JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2004, p. 5189–5198 Vol. 42, No. 11
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.11.5189–5198.2004

Real-Time Quantitative PCR Assays for Detection and Monitoring of Pathogenic Human Viruses in Immunosuppressed Pediatric Patients

F. Watzinger,[1] M. Suda,[1] S. Preuner,[1] R. Baumgartinger,[1] K. Ebner,[1] L. Baskova,[1] H. G. M. Niesters,[2] A. Lawitschka,[3] and T. Lion[1]*

Division of Molecular Microbiology and Development of Genetic Diagnostics, Children's Cancer Research Institute,[1] and St. Anna Kinderspital,[3] Vienna, Austria, and Department of Virology, University Medical Centre Rotterdam, Rotterdam, The Netherlands[2]

Received 15 January 2004/Returned for modification 29 February 2004/Accepted 1 July 2004

A panel of 23 real-time PCR assays based on *Taq*Man technology has been developed for the detection and monitoring of 16 different viruses and virus families including human polyomaviruses BK virus and JC virus, human herpesviruses 6, 7, and 8, human adenoviruses, herpes simplex viruses 1 and 2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, parvovirus B19, influenza A and B viruses, parainfluenza viruses 1 to 3, enteroviruses, and respiratory syncytial virus. The test systems presented have a broad dynamic range and display high sensitivity, reproducibility, and specificity. Moreover, the assays allow precise quantification of viral load in a variety of clinical specimens. The ability to use uniform PCR conditions for all assays permits simultaneous processing and detection of many different viruses, thus economizing the diagnostic work. Our observations based on more than 50,000 assays reveal the potential of the real-time PCR tests to facilitate early diagnosis of infection and to monitor the kinetics of viral proliferation and the response to treatment. We demonstrate that, in immunosuppressed patients with invasive virus infections, surveillance by the assays described may permit detection of increasing viral load several days to weeks prior to the onset of clinical symptoms. In virus infections for which specific treatment is available, the quantitative PCR assays presented provide reliable diagnostic tools for timely initiation of appropriate therapy and for rapid assessment of the efficacy of antiviral treatment strategies.

The employment of PCR techniques for virus detection and quantification offers the advantages of high sensitivity and reproducibility combined with an extremely broad dynamic range. A plethora of qualitative and quantitative PCR virus assays have been described, and commercial PCR kits are available for quantitative analysis of a number of clinically important viruses such as human immunodeficiency virus (14, 21), hepatitis B and C viruses (13, 22), and cytomegalovirus (CMV) (6). In addition to permitting the assessment of viral load at a given time point, quantitative PCR tests offer the possibility of determining the dynamics of virus proliferation, monitoring of the response to treatment, and in viruses displaying persistence in defined cell types, distinction between latent and active infection. Moreover, from a technical point of view, the employment of sequential quantitative PCR assays in virus monitoring helps identify false-positive results caused by inadvertent contamination of samples with traces of viral nucleic acids or PCR products (20).

We have established quantitative virus detection assays based on the real-time PCR (RQ-PCR) technology for 16 different viruses or virus families which play an important role in the clinical surveillance of immunosuppressed children. All assays were designed to run under identical PCR conditions to render the diagnostic work as economical as possible.

The RQ-PCR assays are presented in a ready-to-use format, and clinical applications of quantitative virus analysis in immunosuppressed patients are discussed.

* Corresponding author. Mailing address: CCRI, Kinderspitalgasse 6, A-1090 Vienna, Austria. Phone: 0043 1 40470 489. Fax: 0043 1 40470 437. E-mail: thomas.lion@ccri.at.

MATERIALS AND METHODS

Sample preparation. (i) Nucleic acid extraction. For the isolation of DNA and RNA, commercially available kits were used, essentially as recommended by the manufacturer. DNA extraction from largely cell-free liquids, except urine, and from peripheral blood leukocytes for the detection of intracellular virus particles was performed by using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany). Isolation of virus DNA from urine was done by using the QIAamp viral RNA mini kit, and isolation of virus DNA from stool was done by using the QIAamp DNA stool mini kit (Qiagen). For the isolation of RNA from each of these sources, the QIAamp viral RNA mini kit was used. The only modifications performed included the adjustment of the input and the elution volumes. For nucleic acid extraction, the input volume for all samples was 200 μl for DNA and 140 μl for RNA and the elution volume was 240 μl for DNA and 120 μl for RNA.

(ii) Reverse transcription. For reverse transcription of purified viral RNA, a total of 30 μl of viral RNA eluate and 5 μl of nuclease-free water were mixed with 1 mM concentrations of each of the deoxynucleoside triphosphates and 25 μM $pd(N)_6$, and this mixture was incubated at 72°C for 5 min. The denatured RNA was placed on ice for 1 min before the addition of 12 μl of reaction buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 5 mM $MgCl_2$), 10 mM dithiothreitol, 1.5 μl of RNasin (40 U/μl; Promega, Mannheim, Germany), and 1.5 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Invitrogen, Carlsbad, Calif.). The reaction mixture was incubated at 37°C for 45 min, and finally, the enzymes were inactivated by heating at 98°C for 3 min.

Target sequence selection and primer and probe design. Specific primers and probes were selected and designed by using the Primer Express, version 2.0, software (Applied Biosystems [AB], Foster City, Calif). The oligonucleotide sequences, locations, amplicon lengths, and GenBank accession numbers of the corresponding target genes are displayed in Table 1. As indicated in this table, some of the primers reveal a degenerated code. This was a prerequisite for the detection of viral subspecies differing from each other by single nucleotides.

For the experiments described below, hydrolysis probes labeled with 6-carboxyfluorescein reporter molecules at the 5′ end and 6-carboxy-tetramethylrhodamine quencher molecules at the 3′ end (AB) were used. The optimal concentration of primers was assessed by performing serial PCRs across a concentration range from 50 to 900 nM.

Real-time PCR. All reactions were set up as singleplex PCRs in a total volume of 25 μl containing 12.5 μl of Universal Master mix (2× concentration, including

TABLE 1. Sequence details of all primer-probe combinations used

Virus type	Target	Amplicon length (bp)	Oligonucleotide sequence (5′–3′)[a] { Forward / Probe / Reverse }	Concn (nM)	Nucleotide positions	GenBank accession no.
HHVs						
CMV	MIE protein	76	AAC TCA GCC TTC CCT AAG ACC A	300	2414–2435	M21295
			CAA TGG CTG CAG TCA GGC CAT GG	200	2437–2459	
			GGG AGC ACT GAG GCA AGT TC	300	2470–2489	
EBV	BNT p143	74	GGA ACC TGG TCA TCC TTT GC	300	4679–4698	NC_001345
			CGC AGG CAC TCG TAC TGC TCG CT (AS)	200	4700–4722	
			ACG TGC ATG GAC CGG TTA AT	300	4733–4752	
HHV-6	DNA polymerase gene	74	GAA GCA GCA ATC GCA ACA CA	300	57517–57536	NC_001664
			AAC CCG TGC GCC GCT CCC	200	57544–57561	
			ACA ACA TGT AAC TCG GTG TAC GGT	900	57568–57590	
HHV-7	Major capsid protein	124	CCC AAC TAT TTA CAG TAG GGT TGG TG	300	84230–84255	U43400
			CTA TTT TCG GTC TTT CCA ATG CAC GCA (AS)	200	84258–84284	
			TTT AGT TCC AGC ACT GCA ATC G	900	84332–84353	
HHV-8	ORF[c] 26	111	GTG CTC GAA TCC AAC GGA TT	300	47308–47327	U75698
			TGT TCC CCA TGG TCG TGC C	200	47336–47354	
			CGA TAT TTT GGA GTA GAT GTG GTA CAC	300	47392–47418	
HSV-1[b]	US 4 gene	166	TTC TCG TTC CTC ACT GCC TCC C	900	137279–301	NC 001806
			CGT CTG GAC CAA CCG CCA CAC AGG T (AS)	200	137379–404	
			GCA GGC ACA CGT AAC GCA CGC T	50	137423–445	
HSV-2	Glycoprotein D gene	71	CGC CAA ATA CGC CTT AGC A	300	99–117	AF021342
			CTC GCT TAA GAT GGC CGA TCC CAA TC	200	123–148	
			GAA GGT TCT TCC CGC GAA AT	300	150–169	
VZV	ORF 38	82	AAG TTC CCC CCG TTC GC	300	69313–69329	X04370
			CCG CAA CAA CTG CAG TAT ATA TCG TCT CA	200	69336–69364	
			TGG ACT TGA AGA TGA ACT TAA TGA AGC	300	69368–69394	
Human AdV						
AdV A	Hexon gene	135	GGK CTG GTG CAA TTC GCC	300	17818–17835	X73487
			CCA CGG ACA CCT ACT TCA CCC TGG G	200	17840–17864	
			CAC GGG CAC AAA ACG CA	300	17936–17952	
AdV B	Hexon gene	138	CGC CGG ACA GGA TGC TT	900	45–61	X76549
			AGT CCG GGT CTG GTG CAG TTC GCC	200	73–96	
			CTA CGG TCG GTG GTC AC	900	166–182	
AdV C	Hexon gene	138	ACC TGG GCC AAA ACC TTC TC	300	2884–2903	J01966
			AAC TCC GCC CAC GCG CTA GA	200	2910–2929	
			CGT CCA TGG GAT CCA CCT C	900	2940–2958	
AdV D	VA RNA gene	143	AAA AAC GAA AGC GGT TGA GC	300	2–21	U10675
			CCA ATA CCA CGT TAG TCG CGG CT	200	104–126	
			CGG GTC GAG ACG GGA GT	50	128–144	
AdV E	Hexon gene	75	CAA CAC CTA CTC GTA CAA AGT GCG	900	225–248	X84646
			CGC CCA CGG CCA GCG TGT	200	251–268	
			TAG GTG CTG GCC ATG TCC A	300	281–299	
AdV F	1.5 IV-2 gene	113	CCC GTG TTT GAC AAC GAA GG	300	31277–31296	L19443
			ATC GAC AAG GAC AGT CTG CCA ACA CTA ACG	200	31326–31355	
			TTA GAG CTA GGC ATA AAT TCT ACA GCA	300	31363–31389	
Human polyoma-viruses:						
BKV	VP3 gene	116	TGT ACG GGA CTG TAA CAC CTG C	300	1525–1546	V01108
			TGA AGC ATA TGA AGA TGG CCC CAA C	200	1550–1574	
			TTT GGM ACT TGC ACG GG	300	1624–1640	
JCV	Late mRNA gene	123	TGA ACC AAA AGC TAC ATA GGT AAG TAA TG	900	474–502	NC001699
			TTC ATG GGT GCC GCA CTT GCA	200	523–543	
			AAT CCT GTG GCA GCA G	900	581–596	
PVB 19	VP2 gene	75	TGG CCC ATT TTC AAG GAA GT	300	3017–3036	Z68146
			CCG GAA GTT CCC GCT TAC AAC	200	3040–3062	
			CTG AAG TCA TGC TTG GGT ATT TTT C	300	3067–3091	
Enteroviruses[d]						
	5′ UTR[e] gene	148	CCC TGA ATG CGG CTA ATC C	900	455–473	D00820
			CGG AAC CGA CTA CTT TGG GTG TCC GTG TTT C	200	535–565	
			ARA TTG TCA CCA TAA GCA GCC A	900	581–602	
Respiratory syncytal virus	N gene	149	GGC AGT AGA GTT GAA GG	900	1801–1817	M11486
			ACT TGC CCT GCA CCA TAG GCA TTC ATA AAC AAT	200	1830–1862	
			ACA ACT TGT TCC ATT TCT GC	300	1930–1949	
Influenza viruses						
Influenza A	M[f] gene	132	CAT GGA ATG GCT AAA GAC AAG ACC	900	126–149	U49116
			TTT GTG TTY ACG CTC ACC GTG CCC A	200	184–208	
			CCA TTT AGG GCA TTT TGG ACA	900	237–257	
Influenza B	HA[g] gene	137	AGA CCA GAG GGA AAC TAT GCC C	300	134–155	AB036449
			ACC TTC GGC AAA AGC TTC AAT ACT CCA	200	219–245	
			TCC GGA TGT AAC AGG TCT GAC TT	900	248–270	
Parainfluenza viruses						
PIV-1[h]	HN[i] gene	109	GTT GTC AAT GTC TTA ATT CGT ATC AAT AAT T	900	1191–1220	U70948
			TAG GCC AAA GAT TGT TGT CGA GAC TAT TCC AA	200	1232–1263	
			GTA GCC TMC CTT CGG CAC CTA A	900	1278–1299	

Continued on following page

TABLE 1—*Continued*

Virus type	Target	Amplicon length (bp)	Oligonucleotide sequence (5′–3′)[a] (Forward / Probe / Reverse)	Concn (nM)	Nucleotide positions	GenBank accession no.
PIV-2	HN gene	90	GCA TTT CCA ATC TTC AGG ACT ATG A	900	767–791	D00865
			CCA TTT ACC TAA GTG ATG GAA TCA ATC GCA AA	200	795–826	
			ACC TCC TGG TAT AGC AGT GAC TGA AC	900	831–856	
PIV-3	HN gene	136	AGT CAT GTT CTC TAG CAC TCC TAA ATA CA	900	779–807	L25350
			AAC TCC CAA AGT TGA TGA AAG ATC AGA TTA TGC A	200	828–861	
			ATT GAG CCA TCA TAA TTG ACA ATA TCA A	900	887–914	
Positive controls						
SHV	gB[j] gene	89	GGG CGA ATC ACA GAT TGA ATC	900	267–287	Z68147
			TTT TTA TGT GTC CGC CAC CAT CTG GAT C	200	305–332	
			GCG GTT CCA AAC GTA CCA A	900	337–355	
B2-MG	DNA	105	TGA GTA TGC CTG CCG TGT GA (ex 2)	300	343–362	M17987
			CCA TGT GAC TTT GTC ACA GCC CAA GAT AGT T (ex 2)	200	364–394	
			ACT CAT ACA CAA CTT TCA GCA GCT TAC (intr 2)	300	421–447	
B2-MG	RNA	82	TGA GTA TGC CTG CCG TGT GA (ex 2)	300	343–362	M17987
			CCA TGT GAC TTT GTC ACA GCC CAA GAT AGT T (ex 2)	200	364–394	
			TGA TGC TGC TTA CAT GTC TCG AT (ex 3)	300	1018–1040	

[a] Sequences are for the forward primer, probe, and reverse primer (top, middle, and bottom, respectively).
[b] HSV-1, herpes simplex virus type 1.
[c] ORF, open reading frame.
[d] Includes polioviruses, coxsackie A and B viruses, echoviruses, and enterovirus types 68 to 71.
[e] UTR, untranslated region.
[f] M, matrix protein.
[g] HA, hemagglutinin.
[h] PIV-1, parainfluenza, virus type 1.
[i] HN, hemagglutinin/neuraminidase mRNA.
[j] gB, glycoprotein B.

ROX reference dye and uracil *N′*-glycosylase [UNG]; AB), 50 to 900 nM concentrations of primers, 200 nM *Taq*Man probe (Table 1), and 6 μl of genomic DNA or cDNA template. The mixtures were prepared in 96-well optical microtiter plates (AB), centrifuged for 1 min at 272 × *g* and amplified on the ABI 7700 or 7900 sequence detection system by using the following uniform cycling parameters: 2 min at 50°C (degradation of potentially present contaminating dUTP-containing amplicons by UNG), 10 min at 95°C (inactivation of UNG and activation of Ampli*Taq* Gold DNA polymerase), and 50 cycles of 15 s at 95°C and 60 s at 60°C (amplification of the specific target sequence).

Specificity. All primer and probe combinations were tested for potential cross-reactivity with unrelated viral and other microbial sequences based on the available data by the BLAST alignment software. None of the selected primer and probe combinations displayed significant homologies to any other sequences. Moreover, the theoretically conceivable cross-reactivity with human DNA and RNA sequences has been excluded by testing the primer and probe combinations against preparations of human nucleic acids.

Standardization. For standardization of quantitative virus detection assays, commercially available quantified DNA control panels (Advanced Biotechnologies, Inc., Columbia, Md.), in-house cloned plasmid standards, or high-titer virus preparations derived from culture supernatants were used. The calculation of virus particle numbers was based on spectrophotometric or fluorometric measurement of purified viral DNA or RNA. For the establishment of standard curves, serial logarithmic dilutions covering a range of ≥4 logs were employed, as described in more detail in Results.

Controls. (i) Negative controls. A number of precautions were undertaken to prevent and control the occurrence of false-positive virus tests. Every clinical RQ-PCR test performed included control reactions lacking template (no-template controls) and reactions including nonhomologous template (no-amplification controls) to test for the presence of contamination or the generation of nonspecific amplification products under the assay conditions used. Moreover, to further reduce the risk of false-positive tests resulting from contamination with PCR products, all PCRs were performed by replacing the nucleotide dTTP with dUTP. Prior to amplification, a digestion step with UNG was carried out to eliminate any contaminating PCR product, if present.

(ii) Positive controls. In addition to the DNA and cDNA of the respective control virus strain, the following controls were used in each assay to document efficient nucleic acid extraction and absence of enzyme inhibitors in the template preparation. (a) In largely cell-free clinical samples, such as plasma, serum, cerebrospinal fluid, urine, sputum, bronchoalveolar lavage fluid, or stool, a defined quantity of a nonhuman control virus (seal herpes virus [SHV], kindly provided by H. G. M. Niesters, University of Rotterdam, The Netherlands) was spiked into each sample prior to DNA and RNA extraction. Since constant DNA quantities of the control virus are coextracted even when RNA isolation kits are used (H. G. M. Niesters, personal communication; our own unpublished observations), the virus can also serve as a control in RNA virus detection assays. Under the standardized assay conditions used, constant levels of the seal virus were detected, provided that the nucleic acid extraction was efficient and no inhibitors of reverse transcription or PCR amplification were present (see Results). (b) In clinical samples containing cells, such as peripheral blood, buccal swabs, or biopsy material, a human single-copy housekeeping gene (β2-microglobulin [B2-MG]) (Table 1) (19) was coamplified in parallel with the virus sequence of interest.

In instances in which the cycle threshold (C_t) values of the above controls were off scale (below the expected reading), an appropriate correction factor was applied to the calculation of virus copy number in the corresponding clinical samples to compensate for impaired nucleic acid extraction or amplification efficiencies. Negative virus test results in the presence of low-positive (>1 log below normal) or negative SHV or B2-MG controls were regarded as not interpretable.

RESULTS

The sequence information of primers and probes for 23 real-time PCR virus detection assays is displayed in Table 1, together with an indication of their precise positions within the targeted genes. Moreover, the optimal primer and probe concentrations are indicated for each detection assay. All virus PCR tests presented were designed to be conducted under identical cycling conditions, as outlined in Materials and Methods, to facilitate the molecular diagnostic work.

Efficiency and sensitivity of RQ-PCR virus assays. These parameters were assessed by repeated testing of serial logarithmic dilutions of the standard reference virus strains covering a range of ≥4 logs. The number of virus copies used to prepare the serial dilutions had been determined by spectrophotometric or fluorometric measurement of the genomic DNA and cDNA concentrations of individual virus strain preparations.

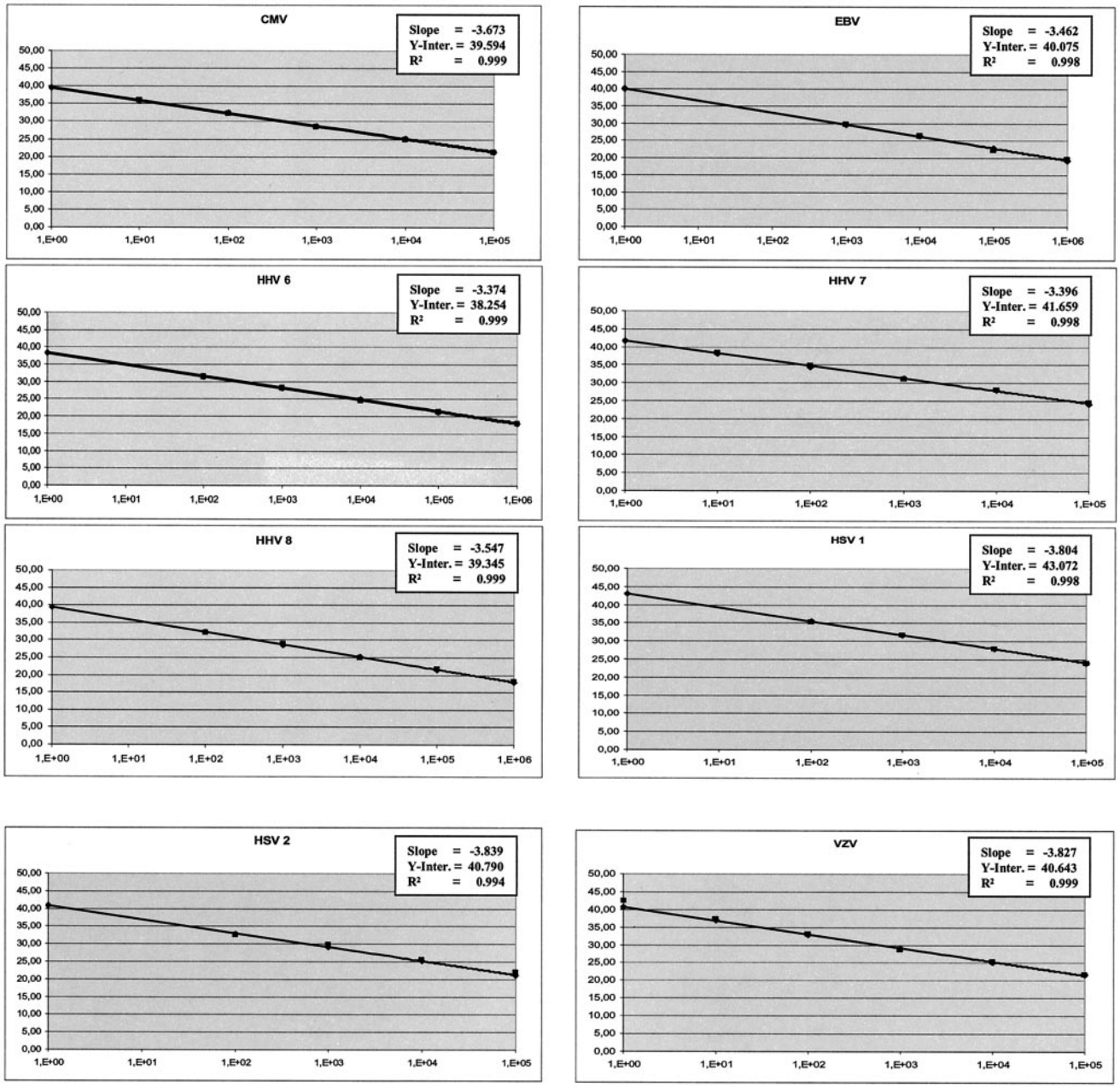

FIG. 1. Standard curves of the RQ-PCR virus assays described. Serial logarithmic dilutions were analyzed by using standard amplification conditions. The C_t (x axis) of each of the dilutions is plotted against the cycle number (y axis). The slope, y-axis intercept (Y-Inter.), and correlation coefficient are displayed in each graph. HSV 1, herpes simplex virus type 1; B19, PVB19; JCV, JC virus; EV, enterovirus; RSV, respiratory syncytial virus; Inf A, influenza A virus; PIV 1, parainfluenza virus type 1.

After PCR amplification, the C_t values (crossing point of the amplification curve with the preset threshold of fluorescence detection) of individual dilution steps were plotted against the initial virus copy number, leading to typical standard curves. The standard curves provided information on the amplification efficiency, the consistency of replicate reactions, and the theoretical and actual detection limits of the assay. The amplification efficiencies, defined by the standard curve slopes, were generally at or around 3.5. The consistency of replicates was measured by the correlation coefficient (R^2), which indicates the linearity of the C_t values plotted in the standard curves. The R^2 indices were higher than 0.990 in all measurements (Fig. 1).

The actual sensitivities of the assays were determined by the lowest standard dilution consistently detectable in replicate reactions. In all assays presented, 1E + 02 virus genomes were reproducibly detected.

Reproducibility of RQ-PCR virus assays. To evaluate the intra-assay variation of the virus tests, control samples across a wide range of virus copy numbers were analyzed concomitantly in triplicate reactions. The coefficients of variation (CVs) were in the range of 0.5% for most of the virus samples analyzed;

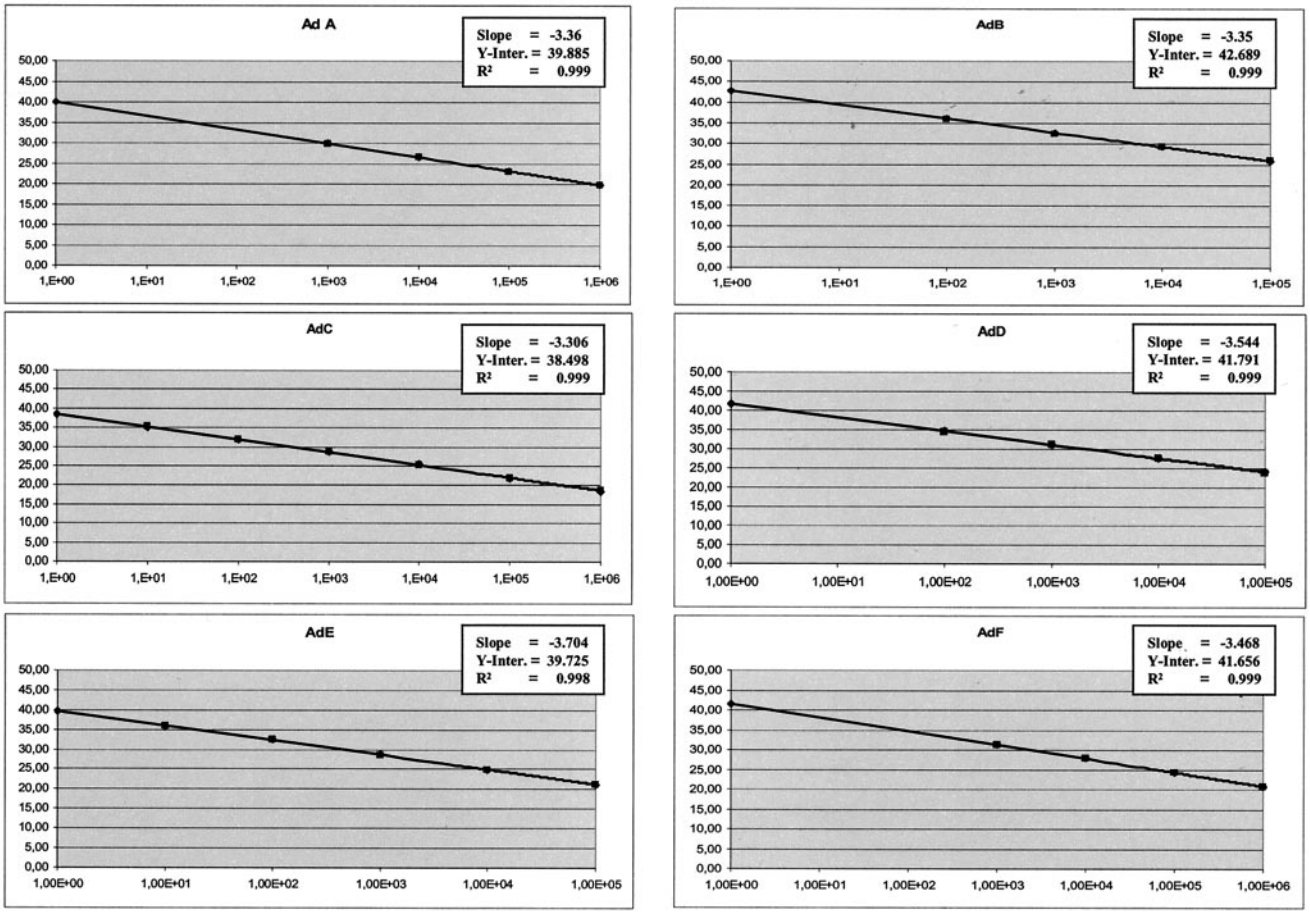

FIG. 1—*Continued.*

only samples containing very low virus genome equivalents (<1.00E + 01) showed consistently higher CVs (around 1%). The interassay variation was assessed by investigating a minimum of three different DNA or cDNA aliquots of individual virus samples in independent assays. Comparison of triplicate tests within different runs revealed CVs in the order of 1.6% (Table 2).

The results obtained document the high precision and low variation within and between individual virus tests.

Quantification of virus copy numbers in clinical samples. The virus loads in individual patient specimens were investigated by testing the most recent and, if available, a previously quantified patient sample in duplicate reactions, together with the appropriate external virus standard preparations. The efficiency of virus DNA and RNA isolation from clinical samples and the possible presence of reverse transcriptase or polymerase inhibitors were monitored by using internal controls, as described in Materials and Methods. These controls permitted appropriate correction in the calculation of virus copy numbers in the specimen investigated. For the calculation of virus particles in the sample tested, the slope (s) and the y-axis intercept (Y) (the y-axis intercept is the point at which the standard curve intersects with the ordinate; it indicates the theoretical detection limit of the reaction by revealing the C_t expected in the presence of a single target molecule in the sample) of the corresponding standard curve and the C_t of the target virus amplification were used according to the following equation: P_0 = Inverse log($C_t - Y/s$), where P_0 is the number of virus copy equivalents in the PCR prior to amplification.

Examples of clinical application in immunosuppressed patients. The panel of virus tests presented covers viral pathogens of well established or supposed importance in pediatric patients with severe immunosuppression. The following examples were derived from virus monitoring of immunocompromised children after allogeneic stem cell transplantation (SCT) and illustrate the clinical utility of the RQ-PCR virus detection assays in this particular clinical setting.

(i) Rapid diagnosis of viral cause of disease symptoms. Severe inflammation of the urinary bladder in immunocompromised patients may occur as a result of infection with a variety of bacterial and viral pathogens. The polyomavirus BK virus (BKV) is a relatively common cause of hemorrhagic cystitis in children undergoing allogeneic SCT. Although no specific antiviral treatment is currently available for this type of infection, detection and monitoring of the virus during the course of disease is of clinical relevance with regard to identification of the cause of the symptoms observed and with regard to differential diagnosis from other pathogens requiring specific treatment. Figure 2 illustrates the detection and surveillance of BKV load in serial urine samples of a patient displaying hemorrhagic cystitis after allogeneic SCT. All tests for other pathogens in the urine were negative. The causative

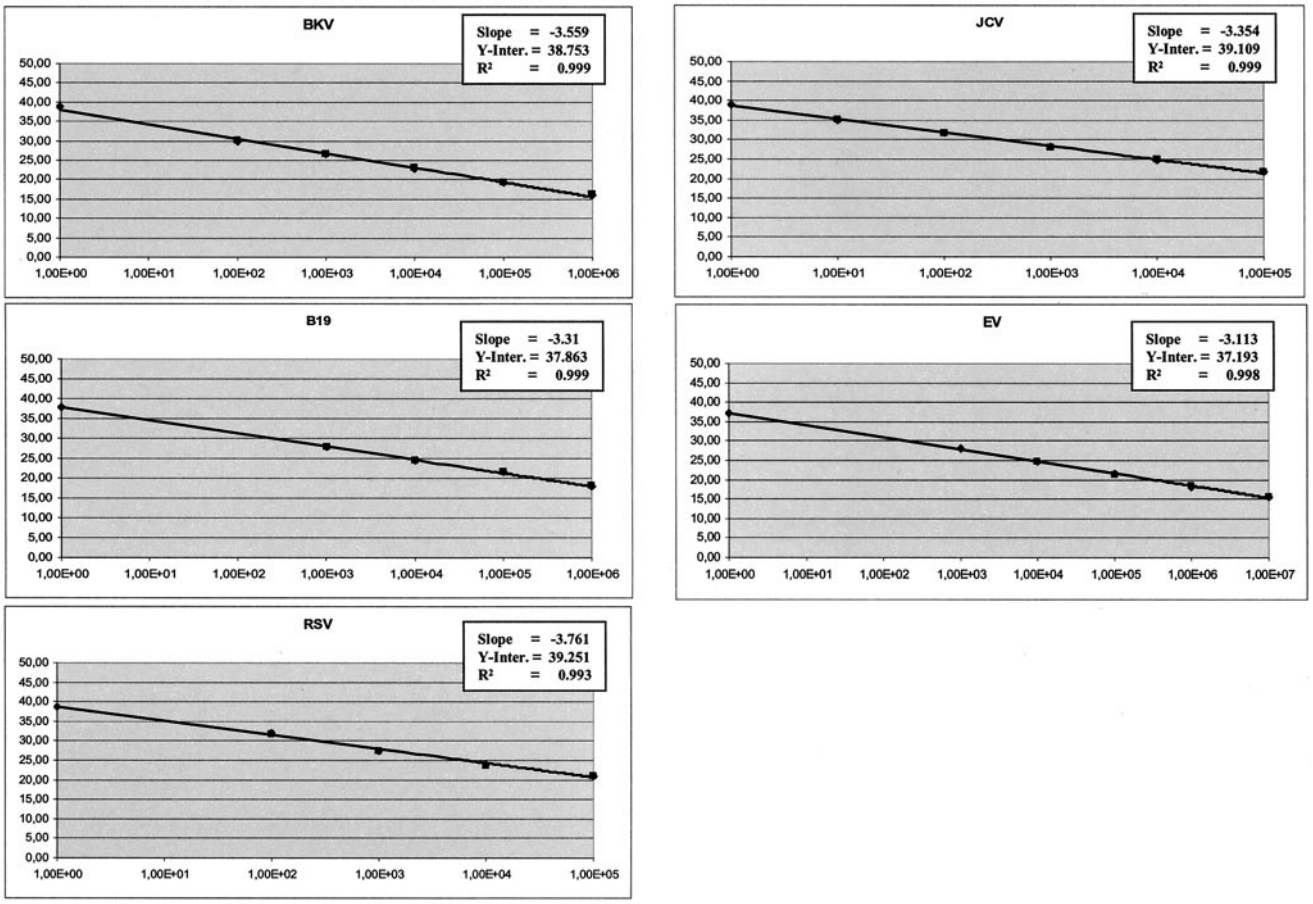

FIG. 1—*Continued.*

agent and the course of infection under symptomatic therapy could be documented by RQ-PCR monitoring.

(ii) Latent virus infection and reactivation. Following primary exposure, a number of viruses, including members of the herpesvirus family and adenoviruses, may persist as latent infections in peripheral blood (PB) leukocytes.

In view of the high prevalence of infections with these viruses, low viral copy numbers documenting persistence are detectable in B cells of the PB in a large proportion of healthy individuals. In immunosuppressed patients, reactivation of latent virus infection, associated with serious clinical symptoms, represents a frequent finding (33). In these instances, the viruses proliferate within the affected cells and are released into the extracellular compartment. Since viral reactivation may lead to life-threatening complications in patients with impaired immune responses, early diagnosis is of major clinical importance to permit timely initiation of appropriate antiviral treatment.

Epstein-Barr virus (EBV) is commonly detectable at low copy numbers in PB lymphocytes when present in the latent state. Reactivation of the virus in immunocompromised patients can lead to a lymphoproliferative disease, which may result in the occurrence of fatal malignant lymphoma (15, 32). In these instances, increasing copy numbers of the virus are detectable within PB lymphocytes and rising levels of free virus are detectable in plasma. Early detection of EBV proliferation kinetics provides a basis for timely initiation of preemptive treatment (30, 31). In the example presented, a retrospective analysis of a child who died from EBV-associated lymphoma is shown (Fig. 3). Increasing levels of the virus in PB were documented by RQ-PCR over a period of several weeks before the lymphoma was diagnosed clinically.

(iii) Early detection of invasive viral infection. Adenoviral (AdV) infections are frequently observed in immunosuppressed children during the posttransplant period (2) and are associated with high morbidity and mortality. Earlier observations made in our laboratory indicate that invasive AdV infections require a very early onset of antiviral treatment (20). In a number of instances, an example of which is shown in Fig. 4, invasive AdV infections with rapid proliferation kinetics can be detected in serial PB samples by RQ-PCR. Detection of the virus and the observation of increasing viral load precede the onset of clinical symptoms by a median of 3 weeks (20).

(iv) Documentation of response to antiviral treatment. In immunocompromised patients suffering from potentially life-threatening virus infections, the surveillance of the success of treatment is of paramount importance for appropriate clinical management, including the choice and duration of appropriate antiviral therapy (4, 23, 27). The example displayed in Fig. 5 shows the documentation of decreasing CMV levels both in plasma and PB leukocytes, followed by elimination of the virus

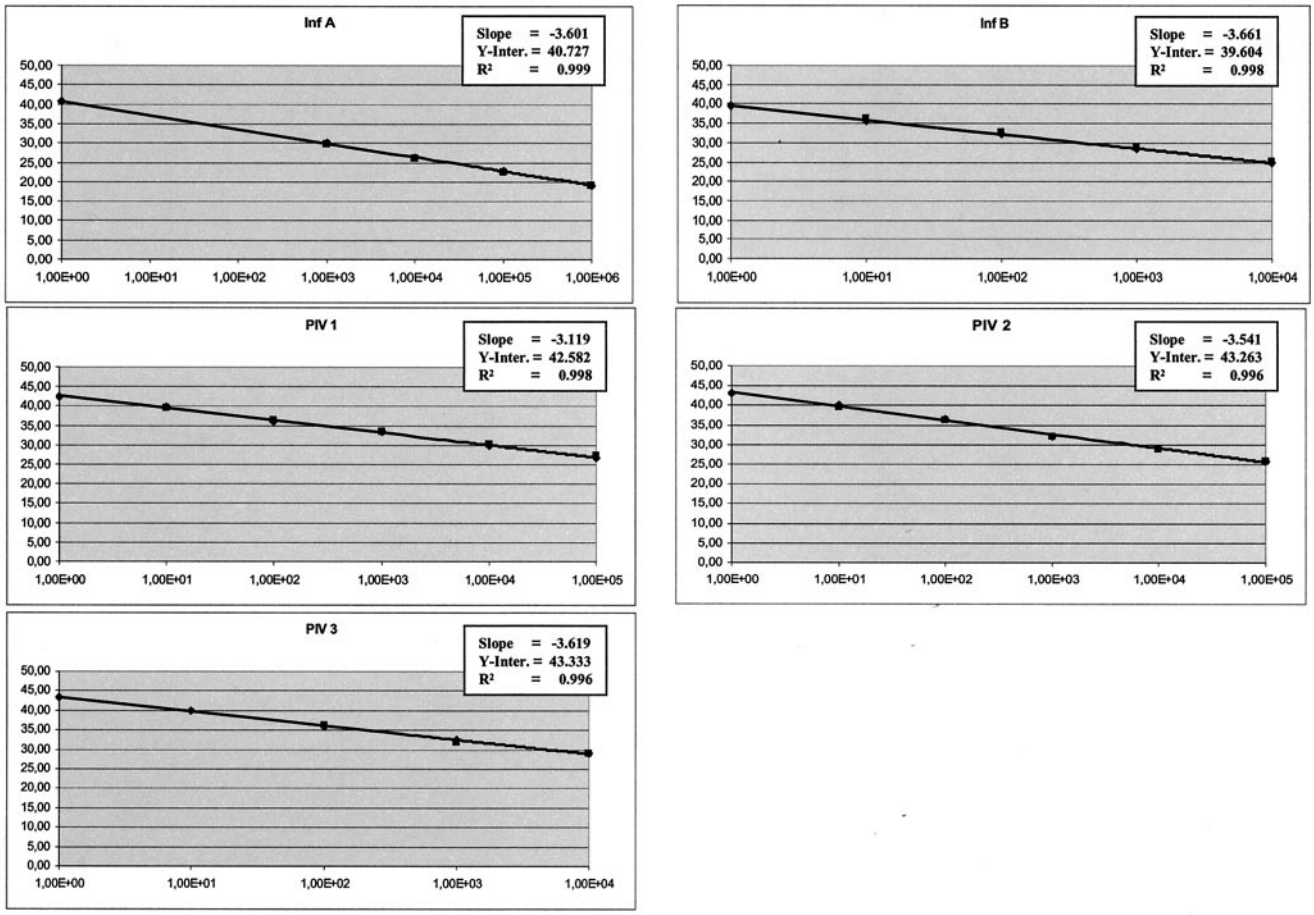

FIG. 1—*Continued.*

below the limit of detection, in response to antiviral treatment with ganciclovir.

DISCUSSION

In this paper, we present real-time PCR assays for qualitative and quantitative analysis of 16 viruses or virus families which appear to be of particular importance in the clinical management of immunosuppressed children undergoing high-dose chemotherapy or allogeneic SCT. Qualitative virus detection revealing merely the presence or absence of a viral pathogen is not sufficient in a variety of clinical situations. In many

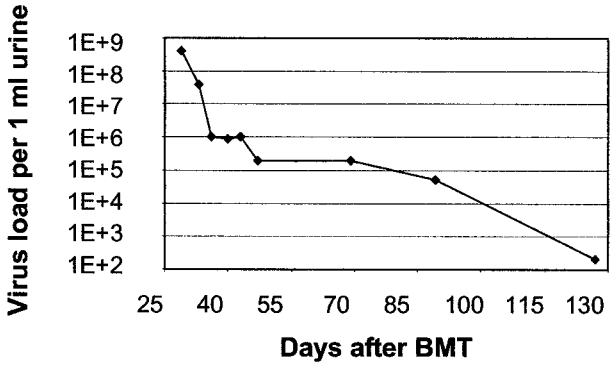

FIG. 2. Kinetics of BKV load during hemorrhagic cystitis. Documentation of BKV infection of the urinary bladder and clearance of the virus by serial RQ-PCR analysis of urine samples during the post-transplant period. The virus load (*y* axis) is plotted against the time after bone marrow transplantation (BMT) (*x* axis).

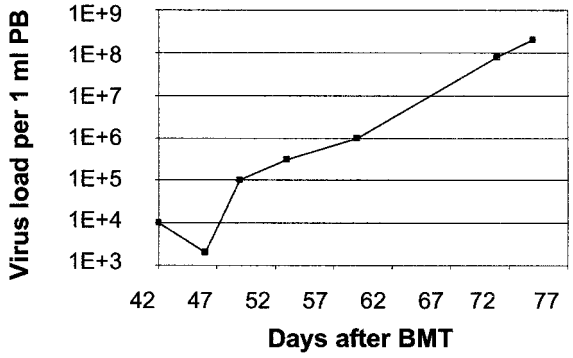

FIG. 3. Kinetics of EBV load in PB in a posttransplant lymphoproliferative disease. Serial RQ-PCR analysis documents the reactivation of a latent EBV infection by revealing constantly increasing virus copy numbers. This retrospective analysis of virus proliferation kinetics, which heralded the development of EBV-associated malignant lymphoma, underlines the potential of molecular detection and monitoring of EBV load to provide a basis for early initiation of preemptive antiviral treatment. BMT, bone marrow transplantation.

TABLE 2. CVs of virus assays described[a]

PCR target	Quantity	Intra-assay variation		Interassay variation (CV2)	PCR target	Quantity	Intra-assay variation		Interassay variation (CV2)
		CV1a	CV1b				CV1a	CV1b	
CMV	1.00E+05	0.22	0.21	2.41	AdV E	1.00E+05	0.18	0.57	1.17
	1.00E+04	0.94	1.09	2.12		1.00E+04	0.17	0.15	0.78
	1.00E+03	0.57	0.47	1.90		1.00E+03	0.63	0.43	0.74
	1.00E+02	0.80	0.26	1.56		1.00E+02	0.32	0.33	0.50
	1.00E+01	0.38	2.12	1.99		1.00E+01	0.45	0.58	1.34
EBV	1.00E+06	0.30	0.63	2.96	AdV F	1.00E+06	0.13	0.30	0.87
	1.00E+05	1.15	0.80	2.80		1.00E+05	0.19	0.32	1.17
	1.00E+04	0.32	0.28	1.90		1.00E+04	0.22	0.15	1.02
	1.00E+03	0.24	0.17	1.83		1.00E+03	0.13	0.27	0.82
HHV-6	1.00E+06	0.27	0.39	1.42	BKV	3.00E+06	0.31	0.21	1.02
	1.00E+05	0.26	0.52	1.63		3.00E+05	0.36	0.46	1.08
	1.00E+04	0.51	0.50	1.00		3.00E+04	0.22	0.27	0.86
	1.00E+03	0.08	0.63	0.84		3.00E+03	0.33	0.39	0.96
	1.00E+02	0.25	1.56	1.60		3.00E+02	0.25	0.16	0.79
HHV-7	1.00E+05	0.85	0.41	2.45	JCV	1.60E+05	0.21	0.18	0.53
	1.00E+04	0.11	0.26	1.89		1.60E+04	0.53	0.30	0.56
	1.00E+03	0.45	0.21	1.69		1.60E+03	0.33	0.27	0.52
	1.00E+02	0.50	0.26	1.73		1.60E+02	0.30	0.24	0.35
	1.00E+01	0.20	0.28	1.24		1.60E+01	0.61	1.15	0.83
HHV-8	1.00E+06	0.52	0.46	1.07	PVB19	1.00E+06	0.56	0.57	2.33
	1.00E+05	0.29	0.44	0.77		1.00E+05	0.35	0.37	2.49
	1.00E+04	0.53	0.11	0.82		1.00E+04	0.38	0.25	2.41
	1.00E+03	0.68	0.58	0.81		1.00E+03	0.43	0.37	2.07
	1.00E+02	0.23	0.20	0.67	Enterovirus	1.00E+07	0.24	0.45	1.58
HSV-1[b]	1.00E+05	0.38	0.10	2.60		1.00E+06	0.43	0.44	1.31
	1.00E+04	0.30	0.20	1.99		1.00E+05	0.63	0.40	1.18
	1.00E+03	0.20	0.19	1.79		1.00E+04	0.62	0.42	1.08
	1.00E+02	0.51	0.22	1.61		1.00E+03	1.75	2.57	2.39
HSV-2	1.00E+05	2.42	0.62	2.05	RSV[c]	1.00E+05	0.67	0.39	3.20
	1.00E+04	0.37	0.12	1.80		1.00E+04	0.55	0.21	2.06
	1.00E+03	0.87	0.36	1.73		1.00E+03	0.39	0.40	1.66
	1.00E+02	0.33	0.36	1.43		1.00E+02	0.58	0.59	2.43
VZV	1.00E+05	0.64	0.46	3.67	Influenza A virus	1.00E+06	0.34	0.25	2.52
	1.00E+04	0.17	0.20	3.09		1.00E+05	0.34	0.20	1.99
	1.00E+03	0.39	0.38	3.17		1.00E+04	0.83	0.50	1.78
	1.00E+02	0.29	0.13	2.69		1.00E+03	0.60	0.22	1.31
	1.00E+01	2.94	1.15	3.91	Influenza B virus	1.00E+04	0.70	0.73	2.78
AdV A	1.00E+06	0.32	0.62	1.16		1.00E+03	0.30	0.31	2.36
	1.00E+05	0.18	0.24	1.08		1.00E+02	0.39	0.40	2.10
	1.00E+04	0.18	0.37	0.85		1.00E+01	1.12	1.16	2.13
	1.00E+03	0.40	0.17	0.84	PIV-1[d]	1.00E+05	0.88	0.26	1.50
AdV B	1.00E+05	0.32	0.07	1.92		1.00E+04	0.18	0.19	1.46
	1.00E+04	0.34	0.06	1.79		1.00E+03	0.43	0.09	1.30
	1.00E+03	0.39	0.26	1.73		1.00E+02	0.34	0.21	1.20
	1.00E+02	0.23	0.17	1.27		1.00E+01	0.52	0.32	1.03
AdV C	1.00E+05	0.50	0.51	0.56	PIV-2	1.00E+05	0.06	0.24	1.84
	1.00E+04	0.40	0.31	0.75		1.00E+04	0.41	0.19	1.22
	1.00E+03	0.07	0.15	0.45		1.00E+03	0.17	0.29	1.33
	1.00E+02	0.21	0.22	0.43		1.00E+02	0.18	0.19	1.20
	1.00E+01	0.75	0.81	0.73		1.00E+01	0.73	0.94	1.25
AdV D	1.00E+05	0.53	0.68	1.79	PIV-3	1.00E+04	0.32	0.26	0.57
	1.00E+04	0.09	0.45	1.92		1.00E+03	0.82	0.57	0.72
	1.00E+03	0.37	0.15	1.49		1.00E+02	0.55	0.50	0.59
	1.00E+02	0.42	0.11	1.08		1.00E+01	0.44	1.56	1.29

[a] CVs observed in RQ-PCR analyses of different virus quantities are presented. The intra-assay variation was calculated by comparing the C_t values of two simultaneously amplified triplicate reactions (CV1a versus CV1b). The interassay variation was assessed by comparing triplicate reactions of different RQ PCR runs (CV2). The mean values for intra-and interassay variation across all virus assays are as follows: CV1a, 0.46; CV1b, 0.43; CV2, 1.55.

[b] HSV-1, herpes simplex virus type 1.

[c] RSV, respiratory syncytial virus.

[d] PIV-1, parainfluenza virus type 1.

instances, the rate of disease development was shown to be related directly to the viral DNA or RNA levels detected in plasma, serum, or PB lymphocytes. The association between disease progression and viral load is well established for infections with a number of viruses, particularly human immunodeficiency virus and hepatitis B and C viruses (3, 11, 22).

In immunosuppressed patients, particularly after allogeneic SCT, the need to quantitatively monitor infections with CMV and EBV has been long appreciated (6, 8, 18). The clinical relevance of a number of other viral infections in this setting is less well established, but there is a growing body of evidence indicating that viruses such as AdV (20, 26), human herpesvi-

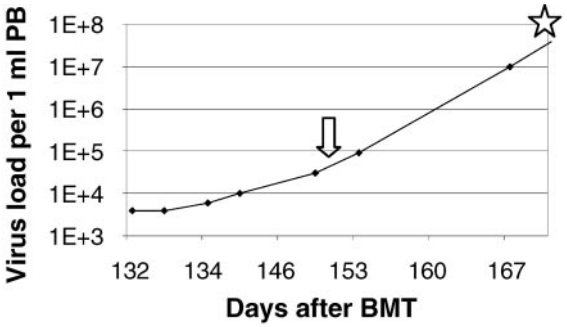

FIG. 4. Kinetics of AdV load in PB. The curve shows the appearance and expansion of AdV in PB. Observation of the first 10-fold increase in virus load (arrow) preceded the onset of clinical symptoms (star) by more than 3 weeks. The monitoring of AdV in PB may therefore serve as a basis for early initiation of preemptive antiviral treatment. BMT, bone marrow transplantation.

rus 6 (HHV-6) (24), varicella-zoster virus (VZV) (12), parvovirus B19 (PVB19) (28), and others (1, 7, 10) merit careful monitoring in these patients. Molecular diagnostic assays, such as the tests presented herein, are therefore of increasing clinical importance.

To render the screening for multiple viruses more practicable, all tests presented were conceived to permit target amplification and quantification under identical PCR conditions. The examples shown represent important paradigms of clinical application of quantitative virus detection assays in immunocompromised patients. As demonstrated, quantitative virus tests are useful when assessing a clinically suspected viral cause of infection because the documentation of rising virus copy numbers, in the absence of other detectable pathogens, provides support for a role of the virus as a causative agent. Moreover, the observation of increasing viral load in sequential assays virtually excludes the possibility of false interpretation of positive PCR results resulting from contamination with amplification products or traces of viral nucleic acids harboring the amplifiable sequence.

Another clinically important application of quantitative virus tests is the possibility of differentiating between latent

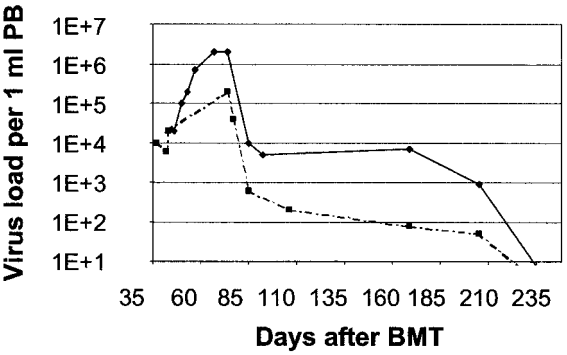

FIG. 5. Kinetics of CMV load in PB in response to antiviral treatment. Monitoring of CMV by RQ-PCR in serial plasma (solid line) and PB leukocyte (dashed line) samples during the posttransplant period reveals viral reactivation by rising levels of CMV DNAemia and subsequent clearance of the virus following antiviral therapy. BMT, bone marrow transplantation.

infection and reactivation. Persisting viruses may occur after primary infection in healthy immunocompetent individuals, as well as in asymptomatic patients (16), and cause universally positive results in qualitative PCR assays. Mere detection of viral pathogens by qualitative PCR may not be relevant to the clinical outcome in these individuals, but consecutive assessment of the virus load seems to play an important role in the diagnosis and prognosis of patients with viral reactivation by providing a basis for timely initiation of appropriate treatment (5, 6, 9, 25, 29). Sequential assessment of viral load by means of RQ-PCR is a helpful parameter for clinical decision making, particularly if molecular detection and documentation of proliferation kinetics of the virus precede the onset of clinical symptoms. This has been demonstrated for a number of virus infections (17, 20, 31).

Finally, the ability of quantitative virus tests to facilitate monitoring of the response to antiviral treatment is an invaluable tool in the clinical care of immunocompromised patients, providing a means of controlling of the appropriate choice and the necessary duration of therapy. Quantitative virus testing has therefore become an indispensable diagnostic instrument in many clinical situations. The real-time PCR tests presented provide a contribution to the rapidly growing field of molecular investigation of viral infections as a basis for improved patient care.

REFERENCES

1. **Abdallah, A., K. E. Rowland, S. K. Schepetiuk, L. B. To, and P. Bardy.** 2003. An outbreak of respiratory syncytial virus infection in a bone marrow transplant unit: effect on engraftment and outcome of pneumonia without specific antiviral treatment. Bone Marrow Transplant. **32:**195–203.
2. **Akiyama, H., T. Kurosu, C. Sakashita, T. Inoue, S. Mori, K. Ohashi, S. Tanikawa, H. Sakamaki, Y. Onozawa, Q. Chen, H. Zheng, and T. Kitamura.** 2001. Adenovirus is a key pathogen in hemorrhagic cystitis associated with bone marrow transplantation. Clin. Infect. Dis. **32:**1325–1330.
3. **Cervia, J., B. Kaplan, S. Schuval, and S. Weiss.** 2003. Virologic testing in the management of perinatal HIV exposure. AIDS Read. **13:**39–47.
4. **Dini, G., E. Castagnola, P. Comoli, M. J. van Tol, and J. M. Vossen.** 2001. Infections after stem cell transplantation in children: state of the art and recommendations. Bone Marrow Transplant. **28**(Suppl. 1)**:**S18–S21.
5. **Emery, V. C., A. V. Cope, E. F. Bowen, D. Gor, and P. D. Griffiths.** 1999. The dynamics of human cytomegalovirus replication in vivo. J. Exp. Med. **190:** 177–182.
6. **Emery, V. C., C. A. Sabin, A. V. Cope, D. Gor, A. F. Hassan-Walker, and P. D. Griffiths.** 2000. Application of viral-load kinetics to identify patients who develop cytomegalovirus disease after transplantation. Lancet **355:** 2032–2036.
7. **Fischmeister, G., P. Wiesbauer, H. M. Holzmann, C. Peters, M. Eibl, and H. Gadner.** 2000. Enteroviral meningoencephalitis in immunocompromised children after matched unrelated donor-bone marrow transplantation. Pediatr. Hematol. Oncol. **17:**393–399.
8. **Gartner, B. C., H. Schafer, K. Marggraff, G. Eisele, M. Schafer, D. Dilloo, K. Roemer, H. J. Laws, M. Sester, U. Sester, H. Einsele, and N. Mueller-Lantzsch.** 2002. Evaluation of use of Epstein-Barr viral load in patients after allogeneic stem cell transplantation to diagnose and monitor posttransplant lymphoproliferative disease. J. Clin. Microbiol. **40:**351–358.
9. **Gustafsson, A., V. Levitsky, J. Z. Zou, T. Frisan, T. Dalianis, P. Ljungman, O. Ringden, J. Winiarski, I. Ernberg, and M. G. Masucci.** 2000. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. Blood **95:**807–814.
10. **Hassan, I. A., R. Chopra, R. Swindell, and K. J. Mutton.** 2003. Respiratory viral infections after bone marrow/peripheral stem-cell transplantation: the Christie hospital experience. Bone Marrow Transplant. **32:**73–77.
11. **Hodinka, R. L.** 1998. The clinical utility of viral quantitation using molecular methods. Clin. Diagn. Virol. **10:**25–47.
12. **Ishizaki, Y., J. Tezuka, S. Ohga, A. Nomura, N. Suga, R. Kuromaru, K. Kusuhara, Y. Mizuno, N. Kasuga, and T. Hara.** 2003. Quantification of circulating varicella zoster virus-DNA for the early diagnosis of visceral varicella. J. Infect. **47:**133–138.
13. **Jungkind, D.** 2001. Automation of laboratory testing for infectious diseases using the polymerase chain reaction–our past, our present, our future. J. Clin. Virol. **20:**1–6.

14. **Kumar, R., N. Vandegraaff, L. Mundy, C. J. Burrell, and P. Li.** 2002. Evaluation of PCR-based methods for the quantitation of integrated HIV-1 DNA. J. Virol. Methods **105:**233–246.
15. **Lankester, A. C., M. J. van Tol, J. M. Vossen, A. C. Kroes, and E. Claas.** 2002. Epstein-Barr virus (EBV)-DNA quantification in pediatric allogenic stem cell recipients: prediction of EBV-associated lymphoproliferative disease. Blood **99:**2630–2631.
16. **Larsson, S., C. Soderberg-Naucler, F. Z. Wang, and E. Moller.** 1998. Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. Transfusion **38:**271–278.
17. **Leruez-Ville, M., M. Ouachee, R. Delarue, A. S. Sauget, S. Blanche, A. Buzyn, and C. Rouzioux.** 2003. Monitoring cytomegalovirus infection in adult and pediatric bone marrow transplant recipients by a real-time PCR assay performed with blood plasma. J. Clin. Microbiol. **41:**2040–2046.
18. **Limaye, A. P., M. L. Huang, W. Leisenring, L. Stensland, L. Corey, and M. Boeckh.** 2001. Cytomegalovirus (CMV) DNA load in plasma for the diagnosis of CMV disease before engraftment in hematopoietic stem-cell transplant recipients. J. Infect. Dis. **183:**377–382.
19. **Lion, T.** 2001. Current recommendations for positive controls in RT-PCR assays. Leukemia **15:**1033–1037.
20. **Lion, T., R. Baumgartinger, F. Watzinger, S. Matthes-Martin, M. Suda, S. Preuner, B. Futterknecht, A. Lawitschka, C. Peters, U. Potschger, and H. Gadner.** 2003. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. Blood **102:**1114–1120.
21. **O'Doherty, U., W. J. Swiggard, D. Jeyakumar, D. McGain, and M. H. Malim.** 2002. A sensitive, quantitative assay for human immunodeficiency virus type 1 integration. J. Virol. **76:**10942–10950.
22. **Pas, S. D., E. Fries, R. A. De Man, A. D. Osterhaus, and H. G. Niesters.** 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. J. Clin. Microbiol. **38:** 2897–2901.
23. **Reusser, P.** 2002. Challenges and options in the management of viral infections after stem cell transplantation. Support. Care Cancer **10:**197–203.
24. **Sashihara, J., K. Tanaka-Taya, S. Tanaka, K. Amo, H. Miyagawa, G. Hosoi, T. Taniguchi, T. Fukui, N. Kasuga, T. Aono, M. Sako, J. Hara, K. Yamanishi, and S. Okada.** 2002. High incidence of human herpesvirus 6 infection with a high viral load in cord blood stem cell transplant recipients. Blood **100:** 2005–2011.
25. **Savoie, A., C. Perpete, L. Carpentier, J. Joncas, and C. Alfieri.** 1994. Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of pediatric transplant patients and risk of lymphoproliferative disease. Blood **83:**2715–2722.
26. **Schilham, M. W., E. C. Claas, W. van Zaane, B. Heemskerk, J. M. Vossen, A. C. Lankester, R. E. Toes, M. Echavarria, A. C. Kroes, and M. J. van Tol.** 2002. High levels of adenovirus DNA in serum correlate with fatal outcome of adenovirus infection in children after allogeneic stem-cell transplantation. Clin. Infect. Dis. **35:**526–532.
27. **Schutten, M., and H. G. Niesters.** 2001. Clinical utility of viral quantification as a tool for disease monitoring. Expert Rev. Mol. Diagn. **1:**153–162.
28. **Segovia, J. C., G. Guenechea, J. M. Gallego, J. M. Almendral, and J. A. Bueren.** 2003. Parvovirus infection suppresses long-term repopulating hematopoietic stem cells. J. Virol. **77:**8495–8503.
29. **Sirvent-Von Bueltzingsloewen, A., P. Morand, M. Buisson, G. Souillet, H. Chambost, J. L. Bosson, and P. Bordigoni.** 2002. A prospective study of Epstein-Barr virus load in 85 hematopoietic stem cell transplants. Bone Marrow Transplant. **29:**21–28.
30. **van Esser, J. W., H. G. Niesters, S. F. Thijsen, E. Meijer, A. D. Osterhaus, K. C. Wolthers, C. A. Boucher, J. W. Gratama, L. M. Budel, H. B. van der, A. M. van Loon, B. Lowenberg, L. F. Verdonck, and J. J. Cornelissen.** 2001. Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. Br. J. Haematol. **113:**814–821.
31. **van Esser, J. W., H. G. Niesters, H. B. van der, E. Meijer, A. D. Osterhaus, J. W. Gratama, L. F. Verdonck, B. Lowenberg, and J. J. Cornelissen.** 2002. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and preemptive rituximab in high-risk patients after allogeneic stem cell transplantation. Blood **99:**4364–4369.
32. **van Esser, J. W., H. B. van der, E. Meijer, H. G. Niesters, R. Trenschel, S. F. Thijsen, A. M. van Loon, F. Frassoni, A. Bacigalupo, U. W. Schaefer, A. D. Osterhaus, J. W. Gratama, B. Lowenberg, L. F. Verdonck, and J. J. Cornelissen.** 2001. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted SCT. Blood **98:**972–978.
33. **Wagner, H. J., M. Wessel, W. Jabs, F. Smets, L. Fischer, G. Offner, and P. Bucsky.** 2001. Patients at risk for development of posttransplant lymphoproliferative disorder: plasma versus peripheral blood mononuclear cells as material for quantification of Epstein-Barr viral load by using real-time quantitative polymerase chain reaction. Transplantation **72:**1012–1019.