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Multiplexed, Real-Time PCR for Quantitative Detection of Human Adenovirus

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Adenovirus infection is becoming increasingly recognized as a cause of morbidity and mortality in the immunosuppressed patient population. While early detection and quantitation of adenovirus in peripheral blood has been suggested as a means of directing and monitoring antiviral therapy in these patients, few methods have been published, particularly with respect to viral quantitation. A multiplexed real-time PCR assay was developed that can quantitatively detect a wide range of known serotypes of human adenovirus, including all of subgroups A to C. This assay was compared to a qualitative, Southern blot-based PCR assay by using 45 peripheral blood specimens from 16 patients. There was 100% concordance between the two tests in terms of qualitative results. The real-time assay detected adenovirus in patient samples at levels from <200 to 266,681 copies/ml of blood. By using control viral samples, sensitivity was demonstrated to less than 10 copies of viral genome per reaction and quantitative linearity was demonstrated from 10 to 10^6 copies of input viral DNA. Equivalent sensitivity and linearity were demonstrated for 15 different reference serotypes of adenovirus. Eleven other viral serotypes have complete target region sequence homology to one or more of the strains tested. No cross-reactivity was noted with other commonly isolated viral species. Sequence analysis showed no significant homology with any other human pathogens (bacterial or viral). This assay allows rapid, sensitive, and specific quantitation of adenovirus and may have a significant impact on the care of immunocompromised patients at risk for disseminated viral infection.

Adenovirus (AdV) has been increasingly shown to play a role in the morbidity and mortality of immunosuppressed patients (12). Allogeneic bone marrow and stem cell transplant patients appear at especially high risk for infection, with some evidence of higher risk among recipients of unrelated or HLA-mismatched transplants, as well as in pediatric age groups (7, 16, 21). AdV infections can affect numerous organ systems and may lead to disseminated disease, with an especially high mortality rate. As many as 95% of children are seropositive for AdV by age 6 (2). Although infection in immunocompetent children is typically mild and self-limited, latent virus remains in lymphoid and renal tissues. Perhaps due to this phenomenon of latency, carriage rates as high as 20% have been demonstrated in patients undergoing bone marrow transplantation. Many of these carriers can develop invasive disease, with mortality rates exceeding 50% in some studies (1, 7, 9, 19). At St. Jude Children's Research Hospital, a previous study showed a 6% rate of infection, with a mortality rate of 54% among those infected (9). High rates of infection in children may be due to a higher primary exposure rate in this population (i.e., increased incidence of primary disease), an increased incidence of recent, pretransplant infection (i.e., a higher incidence of latency), an increased rate of complications due to the use of immunosuppressive regimens in this population, and/or various sources of testing and reporting bias (7, 9, 15). Detection in blood has been associated with a higher incidence of symptomatic disease (5), with some studies suggesting that viral load is predictive of disease outcome and might also be used to monitor response to antiviral therapy (5, 18, 25).

AdVs are nonenveloped, double-stranded DNA viruses (15). Fifty-one serotypes of human AdV have been identified; based on erythrocyte agglutination assays and on DNA sequence homology, serotypes can be further segregated into subgroups (subgenus groups) A to F, with homology within subgroups ranging from 50 to near 100%. Between subgroups there is much less homology (as low as 4%) (25). Most human infections are due to viruses from subgroups A to C, but many other serotypes have been shown to cause occasional illness (12, 15, 25). Associations between specific serotypes and patient groups or clinical disease characteristics are also imperfect.

The mouth, nasopharynx, and ocular conjunctiva serve as the portals of entry for AdV (15). Virus is internalized and multiplies within the host cell nucleus. AdV can persist after symptomatic disease resolution in lymphoid tissue, with latency documented for as long as 2 years after primary infection. As noted above, reactivation may occur in the presence of severe immunosuppression and may account for much of the disease seen in such patients (12). AdVs cause a variety of human disease including respiratory illness, gastroenteritis and related syndromes, and neurologic disease. In immunocompromised patients, sequelae are more severe and may include hemorrhagic cystitis, hepatitis, enteritis, encephalitis, and pneumonia (7, 9, 12, 15, 16, 24).

The laboratory diagnosis of AdV infection has typically relied on the detection of a cytopathic effect in various cell lines (24). These techniques are effective in detecting end organ infection in symptomatic patients but lack the sensitivity necessary to detect low levels of circulating virus in peripheral

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TABLE 1. Control viral serotypes of human AdV

Serotype	Subgenus	Tested[a]	GenBank accession no.	Source
AdV12	A	Y	X73487	ATCC
AdV18	A	Y	Y17249	ATCC
AdV31	A	Y	Y17253	ATCC
AdV3	B	N	X86549	ND[b]
AdV7	B	Y	AF065065	ATCC
AdV7a	B	N	AF065066	ND
AdV11	B	Y	AJ272606	ATCC
AdV14	B	N	AJ272607	ND
AdV16	B	N	AJ272608	ND
AdV21	B	N	AY008279	ND
AdV34	B	N	AJ272601	ND
AdV35	B	N	AJ272611	ND
AdV50	B	N	AJ272612	ND
AdV1	C	Y	Y17244	ATCC
AdV2	C	Y	J01917	ATCC
AdV5	C	Y	J01966	ATCC
AdV6	C	Y	Y17245	ATCC
AdV8	D	N	Y17246	ND
AdV9	D	Y	Y14247	ATCC
AdV10	D	Y	AJ250707	ATCC
AdV17	D	Y	AF108105	ATCC
AdV19	D	N	Y17250	ND
AdV28	D	Y	Y17251	ATCC
AdV37	D	Y	Y17252	ATCC
AdV48	D	N	U20821	ND
AdV4	E	Y	X84646	ATCC

[a] Y, the viral DNA was tested; N, not tested.
[b] ND, not done.

blood samples. Furthermore, the mean time to detection can be as long as 6 days by use of these methods (17). Serologic techniques are of little value for diagnosis of acute disease, due to a high seroprevalence in even the youngest age groups. Detection in paraffin-embedded tissue sections has been accomplished through the observation of characteristic morphological changes and has been significantly enhanced by the use of immunohistochemical methods and in situ hybridization (17, 28). PCR has been increasingly used to detect many pathogenic viruses in human specimens (10, 17, 26). PCRs for AdV have been effective for detection of virus in respiratory specimens, body fluids, and tissue specimens and for subgenus and serotype identification of viral isolates (3, 4, 6, 8, 11, 23, 29). Several assays have recently been used to detect AdV in various compartments of peripheral blood (serum, plasma, and whole blood). The design and validation of these tests are complicated by the genetic heterogeneity of this virus. This issue is of even greater import in the design of quantitative assays, because amplification efficiency must be demonstrated to be equal in all serotypes detected to avoid the necessity of viral typing prior to quantitation.

Recent publications have suggested the clinical utility of measurement of the AdV load in peripheral blood (18, 25). The details of one semiquantitative assay (based on Southern blotting methodology) have been published (25). The development of a rapid, quantitative assay, based on real-time PCR methods and validated against a wide range of viral serotypes, could prove highly useful in the further characterization of the clinical risk status of immunosuppressed patients, the determination of appropriate treatment regimens, and the assessment of therapeutic responsiveness (18, 25). The examples of human immunodeficiency virus and hepatitis C virus, among others, have demonstrated that viral load data can have a profound impact on clinical care. With this in mind, the present study focuses on the development of a real-time PCR assay for the detection and quantification of AdV in immunocompromised pediatric patients.

MATERIALS AND METHODS

Control viral strains. DNA sequence data from 26 serotypes of human AdV, representing viral subtypes A to E, were used for primer and probe design (Table 1; also see below). Fifteen control viral strains, each of a different serotype, were obtained, representing all major phylogenetic groups of AdV (enteric serotypes excluded). Eleven additional serotypes had DNA sequences within the PCR target region identical to those of one or more control strains. All control AdV strains, except for AdV type 5, were obtained from the American Type Culture Collection (ATCC, Manassas, Va.). Known concentrations of AdV type 5 and Epstein-Barr virus (EBV) (both cesium chloride gradient purified and quantified by direct electron microscopic examination) as well as human herpesvirus 6 (HHV-6) and HHV-8 (sucrose density gradient purified) were purchased from Advanced Biotechnologies, Inc. (Columbia, Md.). Other viruses used for cross-reactivity testing included cytomegalovirus (CMV), simian virus 40 (SV40), her-

TABLE 2. Nucleic acid targets for primers and probes

Purpose and name	Product size (bases)	Sequence (5′–3′)[a]	Target[b]
Primers			
DGF	24	AGACATGACHTTYGAGGTGGAYCC	AdV (24 types)
DGR	19	CGCAGGTASACBGYYTCRA	AdV (25 types)
31F	25	AGATATGACATTTGAAGTTGACCCC	AdV31
31R	21	CGCAGATAGACCGCTTCAATG	AdV31
4F	23	AGACATGAATTTCGAAGTCGACC	AdV4
Probes			
98A12	23	TACGCATTCACCAGCCACACCGC	AdV12
98A18/31R	25	CGCACCACGTCGAAAACTTCAAACA	AdV18, AdV31
98B1	22	CAGAGTGCACCAGCCACACCGC	Group B:1 (types 3, 7, 7a, 16, 21, 50)
98B2	23	TCAGAGTGCATCAGCCACACCGC	Group B:2 (types 11, 14, 34, 35)
98C	24	TCCGTGTGCACCAGCCGCAC	Group C (types 1, 2, 5, 6)
98D	21	TCAGAGTGCACCAGCCGCACC	Group D (types 8, 9, 10, 17, 19, 28, 37, 48)
98E4	20	TCCGAGTGCACCAGCCCCAC	Group E (type 4)

[a] IUB codes: B = C, G, or T; H = A, C, or T; R = A or G; S = G or C; Y = C or T.
[b] Hexon gene of human AdV type and subgenus; types whose nucleotide sequences were used to determine the consensus sequence are listed. See Table 1 for GenBank accession numbers.

TABLE 3. Comparison of spectrophotometric methodology to QRT PCR for quantification of PCR amplicons[a]

AdV type	Input PCR amplicon copy no.[b]	QRT PCR copy no.[c]	Mean copy no.	SD	CV
1	6	5.7634 5.7686 5.7924	5.7750	0.0154	0.0027
2	6	5.9031 5.8887 5.9375	5.91031	0.0251	0.0042
4	6	5.6021 5.6232 5.5563	5.5948	0.0342	0.0061
5	6	5.9004 5.8370 5.8513	5.8637	0.0333	0.0057
6	6	5.8692 5.8156 5.8563	5.8476	0.0280	0.0048
7	6	5.6532 5.6902 5.6128	5.6532	0.0387	0.0068
9	6	5.8808 5.7889 5.8129	5.8293	0.0477	0.0082
10	6	5.5798 5.5441 5.5563	5.5603	0.0182	0.0033
11	6	5.8921 5.7853 5.9140	5.8673	0.0688	0.0117
12	6	5.5315 5.4914 5.4624	5.4960	0.0347	0.0063
17	6	5.7559 5.7868 5.7328	5.7591	0.0270	0.0047
18	6	5.4314 5.5185 5.3802	5.4472	0.0699	0.0128
28	6	5.8062 5.7193 5.8028	5.7779	0.0492	0.0085
31	6	5.3802 5.4771 5.5441	5.4723	0.0824	0.0151
37	6	5.7559 5.8299 5.7649	5.7849	0.0404	0.0070
Avg			5.7093		0.0073

[a] All data are expressed in $\log_{10}$ units.
[b] Based on spectrophotometric methodology.
[c] Mean copy number of each of three QRT-PCR runs.

pes simplex virus type 1 (HSV-1), and HSV-2; these were obtained from the ATCC.

Clinical samples. Following Institutional Review Board approval, excess frozen, pelleted peripheral blood mononuclear cells (PBMCs) were obtained from 45 specimens previously tested for AdV by qualitative PCR with detection by Southern blot techniques (SB-PCR; Viromed, Minnetonka, Minn.) between October 2001 and April 2002. Specimens were coded to conceal patients' identities, extracted, and tested by quantitative real-time PCR (QRT PCR).

Preparation of control viral DNA. Stock viral isolates were heat inactivated at 95°C for 30 min prior to purification. Viral genomic DNA was extracted from 100 μl of viral lysate by using a QIAamp Blood Mini Kit (Qiagen Inc., Valencia, Calif.). Control PCR products used for assessment of sensitivity and quantitative linearity were purified by using a QIAquick PCR purification kit (Qiagen Inc.).

Preparation and extraction of clinical samples. PBMCs were separated from 4 ml of whole blood and divided into two equal aliquots. One aliquot (used for SB-PCR assays) was extracted by using phenol-chloroform methodology with ethanol precipitation; the second aliquot was immediately frozen at −70°C. DNA was extracted from the frozen cell pellets by using a QIAamp Blood Mini Kit (Qiagen Inc.) and analyzed by QRT PCR.

Reference PCR assay. The results of QRT PCR performed on clinical specimens were compared with the results obtained from the same specimens by using a qualitative PCR assay based on the modifications of Echavarria et al. (4). Briefly, AdV PCR was performed by using primers specific for the hexon gene. The PCR product was detected on a 2% NuSieve 3:1 agarose gel and visualized with SYBR Green I dye. Southern blotting was performed by electrophoretic transfer to a nylon membrane and specific hybridization with a digoxigenin-11-dUTP-labeled probe.

QRT PCR. (i) Primers and probes. One primer pair degenerate at eight sites (total of 60 individual primer sequences) as well as specific primers for types 31 (forward and reverse) and 4 (forward only) and seven group-specific probes were designed to amplify and detect all targeted viral serotypes (Table 2). The targeted serotypes represented five of the six known AdV subgenera, including all of viral subgroups A to C and E and numerous members of subtype D. Subtype F (enteric serotypes 40 and 41) was excluded during the assay design. Primer and probe sequences were selected from the AdV hexon gene by using Primer Express software (version 2.0; Applied Biosystems, Foster City, Calif.) based on the nucleotide sequences of 26 individual AdV serotypes (see Table 1 for GenBank accession numbers). Primer and probe sequences were assessed for specificity by comparing them to sequences of other prokaryotic and eukaryotic organisms by using Wisconsin Package software (Accelrys, San Diego, Calif.). Primers were synthesized by the Hartwell Center for Biotechnology (St. Jude Children's Research Hospital core facility) or by Applied Biosystems. Probes were 5′ end labeled with 6-carboxyfluorescein (FAM) as the reporter dye and 3′ end labeled with 6-carboxytetramethylrhodamine (TAMRA) as the quencher. All fluorescent probes used in the study were synthesized at Applied Biosystems.

(ii) Standard PCR amplification. A three-step PCR protocol was used to amplify a 123-bp DNA segment from control viral DNA preparations. A PCR mix was prepared by using a *Taq* PCR Core kit (Qiagen), consisting of 10 μl of 10× PCR buffer, 20 μl of 5× Q solution, 1 μl of 10 mM deoxynucleoside triphosphates, 5 U of *Taq* polymerase, 8 μl of 4 pM degenerate forward primer, 8 μl of 16 pM degenerate reverse primer, and 1 μl of target viral nucleic acid, in a final total volume of 100 μl. PCR was performed using a PTC-225 DNA Engine Tetrad (MJ Research, Inc., Waltham, Mass.) under the following conditions: 1 cycle at 95°C for 5 min, followed by 40 cycles of programmed amplification (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s). Products were purified by using a QIAquick PCR purification kit (Qiagen) and detected by agarose gel (4%) electrophoresis. The number of amplicon copies in each extract was determined by spectrophotometric measurements at 260 nm using a BioMate 5 UV-visible spectrophotometer (Thermo Spectronic, Waltham, Mass.).

(iii) QRT PCR master-mix and reaction conditions. The optimized master mix consisted of 1× TaqMan Universal PCR master mixture (Applied Biosystems), 0.2 μM degenerate forward primer mix, 0.8 μM degenerate reverse primer mix, seven probes (each at 100 nM), and 5 μl of target DNA solution in a final total volume of 50 μl. Human genomic DNA (Sigma-Aldrich, Inc., St. Louis, Mo.) was included as a carrier at 100 ng of DNA per reaction when viral DNA was the only template nucleic acid present. TaqMan exogenous internal positive control (EIPC) reagents (Applied Biosystems) were used for inhibition detection. EIPC reagents consist of an exogenously added low-copy-number internal control, coamplified during primary target PCR. Inhibition is defined by the absence of EIPC target amplification. Real-time PCR was performed on an ABI PRISM 7900HT Sequence Detection system (Applied Biosystems) in a 96-well format by using TaqMan fluorogenic detection-based chemistries (14, 20, 22). Briefly, samples were heated at 50°C for 2 min and then at 95°C for 10 min, followed by a 50-cycle, two-step process, with each cycle consisting of denaturation at 95°C for 15 s and annealing or extension at 60°C for 1 min. Data were collected and analyzed using Sequence Detection System software (version 2.0; Applied Biosystems). Each sample was tested three times (separate runs) and was assayed in triplicate during each run (reported results represent the mean value from each run) to allow assessment of both within-run and between-run variability of results (only run-to-run variability is represented in Table 3).

(iv) Sensitivity, specificity, and inhibition studies. The sensitivity of QRT PCR was evaluated against 15 different AdV serotypes by testing serial dilutions of known numbers of viral particles (AdV type 5) and serial dilutions of amplicon generated from standard PCR and quantified by spectrophotometric methods (all other tested serotypes). Dilutions of target were made both in water and by spiking target into human genomic DNA preparations. The potential cross-reactivity of the assay was evaluated by testing other viral respiratory pathogens,

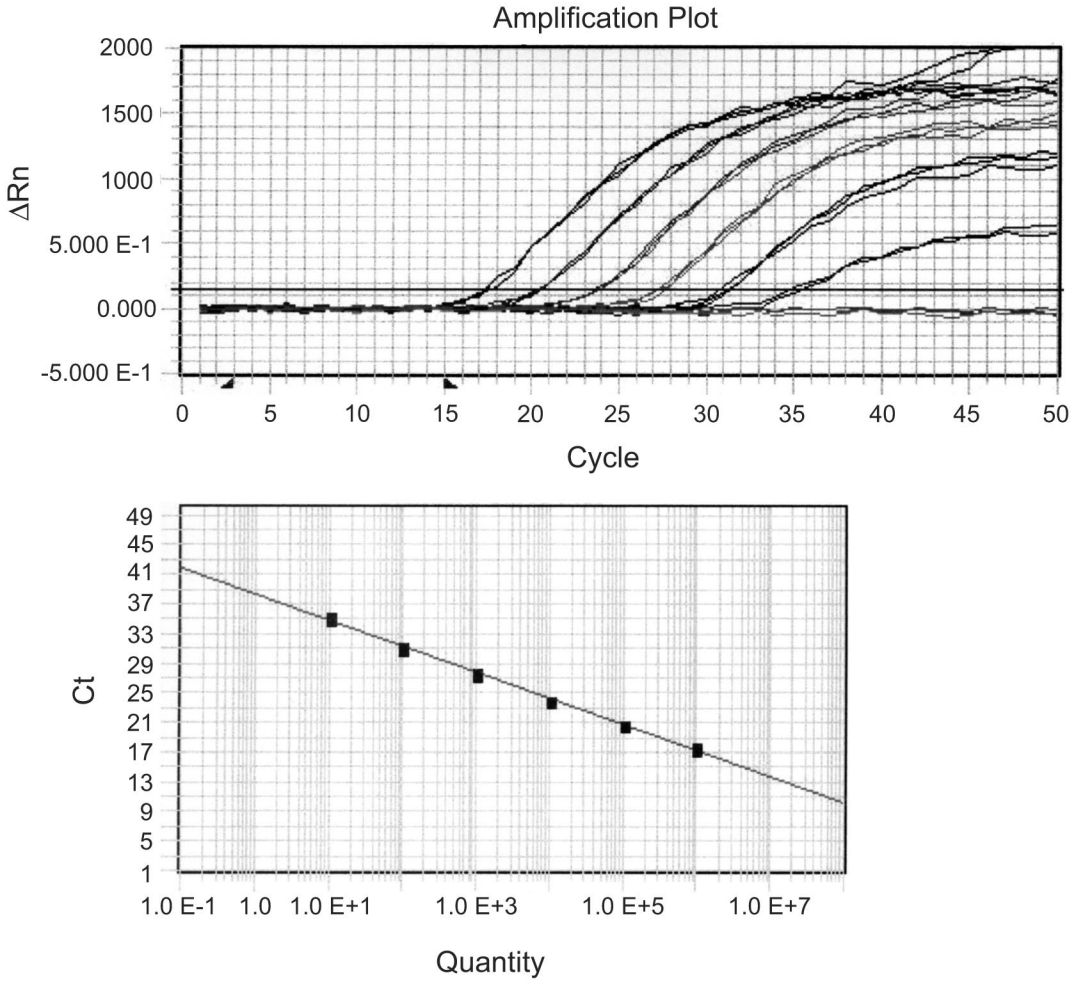

FIG. 1. QRT PCR standard curve. A representative amplification plot (top) and a standard curve plot (bottom) are shown. A 10-fold serial dilution of purified type 5 viral particles, ranging from 10^6 to 10^1 copies, was used to generate both plots.

including EBV, SV40, HSV-1 and -2, HHV-6 and -8, and CMV, and by comparison of target sequences with sequence data from other organisms. Inhibition was assessed in all samples by quantitation of an exogenous internal control (see preceding section). Further assessment of inhibition was performed by spiking serial concentrations of AdV type 5 into 17 AdV-negative patient samples and comparing results to those obtained when the same samples were diluted in sterile H_2O. Inhibition was demonstrated either by a complete loss of signal or by detection of a lower viral copy number in the presence of the negative patient specimens. One-sided 95% confidence intervals (CI) for sensitivity and specificity were computed using the exact binomial distribution.

(v) Quantitative linearity. Tenfold serial dilutions, from 1 to 10^6 copies, of purified AdV type 5 DNA were used for standard curve construction. The standard curve was generated by Sequence Detection System (version 2.0) software, plotting cycle threshold (C_t) versus concentration of viral DNA, expressed as log viral genome copy number. Run acceptability was defined by an R^2 value of $\geq$0.98. Each AdV serotype tested was amplified, quantified by spectrophotometry, and serially diluted in distilled water and in human genomic DNA. QRT PCR results from each serially diluted amplicon were compared with the corresponding spectrophotometry-based theoretical concentrations.

(vi) Comparison to qualitative PCR. QRT PCR was performed on 45 coded clinical specimens previously tested by SB-PCR. Any positive result from the TaqMan assay, irrespective of viral quantity, was regarded as positive for comparative purposes. Negative specimens were subsequently spiked with serial dilutions of control virus for assessment of PCR inhibition (see above).

RESULTS

Validation of quantitative PCR assay. The quantitative PCR assay was tested directly against extracts from 15 different serotypes or strains of AdV (Table 1), with complete sequence homology in the target region to another 11 serotypes. In all cases, sensitivity was demonstrated to $<$10 copies of viral genome/reaction. Quantitative linearity was reproducibly demonstrated in all cases from 10^1 to 10^6 copies (Fig. 1). Accuracy of quantitation was validated against electron microscopic viral particle counts (serotype 5) and against spectrophotometrically quantified PCR amplicons (see Materials and Methods). In all cases, the quantitative results of QRT PCR agreed with those of the reference method to within 0.5 log unit. Each viral extract was assayed in triplicate three times, demonstrating both within-run and run-to-run reproducibility. The results of these experiments are summarized in Table 3. The reproducibility of the experiments is reflected with a between-run log coefficient of variation (CV) ranging from 0.0027 to 0.0151 (mean log CV, 0.0073). The specificity of the QRT PCR assay was examined by a database search and experimentally. A

TABLE 4. Comparison of QRT PCR to SB-PCR with clinical specimens[a]

Case no.	SB-PCR result	QRT PCR result[b] (mean copy no./ml of blood)
1	−	0
2	+	15,415
3	+	80,859
4	+	<200
5	+	<200
6	+	<200
7	+	826
8	+	1,216
9	+	<200
10	+	2,636
11	+	23,890
12	+	<200
13	+	29,516
14	+	226,484
15	+	76,681
16	+	266,681
17	+	<200
18	+	<200
19	−	0
20	−	0
21	−	0
22	−	0
23	−	0
24	−	0
25	−	0
26	+	1,274
27	−	0
28	−	0
29	−	0
30	−	0
31	−	0
32	−	0
33	−	0
34	+	<200
35	−	0
36	−	0
37	−	0
38	+	<200
39	−	0
40	−	0
41	−	0
42	−	0
43	−	0
44	−	0
45	−	0

[a] All specimens consist of PBMCs.
[b] Generated from three independent experiments.

FASTA nucleotide sequence similarity search (GenBank and EMBL) revealed no significant homologous targets, prokaryotic or eukaryotic (data not shown). No cross-reactivity was noted with other respiratory viruses assayed, including EBV, SV40, HSV-1 and -2, HHV-6 and -8, and human CMV.

Clinical specimens. Forty-five specimens from 16 patients were evaluated in this study. As shown in Table 4, QRT PCR showed complete qualitative correlation (sensitivity, 100% [CI, 86 to 100%]; specificity, 100% [CI, 88 to 100%]) with the reference method. In addition, QRT PCR yielded quantitative results not available by use of the Southern blot method. No inhibition was noted in any of the samples tested, based on amplification of an exogenously added internal control and by spiking negative patient samples with viral particles (parallel runs spiked with 10^6 and 10^2 viral particles). The laboratory turnaround time of QRT PCR was approximately 1 day, including specimen preparation, compared to 2 to 3 days for SB-PCR.

DISCUSSION

We report a multiplexed, real-time PCR assay capable of detecting and quantifying a wide range of human AdV serotypes. The assay is composed of five primers (63 primer sequences including degenerate positions) and seven probes and can detect all serotypes from subgroups A, B, C, and E, as well as eight serotypes from subgroup D. Subgenus D consists of 31 serotypes and displays a 94 to 99% intrageneric DNA homology (27), suggesting that more serotypes from subgenus D could be detected and quantified by using this assay. This would need to be proven using actual viral isolates and/or verified with nucleotide sequence information. Sensitivity was uniformly demonstrated to <10 viral genome copies for all viral types tested. A wide dynamic linear range of quantitation was shown for these targets, with a high degree of reproducibility and no evidence of cross-reactivity with other common respiratory viruses. Absolute quantitation was equivalent across all viral types tested to within 0.5 log unit, i.e., viral type did not need to be determined prior to interpretation of quantitative results.

Comparison of the results of this assay to those of SB-PCR showed the new test to be equivalent in sensitivity, with complete agreement of both positive and negative results between the two methods. The new assay has the added benefit of providing quantitative data. Such data have been shown to be helpful in patient management, with some studies showing a link between viral load and clinical disease state (18, 25). The routine availability of quantitative results will allow further study of this association. The usefulness of viral load data for prognostic purposes has been shown in other types of viral infection (13) and promises to be of interest for systemic AdV infection as well. The availability of this test, in conjunction with the increasing availability of antiviral agents, may allow physicians to monitor patients for responsiveness to therapy and make rapid adjustments in treatment regimens. Such potential clinical applications are the subject of ongoing research at this and other institutions.

Other important aspects of this methodology relate to the use of real-time amplification. Such homogeneous techniques (i.e., amplification and detection in a single reaction chamber) allow a high rate of assay throughput with reduced hands-on technologist time. As more and more viral genomic detection assays are used routinely for the surveillance of immunosuppressed patients, rapid processing and scalable platforms will become increasingly critical. Such advantages are compounded when one combines currently available automated extraction methodologies with real-time PCR. Not only do these methods make more efficient use of highly trained personnel; they may also improve turnaround time and assay reproducibility.

The sensitivity of this assay across different viral serotypes was determined through extensive testing of viral reference strains. The latter were assayed in serial dilution, as pure suspensions of viral particles, as lysates of viral culture, and spiked into a human lymphocyte cell line suspension, in order to

simulate actual patient samples. As noted above (see Materials and Methods), at least one viral strain from every nonenteric subgroup was tested. In cases for which a given viral subgroup had greater sequence heterogeneity, more serotypes were tested. For instance, the three serotypes in subgenus A have only a 48 to 69% homology in their DNA sequences and were tested individually in the experiment. The nucleotide sequences of all 10 serotypes in viral subgroup B are identical to one of the two probe sequences used to target this group. Therefore, only serotypes 7 and 11 in subgenus B were tested in these experiments. Other clinically prevalent serotypes, including all subgroup C serotypes (serotypes 1, 2, 5, and 6) and five subgroup D serotypes (serotypes 9, 10, 17, 28, and 37), were also tested by the new assay. The reliability of this assay was also evaluated by monitoring all reactions for PCR inhibition. This was done by use of exogenously added internal control reagents, as well as by spiking negative patient samples with known quantities of AdV. No evidence of inhibition was demonstrated in any of the specimens tested.

The development of an assay for detection and quantitation of a virus with such a high degree of genetic heterogeneity presented a unique diagnostic and developmental challenge. The resultant test made use of an unusually high number of degenerate primers and probes in order to detect all of the necessary viral genotypes. An extensive validation effort was undertaken, not only to ensure amplification of all targeted viral serotypes but also to demonstrate accurate quantitative results across all serotypes, such that serotype identification was not necessary for quantitation. The techniques employed in this situation hold promise for the development of other highly multiplexed diagnostic assays. In the specific case of AdV, the implementation of this test has allowed the routine and rapid screening of immunosuppressed pediatric patients for the presence of AdV infection. In the future, it promises to allow further study of the association of viral load with clinical course and prognosis and may serve as a valuable tool in the clinical management of these patients.

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