovirus viral load determinations on EDTA-whole blood revealed a high level of viremia on illness days 9 and 11, which gradually fell to 1.6×10^6 /ml on illness day 14. The decrease coincided with clinical improvement including resolution of oxygen requirement and defervescence. Comparison of viral load in EDTA-whole blood and serum from the same phlebotomy on illness day 13 revealed 1.4×10^7 and 1.9×10^7 copies/ml, respectively, thus demonstrating that the majority of the virus was extracellular.

DISCUSSION

We report for the first time a quantitative study of adenovirus genome during acute adenovirus infection in previously healthy children. The quantitative PCR assay generated new information regarding viral load in various clinical samples. The kinetics of adenovirus viremia was followed in a single patient and revealed substantial numbers of cell-free virus. The assay was robust for amplification of adenovirus from pharynx, blood and urine. Once DNA was extracted from the clinical sample, the PCR preparation time was <15 minutes, and the assay run time was 2 hours with a result available at the end of the amplification step. The reagent and technologist costs of performing similar types of diagnostic in-house PCR-based assays has been reported as <\$10 per test.¹⁹ The only currently available rapid clinical test for adenovirus is DFA, which failed to detect adenovirus in 3 of 5 virus culture-positive patients, consistent with results published in the literature.²⁰

The 6 adenovirus isolates were all genotyped as group B adenovirus, Ad7h. Our genotyping results were unlikely to be due to PCR contamination because other clinical samples not included in the current study were typed as Ad7 (pediatric isolates) or group C or E adenovirus (adult isolates). Our study included only patients who were ill enough to require laboratory evaluation and Ad7h has been linked to more severe clinical disease in children.⁸ Only 1 of the 6 Ad7h cases fulfilled the clinical criteria for Kawasaki disease. Thus, only 1 of 14 (7%) febrile children was diagnosed with Kawasaki disease.

One of 6 patients (case 1) was viremic with severe clinical manifestations including lower respiratory infection, conjunctivitis and hepatitis. She required prolonged hospitalization, and the improvement in her clinical course corresponded with clearance of viremia. It is likely that this was her first encounter with Ad7h and that she had no transplacental antibody because the mother was suspected to be concurrently infected. The first blood sample (illness day 9) contained 1.8×10^8 copies/mL, which is comparable with previously reported adenovirus loads in symptomatic immunocompromised individuals.^{11,12,22,23}

The large copy number of adenovirus genome detected in the urine of case 3, but not in a concomitant serum sample, suggests that adenovirus was replicating in the urinary tract and was shed directly into the urine. Despite the high level of viruria, the patient had a normal urinalysis and no urinary tract symptoms. Ad7 has been occasionally associated with hemorrhagic cystitis.²⁴ Although numerous studies have examined adenovirus shedding in the urine of immunocompromised hosts²⁵ and in preterm infants,²⁶ we were unable to find previous reports of quantitative PCR on clinical samples, including urine, from normal children with acute, uncomplicated adenovirus infection. Further studies with larger numbers of subjects will be necessary to assess the frequency and magnitude of viruria in immunocompetent adenovirus-infected children.

The number of adenovirus genomes recovered from a throat swab is likely to be influenced by a number of factors including the swabbing procedure and the efficiency of extracting the virus from the cotton swab. Despite these technical issues, high copy numbers of adenovirus genome, 1.6×10^6 – 6×10^7 , were recovered from all 4 throat swabs collected from children whose clinical features were consistent with acute adenovirus infection. Conversely we found small amounts of adenovirus genome (800 copies) in throat swabs from 2 of 32 adenovirus culture-negative children who were diagnosed with other conditions. The discrepancy between viral culture and PCR in these 2 patients may be the difference in sensitivity between the 2 procedures or reduced infectivity of adenovirus during latency.²⁷ After primary acute infection, prolonged viral shedding and recrudescence excretion has been detected by viral culture.²⁸ Persistence of adenovirus genome in adenoidal and tonsillar lymphocytes has also been detected by PCR.²⁷ The 2 children with negative adenovirus culture but low levels of adenovirus genome in the throat may represent recent, antecedent adenovirus infection, viral persistence or recrudescence of viral shedding.²⁸ Quantitative PCR might help to differentiate between acute infection with high levels of adenovirus replication and viral persistence. Further study of viral genome load in blood, urine and throat swabs from children with suspected adenovirus infection and from well-characterized, febrile control children will help to define the natural history of adenovirus infection and the optimal clinical samples for diagnostic testing.

Acknowledgments

DNA sequencing was performed by the Molecular Pathology Shared Resource, University of California, San Diego Cancer Center.

Supported by a grant from the National Institutes of Health (NHLBI-HL69413) to Dr Burns and by National Cancer Institute Cancer Center support grant 5P0CA23100-16 to the Molecular Pathology Shared Resource, University of California, San Diego Cancer Center.

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FIGURE 1.

Case 1. Detection of adenovirus genome copies in whole blood samples. CRP indicates C-reactive protein.

TABLE 1

Demographic and Clinical Data for Adenovirus Culture-Positive Patients

Case	Sex	Age (mo)	Ethnicity/Race	Duration of Hospital Admission (Days)	Clinical Signs/Symptoms
1	F	6	Hispanic	8	Fever, dyspnea, conjunctival injection, hepatomegaly
2	F	31	Hispanic	1	Fever with febrile seizure, conjunctival injection, rhinorrhea, otitis media
3	F	58	Hispanic/Caucasian	0	Fever, conjunctival injection, rhinorrhea, cough, rash, emesis, diarrhea
4	M	65	Caucasian/Filipino	0	Fever, conjunctival injection, rhinorrhea, emesis, diarrhea
5	M	67	Hispanic	2	Fever, conjunctival injection, cough, rash, otitis media, cervical lymph adenopathy, strawberry tongue
6	M	77	Caucasian	1	Fever, conjunctival injection, rhinorrhea, rash, diarrhea

TABLE 2

Virologic Data for Ad7 h Culture-Positive Patients

Case	Illness Day of Sample Collection*	DFA	Viral culture			Ad Genome Copy No.		
			NP	Rectal	Throat (/Swab)	Blood (/mL)	Urine (/mL)	
1	9	ND	+	+	ND	1.8×10^8	ND	ND
2	5	+	+	+	1.6×10^6	–	ND	ND
3	6	+	+	+	1.2×10^7	–	1.0×10^9	ND
4	6	–	+	ND	6.0×10^7	–	ND	ND
5	6	–	+	ND	ND	–	ND	ND
6	6	–	+	+	3.1×10^6	–	ND	ND

* Illness day 1 is the first day of fever.

ND indicates not done.