

Internally Controlled Real-Time PCR Monitoring of Adenovirus DNA Load in Serum or Plasma of Transplant Recipients

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Adenoviruses have been recognized as important pathogens in immunocompromised hosts. Particularly in pediatric allogeneic stem cell transplant recipients, the morbidity of the patients and mortality in those patients with disseminated infections have been found to increase over the last few years. Severe infections are predominantly but not exclusively caused by subgroup C adenoviruses. A multiplex real-time PCR assay using molecular beacons as probes was developed to enable monitoring of adenovirus DNA in those patients with simultaneous identification of subgroups. An internal control was coamplified in the multiplex PCR to check for the DNA isolation procedure as well as the presence of inhibitors in the clinical samples. The assay has been applied retrospectively in patient groups with different clinical outcomes of infection. In fatal cases, significantly higher adenovirus loads developed, exceeding even 10^{11} copies/ml of serum or plasma. Patients with viral loads over 10^6 copies/ml appear to have an increased risk for fatal complications. This quantitative real-time PCR assay has been prospectively used clinically since 2002 to study the course of adenovirus infection. In addition, the assay provides objective start and end points of therapeutic interventions, including the clinically important evaluation of antiviral drugs.

Human adenoviruses (HAdV) are a group of nonenveloped, double-stranded DNA viruses which are endemic in the pediatric population in particular. Most infections occur in children before the age of 5, but older age groups are affected as well. At present, 51 serotypes have been identified. In the immunocompetent individual, adenoviruses can cause infections of the conjunctiva, respiratory tract, and gastrointestinal tract with a variety of clinical manifestations. Although the infections are usually self-limiting, in immunocompromised patients dissemination may occur, potentially resulting in severe complications and even death (38). This occurrence of viral infections or reactivations causing posttransplant complications is reminiscent of cytomegalovirus (CMV) and Epstein Barr virus (EBV) infections (8, 11, 25, 36). Adenoviruses are now also recognized as important pathogens causing severe morbidity and mortality, especially in patients receiving allogeneic bone marrow transplants (3, 4, 6, 13, 15, 17, 18, 38). Infections have been reported in 5 to 30% of these patients, with higher morbidities in children, where mortality can be as high as 50% (1, 9, 31, 38). Most likely, this results from reactivation of latent adenoviruses, but primary infections cannot always be excluded (38). Species C viruses comprising subtypes 1, 2, 5, and 6 have been predominantly found in disseminated adenovirus infections in children. However, subgroup A and B viruses have been detected as well, mainly in solid organ and adult bone marrow transplant patients (15, 18).

Several quantitative real-time PCR assays for monitoring herpesvirus DNA load have been used in the follow-up for patients posttransplantation, resulting in successful implementation of preemptive therapy for CMV and EBV infections (2, 26, 32). Our preliminary studies have shown that adenovirus DNA in serum from immunocompromised patients may be predictive for the outcome of disease (7). Application of a semiquantitative PCR assay for the detection of adenovirus DNA in the plasma of transplant patients suggested an association of the viral load and fatal outcome of the disease (29).

Recently, real-time PCR applications for the detection and quantification of adenovirus have been described for species C viruses in transplant patients (10, 34) and adenovirus type 4 for respiratory diagnosis (16) and also for broadly reactive adenovirus multiplex assays on a variety of clinical samples (12, 14, 23, 24).

In this study, an internally controlled, multiplex real-time PCR that enabled efficient quantification of species A, B, and C adenoviruses was developed. An internal control is coamplified to monitor the integrity of the DNA extracted from clinical samples as well as the presence of PCR inhibitors. The heterogeneity of the adenovirus family complicates the design of an adenovirus real-time PCR. Mismatches in primer and probe sequences can significantly affect the kinetics of a real-time PCR and thus quantification. Our approach has focused on a PCR that amplified DNA of all relevant adenovirus serotypes by using primers selected from the 3' end of the hexon gene (5, 6) and the detection of the amplified products by using molecular beacons as probes (35). By using molecular beacons carrying different fluorophores, species of adenoviruses could be differentiated in the assay (37). Laboratories lacking multicolor

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TABLE 1. Primers and probes for real-time PCR detection and amplification of adenoviruses

Primer or probe	Sequence 5'-3' ^a
Primer	
ADVs	CATGACTTTTGAGGTGGATC
ADVas	CCGGCCGAGAAGGGTGTGCGCAGGTA
ADV31s	TATGACATTTGAAGTTGACC
Probe	
ADV-MB-AC-FAM	<u>GCTGCG</u> GAGCCYACCTTCTTTATGT <u>CGCAGC</u>
ADV-MB-B-HEX	<u>CGTGCG</u> GAGCCCACCTGCTTTATCT <u>CGCAGC</u>
ADV-MB-F-TXR	<u>CGTGCG</u> GAGCCCACACTTCTYTATGT <u>CGCAGC</u>
ADV-MGB	AGCCCACCCTKCTTTAT

^a Underlined nucleotides indicate the stem structure of the molecular beacon. MB, molecular beacon; TXR, Texas red. The oligos are located at the 3' end of the hexon gene.

real-time PCR detection can apply the assay with the same primers but using a minor groove binder (MGB) probe (19). This new assay has been applied retrospectively on a series of pediatric allogeneic stem cell transplant (SCT) patients previously tested by semiquantitative analysis (29). Since 2002, this assay has also been prospectively applied to monitor adenovirus DNA in allogeneic SCT patients (20) and to evaluate the efficacy of ribavirin treatment of this condition (21). This assay contributes to better management of adenovirus infections in transplant patients.

MATERIALS AND METHODS

Viruses and plasmids. Recombinant adenovirus strains of all species, which were purified with cesium chloride and titrated by high-performance liquid chromatography (HPLC) (30), were a kind gift from M. Havenga, Crucell, The Netherlands, who also provided DNA from 51 adenovirus serotypes for specificity testing. The virus stocks were diluted in Basematrix (BBI Diagnostics, Boston, Mass.) and stored in aliquots of 10⁸ virus particles (VP)/ml at -80°C.

An adenovirus type 5 stock of 2.34 × 10¹¹ particles/ml as counted by electron microscopy was purchased from Advanced Biotechnologies Inc, Columbia, Md.

Two DNA plasmids containing an insert of the 3' end region of the hexon gene of adenoviruses type 5 (IQL 103) and type 35 (IQL 104) were constructed (IQ Products, Groningen, The Netherlands). The concentration was 10⁸ copies of DNA/ml.

Primers and probes. The primers previously published by Echavarría et al. (5) were adjusted to better suit real-time PCR kinetics. These primers are selected from the 3' end of the adenovirus hexon gene and resulted in a PCR product of 137 bp. Species-specific molecular beacons carrying different fluorophores were designed for detection and quantification of the different species of adenoviruses. Primers and probes are shown in Table 1.

The primers and the Cy5 (indodicarbocyanine)-labeled molecular beacon for the amplification and detection of the Phocine herpesvirus (PhHV) internal control have been described previously (33). This target is coamplified in the adenovirus PCR as an internal control. Oligonucleotide primers were synthesized by Eurogentec, Seraing, Belgium. The molecular beacons were synthesized by Biolegio, Malden, The Netherlands.

In addition, a minor groove binder (MGB) probe was designed. This probe can be used instead of the molecular beacons for general detection and quantification of the same adenovirus PCR products, without differentiation of the species. The MGB probe was synthesized by Applied Biosystems Inc, Nieuwerkerk aan den IJssel, The Netherlands.

Patients. Allogeneic SCT recipients at the Department of Pediatrics in the Leiden University Medical Center were included in the study, and a unique patient number (UPN) identified the patients. The original diseases necessitating the transplantation included acute myeloblastic leukemia, acute lymphoblastic leukemia, myelodysplastic syndrome, severe combined immunodeficiency disorder,

severe aplastic anemia, Fanconi aplastic anemia, and chronic myelomonocytic leukemia. Occurrence of adenovirus infections was not related to the original disease.

In a retrospective study of 328 allogeneic SCT patients, 36 patients had a positive adenovirus culture from stool, urine, or a throat swab and were selected for further analysis as described previously (29). These patients were divided into three groups based on their clinical presentation. Group I had positive adenovirus cultures but no clinical symptoms (*n* = 17). Group II had adenovirus-related diseases but survived (*n* = 12). Ten of these patients had enteritis, one patient had hemorrhagic cystitis, and one patient had enteritis and hemorrhagic cystitis. Group III comprised patients with a fatal outcome that clinically was attributed to a disseminated adenovirus infection (*n* = 7). All patients but one had enteritis in combination with hepatitis or hemorrhagic cystitis. The remaining patient had pneumonia.

Subsequently, in 2001 and 2002, 48 allogeneic transplant recipients were monitored prospectively for 6 months after SCT.

Extraction of DNA. DNA was isolated from sera, plasma samples, and controls by using spin columns of the QiaAmp blood kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

For the prospective study, DNA was isolated from clinical samples, usually plasma, by the Total Nucleic Acid isolation kit and the automated MagNApure LC DNA extraction system (Roche Diagnostics, Mannheim, Germany). No significant differences were observed between the results obtained by column isolation and those obtained by automated extraction in the MagNApure system (data not shown).

Real-time PCR assay. Real-time PCR was performed using the iCycler IQ multicolor real-time PCR detection apparatus (Bio-Rad, Veenendaal, The Netherlands), which enables simultaneous detection of four different fluorophores. Binding of the specific probes to the PCR products generates fluorescence, which is detected by the iCycler IQ. The amplification cycle at which this fluorescence crosses the threshold line is called the threshold cycle or *C_T* value.

The PCR is performed in a 50-μl reaction volume containing 2× HotStar Taq Master mix (QIAGEN GmbH) as a multiplex assay with three primers (0.3 μM concentrations of each primer [ADVs, ADV31s, and ADVas]), two probes (a 3 μM concentration of 6-carboxyfluorescein [FAM]-labeled ADV-MB-AC for species A and C virus detection and a 3 μM concentration of hexachlorofluorescein [HEX]-labeled ADV-MB-B for species B virus detection), and a final concentration of 4 mM MgCl₂. Addition of 10 μl of extracted DNA completed the reaction mixture. The amplification protocol consisted of an enzyme activation step of 95°C for 15 min followed by 50 cycles of amplification (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s). A fixed amount of PhHV was added to the lysis buffer prior to extraction and was coamplified in the reaction to check DNA isolation and inhibition as described previously (33).

Quantification of the adenovirus load. The extraction of adenovirus control of 10⁸ VP/ml was identical to that of the patient samples, and dilution series of 10² to 10⁷ VP/ml were applied to real-time PCR. The obtained *C_T* values were used to establish a standard curve, and subsequently the viral load of the clinical samples was determined by entering the *C_T* value of the sample in the standard curve. The software of the iCycler IQ multicolor real-time PCR detection system performed the calculations.

Sequence analysis. Two additional primers (sense, GAAACTTCCAGCCCA TGAG; antisense, TTATGTGGTAGCGTTRC) were used for nucleotide sequence analysis of an approximately 450-bp fragment of the 3' end hexon gene. In this way, the primer and probe regions of adenovirus serotypes were analyzed to define the appropriate primers, and in addition, this method was used for genotyping the viruses. The PCR products were subjected to sequence analysis with the Big Dye-Terminator Cycle Sequencing ready reactions kit containing Ampli-Taq DNA polymerase FS (Applied Biosystems, Inc., Foster City, Calif.). Samples were analyzed on an ABI model 310 DNA sequencer (Applied Biosystems, Inc.), and genotyping was performed by BLAST analysis on the NCBI website (www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Multiplex real-time PCR. The primers targeting the 3' end of the hexon gene that were used in previous studies (5, 6, 29) successfully amplified 100 pg of DNA of all 51 adenovirus serotypes in a conventional PCR assay with an annealing temperature of 52°C. However, testing dilution series showed that a subpicogram-level sensitivity could not be obtained for all serotypes. For the selection of appropriate primers that enable

TABLE 2. Nucleotide sequences of the primer and probe regions at the 3' end of the hexon gene of the subgroup A, B, and C serotypes of adenovirus

Subgroup	Serotype	ADV _s ^a	ADV _a	Probe
		CATGACTTTTGAGGTGGATC	CCGGCCGAGAAGGGTGTGCGCAGGTA	GAGCCACCCCTTCTTTATGT
A	12	-----A-----	--C-----C-----	-----T-----
A	18	-----A-----	-----C-----	-----
A	31	T-----A-----A--T--C-	-----A--	-----
B	7	-----	-----C-----	-----G-----C-
B	11	-----C-----	-----C-----A-----	-----G-----C-
B	34	-----C-----	-----C-----A-----	-----G-----C-
B	35	-----C-----	-----C-----A-----	-----G-----C-
C	1	T-----	-----C-----	-----
C	2	-----	-----C-----	-----
C	5	-----	-----C-----	-----
C	6	-----	-----C-----	-----

^a Primer sequences are given under primer names.

an accurate quantification of the serotypes that are known to cause complications in transplant patients (15, 38), sequence analysis was performed (Table 2). This resulted in the primers ADVs and ADVa, which amplified all serotypes of the subgroup A, B, and C viruses (Table 1).

The five mismatches of the ADVs primer with the HAdV 31 serotype sequence (Table 2) result in inefficient amplification and quantification of this serotype. This is shown in Table 3, where dilution series of HAdV 31 DNA were clearly underquantified after amplification using HAdV 5 or HAdV 18 as standards. On the other hand, when using HAdV 31 as a standard (Table 3), only the first two dilutions of this serotype used as experimental samples were amplified. In addition, the HAdV 5 and HAdV 18 dilutions were overquantified. Therefore, an additional primer, ADV31s (Table 1), was added so

that HAdV 31 DNA was also effectively quantified. Two different probes (Table 1) were used for the quantification of species A and C viruses (FAM-labeled probe ADV-MB-AC) and species B viruses (HEX-labeled probe ADV-MB-B). The assay was optimized according to standard procedures to reach the highest efficiency.

As in some cases subgroup F viruses are suggested to be involved in fatal cases of adenovirus dissemination (24, 29), a Texas red-labeled ADV-MB-F probe was designed as well (Table 1). This probe was not added to the multiplex reaction but was used to quantify the DNA from a patient that previously was shown to be HAdV 41 positive (29).

An internal control reaction was coamplified in the assay to check the procedure for efficient DNA isolation and for inhibition. A fixed amount of PhHV was added to the clinical samples prior to DNA isolation and coamplified in the real-time PCR using a Cy5-labeled probe for detection. Only if the C_T value for this spike was within two cycles of the average of uninhibited samples was the result of the specimen considered a valid result.

Quantification and species. The heterogeneity in the adenovirus family complicates standardized quantification, as shown in Table 3, due to primer mismatches. When analyzing dilution series, differences were observed in the C_T values of similar concentrations of HAdV 5 (species C) and HAdV 35 (species B) HPLC-titrated virus standards. This result most likely relates to the fact that in HPLC titration, the quantity is based on particles rather than DNA copies. For that reason, plasmid DNA standards were prepared and it was shown that the amount of DNA of this plasmid stock of HAdV 5 was comparable to the values for the HAdV 5 virus standards. This was further confirmed by obtaining a commercial batch of HAdV 5, which consisted of purified virus, that was quantified by electron microscopy (Advanced Biotechnologies). Therefore, the subgroup C standard was used routinely in the assay and allowed for accurate quantification of all serotypes of the A and C subgroup viruses, which are predominantly associated with adenovirus complications.

When a subgroup B virus was detected in the multiplex PCR, the sample was retested with the specific, less well standardized standard curve of that subgroup.

The MGB probe can be used for quantification of adenovi-

TABLE 3. Inefficient amplification of HAdV 31 shown by heterogeneity of viral DNA loads of experimental dilution series of adenovirus and heterologous serotypes as standard^a

Standard	No. of copies/ml (10 log)		
	HAdV 5 (C)	HAdV 18 (A)	HAdV 31 (A)
HAdV 5 (log)			
7	7.2	6.9	5
6	6	5.7	4
5	5	4.6	0
4	4.2	3.6	0
3	3.1	2.7	0
HAdV 18 (log)			
7	7.3	7	5.1
6	6.1	5.9	4.2
5	5.1	4.8	0
4	4.4	3.8	0
3	3.2	3	0
HAdV 31 (log)			
7	10.4	9.9	7.2
6	8.6	8.3	5.8
5	7.2	6.7	0
4	6.2	5.2	0
3	4.8	4	0

^a The standard DNA load values are dilution series from 10^7 (log 7) to 10^3 (log 3) copies/ml from titrated stocks. Subsequently, the same dilution series are quantified by extrapolating the experimental C_T values into the standard curves.

TABLE 4. Retrospective monitoring of adenovirus DNA in serum samples of SCT recipients pre and posttransplantation

Group and UPN	Serotype	Viral load (10 log no. of copies/ml) in week ^a :																									
		-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
Group I																											
168	31			7.2		6.6	6.6	7.3	6.3	4.2	2.9		2.0														
286	2										6.4		4.1	4.9		-											
295	2							3.2	3.1	3	3.9	5.7	2.1	3.2													
311	2			3.9		4.2					3.7	4															
384	5		-	4.6		4.6	3	-					4.3		-		4.6					-			-		
Group II																											
338	5												3.8		-	-											
361	31												-	-	2.4	4.3											
Group III																											
246	1																										
277	5																										
316	2																										
321	5																										
369	31			4.1	-																			7.4	9.7	12	D
376	31	3.7																									D

^a The SCT was performed at week 0. Weeks before transplantation are indicated with a - (i.e., -4). Group I was asymptomatic, group II was symptomatic and survived, and group III was fatal cases, where the week of death is noted with a D. - indicates a negative PCR result.

ruses as well. No significant difference in viral loads was obtained when using this probe instead of the molecular beacons, provided that the probe was also used for the standard curve amplification.

Sensitivity and specificity. The sensitivity of the assay was determined by using the titrated HAdV 5 and HAdV 35 stocks and the two plasmids containing the 3' end of the hexon genes of these subtypes. Dilution series of these standards were subjected to real-time PCR amplification. The C_T value was plotted against the dilution to generate a standard line that can be used for the quantification of unknown samples by C_T value. The sensitivity of the assay was shown to be between 50 and 250 copies/ml, which correlates to 1 to 5 copies in the reaction tube (data not shown).

The amplification was also shown to be specific for adenoviruses. No amplification signal was obtained when nucleic acids from CMV, EBV, human herpesvirus 6, parvovirus, hepatitis B virus, hepatitis C virus, human immunodeficiency virus, and BK virus were used as targets.

Retrospective analysis of patients. Pediatric stem cell transplant recipients from a retrospectively analyzed cohort with documented adenovirus infections had been divided into groups I, II, and III based on increasing severity of infection by clinical interpretation (29). DNA was extracted from serum samples and subjected to real-time PCR. Results of the DNA load in the samples from the adenovirus-positive patients are shown in Table 4. In four out of six patients with fatal disseminated infection (group III), a steep increase of the adenovirus DNA load in serum could be observed in the month preceding their death, with a maximum of 10¹² copies/ml in UPN 321. Of these four patients, two suffered from combined enteritis and hepatitis, one had enteritis and hemorrhagic cystitis, and one had encephalitis. Unfortunately, no serum samples were available from UPN 369 (pneumonia) and UPN 376 (enteritis and hepatitis) from the weeks preceding their deaths.

One patient (UPN 114) from group III, not included in Table 4, had a period of species F (HAdV 41) viremia, which

started 50 weeks posttransplantation and lasted for 3 months. The load never exceeded 1,000 copies/ml, and the patient died in week 66. A plasma sample taken 1 day before his death was negative, and therefore the clinical significance (enteritis only) of the adenovirus viremia with respect to his death remains unclear.

In 2 out of 12 nonfatal cases (group II) and even in 5 out of 17 asymptomatic patients (group I), adenovirus DNA could be occasionally detected in serum as well, sometimes even before transplantation. Usually the load did not exceed 10⁵ copies/ml, but in the asymptomatic patients UPN 168 and UPN 286, DNA loads of 10⁶ to 10⁷ copies/ml were observed.

Apart from one case of HAdV 41 viremia, only adenovirus species A (comprising serotypes 12, 18, and 31) and species C (serotypes 1, 2, 5, and 6) have been detected in this patient group, with the latter being more prevalent (Table 4).

Prospective study of adenovirus DNA loads. Using the real-time PCR protocol, 48 pediatric patients receiving transplants from January 2001 until December 2002 were monitored prospectively. The available plasma samples were checked for adenovirus DNA for at least 6 months after transplantation. In less than 0.5% (n = 4) of over 800 specimens, inhibition of the PCR was observed, as shown by reduced PhHV internal control amplification.

In 12 of these 48 patients, a positive PCR result was found at least once. Six of these patients developed a disseminated infection, defined as at least two consecutive positive samples, and three of these patients died (Table 5). One patient (UPN 497) died because of multifactorial complications not specifically caused by adenovirus. UPN 484 and UPN 510 had adenovirus-related complications as causes of death. Species C adenoviruses were detected in five of the six patients, and in one of the fatal cases HAdV 18 was the cause (Table 5). Patients with a viral load over 10⁶ to 10⁷ copies/ml may be considered at risk for severe complications.

This method provides clear data on the kinetics of the HAdV DNA load in these SCT patients (Fig. 1). The effect of

TABLE 5. Prospective monitoring of adenovirus DNA in plasma samples of SCT patients in the first weeks after transplantation

UPN	Sero-type	Viral load (10 log no. of DNA copies/ml) at week ^a :												
		0	1	2	3	4	5	6	7	8	9	10	11	
468	1		3.9	6.3	6.7	3.7								
484	18	3	5.1	4.5	5.7	6.5	8.1	8.3	10.8	10.3	11	D		
485	6			5.9	7.7	7.9		7.7	4.6					
490	5								5.3	5	3.9			
497	2								3.5	4.2	4.3	2.9	D ^b	
510	5					2.4	4.6	4.4	7	7.6	D			

^a SCT took place at week 0. The six patients with disseminated infections are shown. Two patients died from adenovirus complications, and one died from multifactorial causes. —, negative PCR results; D, week of patient's death.

^b Patient died of multifactorial causes.

therapeutic interventions in patients receiving cidofovir, ribavirin, or donor lymphocyte infusions can be objectively assessed by quantitative viral DNA detection.

DISCUSSION

Adenovirus infections can result in major complications and even death in the immunocompromised host. Enteritis, hemorrhagic cystitis, and hepatitis occur in these patients, and mortality in allogeneic SCT recipients with a disseminated adenovirus infection is high (1, 3, 4, 9, 13, 18, 31). Adenoviruses can be easily detected in throat swabs and stool and urine specimens by isolation in cell culture. In 5 to 20% of allogeneic SCT patients, adenovirus infections have been detected by conventional assays (22, 38). Recently, our group has shown

that detection of HAdV DNA in serum or plasma can specifically identify and differentiate SCT patients with a disseminated infection who carry an increased risk for clinical complications (7, 20, 29).

In the present work, the development of a single-tube, internally controlled multiplex real-time PCR assay for monitoring the adenovirus DNA load in SCT patients is described. The heterogeneity of the adenovirus family substantially complicated the design of a quantitative PCR assay, as the possibilities for selecting general primers and probes are limited. Lion et al. (24) and Gu et al. (12) recently described a real-time PCR procedure using six different primer sets and probes for individual detection of all species. Heim et al. (14) described a broadly reactive adenovirus assay using a multiplex PCR.

Our assay has been designed to detect and quantify the serotypes involved in disseminated adenovirus disease. The HAdV DNA load measurement in the patients from a retrospective study generally confirmed the initial, semiquantitative PCR results (29). In one case, adenovirus type 41, which previously had been implicated in the fatal course of the disease, was found. However, the low viral loads observed in this patient make this conclusion questionable. In retrospect, Lion et al. (24) also detected a species F virus in a fatal case, and thus the role of species F adenoviruses will require further investigation.

The assay with the MGB probe enabled general detection and quantification of HAdV by using the same primers but without differentiation of species. The C_T values with this probe are in general a little lower than when molecular beacons are used, but when the standard curve is also generated

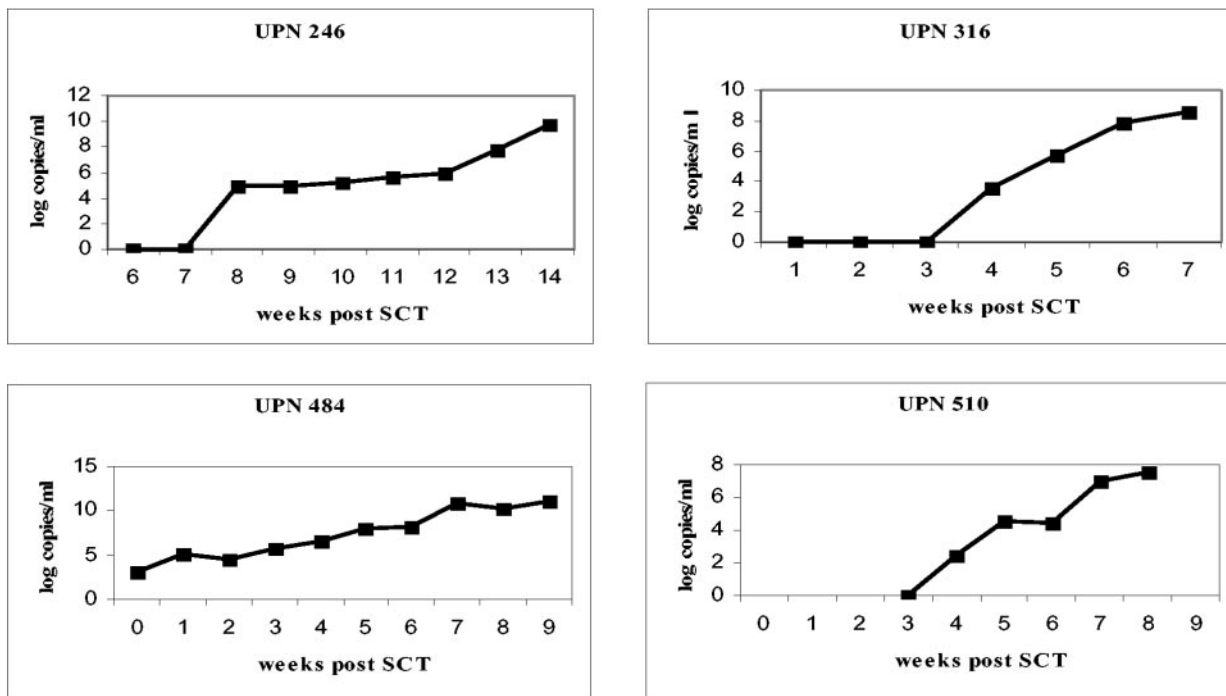


FIG. 1. Course of the adenovirus DNA load in four fatal cases of disseminated adenovirus infection. UPN 246 and 316 were from the retrospective study; UPN 484 and 510 were prospectively monitored. UPN 484 was treated with ribavirin starting in week 3, without a significant effect. The others were not treated.

with the MGB probe, no significant differences in viral loads are obtained (data not shown). Therefore, this procedure can be used in laboratories where no multicolor real-time PCR detection system is available.

An internal control reaction is included in the multiplex PCR assay to monitor appropriate DNA isolation and check for inhibition. Although few samples were inhibitory (less than 0.5%), this control is important for the application of PCR to clinical samples, especially as the effect of drugs in the blood of these patients on the PCR is unknown.

In our and other pediatric patient populations, species C adenoviruses have been most frequently detected after transplantation. Interestingly, species C viruses have also been detected in the majority of tissue samples taken after tonsillectomy or adenoidectomy (10). Whether the increase in infections can be correlated with abandoning the practice of tonsillectomies is not clear yet. Species B adenoviruses, especially subtype 7, 11, 34, and 35 infections, have been described for transplant recipients as well and are the predominant species in solid organ transplant recipients (15, 18).

Clinical application of the assay for pediatric SCT recipients with fatal disseminated adenovirus infections showed adenovirus DNA loads of up to 10^{12} DNA copies/ml of plasma. In the nonfatal cases, the DNA load never exceeded 10^7 copies/ml except in a single case (UPN 485). These data suggest that patients with HAdV DNA loads of over 10^6 to 10^7 copies/ml in plasma or serum may be considered at risk for severe complications. Further studies applying preemptive treatment based on DNA loads should lead to a well-defined threshold for the start of therapeutic interventions. The potential role of the preemptive treatment strategy in these cases is one of the main issues in a multicenter study that has been recently initiated.

An important point of concern in defining thresholds for the initiation of treatment is the standardization of the HAdV DNA load results. The exact number of complete DNA copies in the HPLC-titrated standards used is unclear. Without standardized adenovirus stocks, the thresholds for preemptive therapy have to be defined locally. For general recommendations to identify patients at risk, true standardization by international reference materials has to become available, as recently has been established for nucleic acid testing of several viral targets in blood products (27, 28).

Ribavirin and cidofovir have been reported to be effective against adenovirus infections. However, in cases in which improvement of patients after treatment has been reported, no data on the viral load were available. Moreover, without any intervention, adenovirus viremia can be controlled as a result of immunological recovery of the patient. With quantitative HAdV DNA PCR, tools have become available to enable timely preemptive treatment initiation. Recently, our group has shown that ribavirin did not have convincing antiviral effects in SCT patients (21), and currently the efficacy of cidofovir is being evaluated in the aforementioned multicenter study.

The quantification of adenovirus DNA as described here will be used for the early detection of disseminated infections and as an objective instrument to evaluate therapeutic interventions. This approach should eventually lead to the definition of optimal treatment protocols for this serious complication of immunocompromised children.

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