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Rapid and Quantitative Detection of Human Adenovirus DNA by Real-Time PCR

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Rapid diagnosis of human adenovirus (HAdV) infections was achieved by PCR in the recent years. However, conventional PCR has the risk of carry-over contamination due to open handling with its products, and results are only qualitative. Therefore, a quantitative ''real-time'' PCR with consensus primer and probe (dual fluorescence labelled, ''TaqMan'') sequences for a conserved region of the hexon gene was designed and evaluated. Real-time PCR detected all 51 HAdV prototypes. Sensitivity of the assay was $\leq$15 copies/run and the linear range of quantitation 1.5×10^1 to 1.5×10^8 copies/run. TaqMan PCR gave identical results compared to an established conventional one-step PCR protocol in 218 (38 positive and 180 negative) of 234 clinical samples including blood, serum, eye swabs, and feces, and had divergent results in 16 samples (15 positive only in TaqMan PCR, all with low copy numbers, and one positive only in conventional PCR), indicating a higher sensitivity of TaqMan PCR. Adenovirus viremia was detected by TaqMan PCR in 4 of 27 (14.8%) paediatric and 8 of 93 (8.6%) adult stem cell transplant recipients but only in 5 of 306 healthy controls (blood donors, 1.6%). Virus loads of pediatric patients (median 1.7×10^5) were significantly higher than in adult patients (median 2.3×10^3) and than in controls (all samples $\leq 1.7 \times 10^3$ copies/ml). A few immunosuppressed children had very high virus loads (up to 1.1×10^{10} copies/ml), which were associated with symptoms of disseminated disease. In conclusion, real-time PCR is a sensitive and quantitative procedure for the detection of adenovirus infections. ***J. Med. Virol. 70:228–239, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: immunosuppression; disseminated infection; virus load; Adenoviridae; polymerase chain reaction

INTRODUCTION

The six species of Human Adenoviruses (HAdV, Genus *Mastadenovirus*, Family Adenoviridae) with their 51 types are associated with a variety of diseases affecting all organ systems [Wadell et al., 1999]. For example, acute respiratory diseases in small children are often caused by the types HAdV-1, -2, and -5 of the species HAdV-C, and these viruses persist as asymptomatic infections of the adenoids for months and years with occasional virus shedding. Severe pneumonia in young military recruits is caused by the types HAdV-4 (species HAdV-E) and HAdV-7 (species HAdV-B). The latter two types are also frequent causative agents of pharyngoconjunctival fever (PCF), as is HAdV-3 (species HAdV-B), which is also associated with meningitis. Epidemic keratoconjunctivitis (EKC), a more severe and highly contagious eye infection, is predominantly caused by HAdV-8, -19, and -37 (species HAdV-D). HAdV-19 and -37 are also sexually transmitted and associated with ulcerative genital lesions, cervicitis, and urethritis. Cystitis is caused by HAdV-11, -21, -34, and -35 (species HAdV-B), and these types tend to persist as asymptomatic infections of the urinary tract. HAdV-40 and HAdV-41 (species HAdV-F) are considered to be second to rotaviruses as a cause of gastroenteritis in young children. HAdV-31 (species HAdV-A) has been isolated from infants with gastroenteritis in small outbreaks.

Recently, several studies have demonstrated the clinical significance of life-threatening HAdV infections in immunocompromised hosts, for example, hematopoetic stem cell transplant recipients [Hierholzer, 1992; Echavarria et al., 1999; Howard et al., 1999]. In immunodeficient hosts, HAdV infections are frequently disseminated as a "sepsis" and are associated with one or several organ manifestations as pneumonia, hepatitis, meningitis, diarrhea, rash, and cystitis. Considering the HAdV types (HAdV-1, -2, -5, -11, -34, and -35) frequently detected in immunosuppressed bone marrow transplant recipients, it seems probable that adenovirus disease

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starts by generalisation of a persistent asymptomatic ("latent") infection of the adenoids or the urogenital tract.

Virus isolation is the gold standard for diagnosis of HAdV infections and permits subsequent typing of the isolate. However, it may take up to 3 weeks until a cytopathic effect (CPE) develops, and some HAdV types are fastidious with slow and ineffective growth in culture or require special cell lines such as 293 Graham cells for isolation. For these reasons, several groups have developed PCR protocols for the detection of HAdV in clinical samples [Allard et al., 1990; Pring-Akerblom and Adrian, 1994; Echavarria et al., 1998]. Although PCR was a major improvement for the rapid and reliable diagnosis of localised HAdV infections, detection of adenovirus DNA in blood samples of immunosuppressed patients has only a low predictive value for disseminated adenovirus disease as occasional HAdV DNA-emias are also observed in healthy, persistently infected individuals [Horvath et al., 1986; Carrigan, 1997; Flomenberg et al., 1997]. Quantitation of virus load in blood samples may solve this diagnostic problem [Lankester et al., 2002]. Real-time detection of PCR amplicons during thermal cycling by hybridisation with a double fluorescence labelled probe ("TaqMan" principle) which is cleaved by the 5′-3′ exonuclease activity of Taq DNA polymerase permits simple and reliable quantitation of viral nucleic acids as the number of amplification cycles required for increase of fluorescence over a threshold level is correlated with the template concentration [Holland et al., 1991; Kawai et al., 1999; Ryncarz et al., 1999; Weinberger et al., 2000; Stamey et al., 2001]. Although the TaqMan principle was adapted successfully for the diagnostic detection of various human pathogenic viruses by PCR [Morris et al., 1996; Hawrami and Breuer, 1999; Kawai et al., 1999; Laue et al., 1999; Mercier et al., 1999; Ryncarz et al., 1999; Schweiger et al., 2000; Weinberger et al., 2000; Stamey et al., 2001], the "generic" detection of all six HAdV species has not yet been described, probably due to the high sequence diversity of HAdVs, which makes it difficult to find a well-conserved sequence for probe hybridisation flanked by appropriately well-conserved sequences for primer binding. The application of the TaqMan principle is described for the generic detection of all 51 HAdV types and the evaluation of this method for diagnostic purposes, including the quantitative detection of HAdV in blood samples of immunosuppressed patients, is reported.

MATERIALS AND METHODS

HAdV Quantitation Standard and Panel of Viruses

For preparing a positive control standard, a HAdV-2 PCR amplicon (nt. 18856–19137 of the HAdV-2 sequence) was cloned in a pGEM-T Easy plasmid vector (Promega, Madison, WI). Plasmid DNA was purified from *E. coli* using the Nucleobond 100 kit (Macherey and Nagel, Germany), and it was confirmed by sequencing that the cloned HAdV-2 sequence was identical to the HAdV-2 prototype Genbank sequence (number J01917). Concentration of the plasmid was determined by photometry at 260 nm and calculated as genome equivalents (copies)/ml as the molecular weight of the plasmid was known. A serial dilution of the HAdV quantitation standard plasmid was stored at −20°C in aliquots to avoid repeated freeze/thaw cycles. For testing a panel of HAdV types, A549 cells (Graham 293 cells in case of HAdV-40 and HAdV-41) were infected with HAdV prototype strains. At about 50% CPE, cells were freeze-thawed and DNA was extracted from 200 μl of the lysate with the Qiagen Blood kit (Qiagen, Hilden, Germany). Prototype virus stocks (types HAdV-1, to -21, -23, -25, -27, -28, -30 to -41, -43) of the German national reference laboratory and of the American Type Culture Collection (Manassas, VA) (HAdV-3, -5, -7, -12, -18, -22, -24, -26, -29, -30, -35, -36, -42, -44 to -49 and the proposed types -50 and -51) were included in the panel (for some HAdV types, prototype strains from both origins were tested). HAdV-2, -3, -4, -5, -7, -11, -21, -31, -34, -41, and -48 were quantified as $TCID_{50}$ (50% tissue culture infective dose)/ml and tested by PCR in serial dilutions to evaluate the sensitivity of detection and precision of quantitation for HAdV types with varying numbers of mismatches in the binding sites of the primers and the probe.

Clinical Samples

DNA was extracted from clinical samples as blood, serum, feces, sputum, urine, eye swabs, and nasopharyngeal swabs with the Qiagen Blood kit (Qiagen, Hilden, Germany). In case of liquid samples, 200 μl sample was used according to the manufacturer's instructions. Urine samples with a volume >200 μl were concentrated by centrifugation through Ultrafree-15 Biomax 100 filters (Millipore, Eschborn, Germany) prior to DNA extraction. For the detection of HAdV viremia in immunosuppressed bone marrow transplant recipients and a control group of blood donors the extraction protocol was modified as follows in order to increase the concentration of the purified DNA: elution of DNA was carried out with 50 μl instead of 200 μl of the provided elution buffer (prediluted 1:5 with nuclease free water). DNA was extracted from cerebrospinal fluid samples with the Qiagen viral RNA kit.

Conventional PCR

Two hundred thirty-four patient samples were tested by real-time and conventional PCR protocols. DNA was extracted from the sample as described above, and the eluate that contained DNA was divided for conventional and TaqMan PCR. Conventional adenovirus PCR protocol was performed with the generic primers hex1deg and hex2deg and cycling conditions as described previously [Wadell et al., 1999; Allard et al., 2001]. The protocol was modified slightly by using a ready made master mix with a "Hot-start" DNA polymerase (Qiagen HotStarTaq Master Mix). In addition, all samples with

divergent results between TaqMan and conventional PCR were retested with another conventional PCR protocol using the generic adenovirus primer set Ad-1 and Ad-2 as described by Xu et al. [2000]. After amplification, gel electrophoresis of PCR products (10 μl) was performed on 2% agarose gels, stained with ethidium bromide, and visualised by UV illumination.

Molecular Typing of HAdV

A multiplex PCR that amplified the fiber gene region was carried out with HAdV DNA positive samples to achieve identification of the HAdV species [Xu et al., 2000]. Amplicons of conventional PCRs were sequenced directly with rhodamine-labelled dideoxynucleotide chain terminators (DNA Sequencing Kit, ABI, Foster City, CA) on an ABI-Prism 310 automatic sequencer. Both conventional PCR protocols amplify regions of the hexon gene [Xu et al., 2000; Allard et al., 2001]. The sequences obtained permit identification of the HAdV type in some cases using BLAST and FASTA programs (criteria: sequence homology >99% and next, less homologous databank sequence <97% homology), if sufficient sequence data is available in the Genbank database. As hexon regions of several HAdV-D types were not yet sequenced, sequencing does not permit identification of these HAdV types, but by multiple alignments with databank sequences and clustering identification is easily achieved at the species level.

TaqMan PCR

Taqman PCR was carried out with help of the LightCycler (LC, Roche Diagnostics, Mannheim, Germany) in sealed glass capillaries with a total reaction volume of 20 μl. The FastStart Hybridization Kit (Roche Diagnostics) was used to prepare a PCR master mix. Adenovirus specific primer sequences were as follows: 5′-GCC-ACG-GTG-GGG-TTT-CTA-AAC-TT-3′, Adenoquant 1 (AQ1) and 5′-GCC-CCA-GTG-GTC-TTA-CAT-GCA-CAT-C-3′, Adenoquant 2 (AQ2). The sequence of the probe was 5′-TGC-ACC-AGA-CCC-GGG-CTC-AGG-TAC-TCC-GA-3′ (Adenoprobe, AP) with FAM labelled as a fluorescent dye on the 5′ end and TAMRA as a fluorescence quencher dye labelled to the 3′ end. All oligonucleotides were synthesized, labelled and purified by Eurogentec (Seraing, Belgium). The probe, the primers, and $MgCl_2$ were added to the master mix to achieve a final concentrations of 0.4 mM, 0.5 mM (each primer), and 3 mM, respectively. Heat labile Uracil-DNA Glycosylase (UNG, 1 U/reaction, Roche, Mannheim, Germany) was added to the master mix; 8 μl of the master mix and 12 μl of the DNA template were added in each capillary. Sealed capillaries were centrifuged in a microcentrifuge and placed into the LC. Reaction conditions were 5 min 35°C for Uracil-DNA-Glycosylase incubation followed by 95°C for 10 min to activate the "hot start" Taq-polymerase. Forty-five cycles that consisted of denaturation at 95°C for 3 sec, annealing at 55°C for 10 sec, and extension at 65°C for 60 sec were performed with a temperature increase of 0.5°C/sec between the annealing and extension step. After initial evaluation of TaqMan PCR, the number of cycles was reduced to 40 for the clinical evaluation of the assay. Fluorescence data was acquired at the end of each extension step in the channel F1 of the LC instrument (acquisition mode "single"). The crossing point (CP), the cycle number corresponding to an increase of the fluorescence over a threshold, was calculated automatically by the lightcycler software (version 3.5c, settings: proportional baseline adjustment, threshold = baseline +6 SD of baseline, set point calculus with two points). After the final cycle, the tubes were cooled down to 30°C and disposed without opening the capillaries.

HAdV Viremias in Immunosuppressed Bone Marrow Transplant Recipients and Healthy Controls

Frequency of HAdV DNA detection and virus load was compared by using whole blood (EDTA blood) samples that were sent in from three groups: (I) adult bone marrow recipients (421 samples of 93 patients), (II) pediatric bone marrow recipients (117 samples of 27 patients), and (III) healthy adults as a control group (306 samples of 306 healthy donors). This control group consisted of adults who donated blood during the study period (February 2001 to July 2002) at the local blood bank. In the two patient groups, all EDTA blood samples were included that were available to us during the study period (February 2001 to July 2002) and originated from immunosuppressed bone marrow transplant recipients (allogeneic and autologous). In some of these patients a complete follow-up for several weeks after BMT was possible; in other patients only a few samples or a single sample immediately after BMT or before leaving the BMT ward were available.

Statistical Analysis

The frequencies of HAdV viremia in bone marrow transplant recipient groups (93 adults and 27 children) were compared with the control group (306 blood donors) using Fisher's exact test (two-sided). Differences in virus loads of these groups were tested using the Mann-Whitney test (two-sided). This nonparametric test was used because on nonequal variances and nonequal sample sizes of groups.

RESULTS

Design of Primers and Probe

Oligonucleotide primers were designed to achieve DNA amplification of all 51 types of the genus HAdV. As nucleic acid sequences of human adenoviruses are highly divergent, all five completely sequenced human adenoviruses, type 2 (species HAdV-C, Genbank #J01917), type 5 (species HAdV-C, #M73260), type 12 (species HAdV-A, #X73487), type 17 (species HAdV-D, #AF108105), and type 40 (species HAdV-F, #L19443), were aligned using the clustalX software (version 1.8) [Thompson et al., 1994]. Several highly conserved

regions, which may allow annealing not only of the primers but also of the labelled probe, were found in the hexon gene. As additional hexon sequence data of several HAdV types are available, a multiple alignment of the hexon gene including sequences of all 6 HAdV species was generated and used for primer design (Fig. 1). Amplification primers were selected according to the guidelines set up for Taqman PCR [N.N.], spanning a third highly conserved region that served as a putative binding site for the probe. In spite of the selection of conserved regions, there are minor sequence diversities in the binding sites of the primers. Consensus primer sequences were designed to balance the maximum number of mismatches by calculating the melting temperatures for the interaction of both primers with each adenovirus sequence with help of the Metcalc software (Fig. 1) [Schütz and von Ahsen, 1999]. Successful amplification of all 51 HAdV prototypes (including the proposed types 50 and 51) using the primers AQ1 and AQ2 in a conventional PCR indicated that the primer set was capable to amplify all human adenoviruses (data not shown). Compared to conventional PCR, real-time detection of PCR amplicons with a Taqman probe requires an almost complete hybridisation of a double fluorescence-labelled probe to achieve digestion of the probe by the nuclease activity of Taq polymerase. The design of the probe followed guidelines set up for Taqman probes [N.N.], and the number of mismatches for binding to each adenovirus sequence of the multiple alignment was minimised similar to the strategy used for the primers (Fig. 1). The melting temperatures of the probe and the primers for hybridisation to each adenovirus sequence were calculated and reaction conditions in real-time PCR were adjusted to permit amplification and detection of probably all human pathogenic adenoviruses. Real-time PCR gave positive results for all prototype strains of the genus HAdV including the isolated, proposed new types HAdV-50 and HAdV-51. CP values (defined as the cycle number with fluorescence increasing over background) were low (<20) for all prototype strains suggesting effective amplification and sensitive detection. Moreover, 17 clinical isolates [HAdV-1 (two isolates), -2 (three isolates), -3 (two isolates), -4 (three isolates), -5 (two isolates), -7 (two isolates), -31, -34, and -41] were tested by TaqMan PCR, and all isolates were positive with low CP values (<20), indicating efficient amplification. Real-time PCR was always negative with 100 ng DNA (hDNA) extracted from cultured human cells (MRC5) or blood of an healthy adult as a template ($n = 38$).

Sensitivity and Dynamic Range of Quantitation

A plasmid DNA containing a partial HAdV 2 hexon sequence was serially diluted and used as a template for real-time PCR (1.5×10^8 to 1.5×10^{-1} HAdV 2 genome equivalents (copies)/run) in ten repeated runs to establish the sensitivity of TaqMan PCR. As few as 1.5×10^1 copies were reliably detected by real-time PCR ($n = 10$), whereas 1.5×10^0 copies were only occasionally detected (four of ten runs) and higher dilutions were negative, as were negative controls containing only distilled water (Table I). The serial dilution of plasmid DNA was also used for determining the dynamic range of quantitation. Regression analysis of the crossing points vs. log HAdV DNA concentration resulted in a very high correlation coefficient (0.99 to 1.0) for the concentration range between 1.5×10^1 to 1.5×10^8 copies HAdV DNA. The resulting regression lines ($n = 10$) had a slope of -3.50 ($SD = 0.075$), indicating that a 10-fold increase of the template (quantitation standard) concentration is directly related to an increase of 3.5 cycles in the CP. Amplification efficiency of the PCR was high with a value of 1.93 (calculated with the formula: efficiency $= 10^{-1/\text{slope}}$, theoretical maximum 2.0 that stands for a doubling of the DNA with each PCR cycle). Intraassay variability (standard deviation of CP values) was low, for example 2.7% for 1.5×10^7 copies/run and 1.1% for 1.5×10^4 copies/run. Interassay variability was slightly higher; Table I gives the average crossing point (CP) and standard deviation (SD) of the CP determined in ten different runs on different days. In each experiment, HAdV DNA concentrations of the plasmid serial dilution were set as a standard and HAdV DNA concentrations of each point were calculated automatically by the LC software (version 3.5c) under the assumption of a semi-logarithmic relation between crossing points and HAdV DNA concentration. Calculated HAdV DNA concentrations and SD of calculated concentrations indicate a dynamic range of HAdV DNA quantitation of at least 6 logs (1.5×10^8 to 1.5×10^2 copies HAdV DNA), whereas with lower template concentrations (for example 1.5×10^1 copies/run) the standard deviation increased and with even lower template concentrations results were only qualitative (positive or negative) and Poisson distributed (Table I). As quantitation of HAdV DNA in clinical samples was the main goal of our study and this goal was achieved with less than 40 amplification cycles (Table I), it was decided to carry out only 40 cycles for the evaluation of the assay with clinical samples, restricting the sensitivity to about 1.5×10^1 copies/run. Spiking of the HAdV-2 DNA serial dilution with human genomic DNA (500 ng/run) did not interfere with the sensitivity and quantitation of HAdV DNA detection as the increase of fluorescence over threshold (CP values) was unchanged by hDNA. In contrast to CP values, end point fluorescence data indicated some negative interference of 500 ng hDNA with amplification of HAdV DNA. Serial dilutions 11 HAdV serotypes representing the six HAdV species were used to determine whether HAdV types with more mismatches to the consensus primer and probe sequences than HAdV-2 were amplified as efficiently as HAdV-2 and quantified precisely using the HAdV-2 plasmid as a quantitation standard (Fig. 2, Table II). For example, the HAdV-3 and HAdV-7 have mismatches comparatively close to the 3′ end of primer AQ1 and HAdV-41 has three mismatches compared to the sequence of primer AQ1 which may result in inefficient amplification (Fig. 1). Nevertheless, the sensitivity of detection of these viruses was in the

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                18861                                                  18911
HADV-2   (C)    TGCCGCAGTG GTCTTACATG CACATCTCGG GCCAGGACGC CTCGGAGTAC CTGAGCCCCG
HADV-3   (B1)   ....C..A.. .G.A...... ......G.C. .A.....T.. T....G.... ..C..T..G.
HADV-4   (E)    ....C..... .G.G...... ......G.C. .A........ T......... .....T..G.
HADV-5   (C)    .......... .......... .......... .......... .......... ..........
HVDV-7   (B1)   ....C..A.. .G.A...... ......G.C. .A.....T.. T......... .....T..G.
HADV-12  (A)    .......... .......... ......G.C. .T.....T.. .......... .....T....
HADV-16  (B1)   ....C..A.. .G.A...... ......G.C. .A.....T.. T......... .....T..G.
HADV-17  (D)    .......... .G.G...... ......G.C. .G........ .......... ........G.
HADV-21  (B1)   ....C..... .G.A...... ......G.C. .A.....T.. T......... .....T..G.
HADV-34  (B2)   ....C..... .G.A...... ......G.C. .A.....T.. T......... .....T..G.
HADV-40  (F)    .......A.. .......... ......G.C. .G........ .......... ........G.
HADV-41  (F)    .......A.. .......... ......G.C. .G........ .........T ........G.
HADV-48  (D)    .......... .G.G...... ......G.C. .G........ .......... ........G.
consensus       ...C..... .......... ..... (=AQ2)          ..........  ........G.

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                18921                                                  18971
HADV-2   (C)    GGCTGGTGCA GTTTGCCCGC GCCACCGAGA CGTACTTCAG CCTGAATAAC AAGTTTAGAA
HADV-3   (B1)   .T........ ...C.....T ..A..A..C. .C........ TA..GGG... ..A.......
HADV-4   (E)    .T........ ...C...... .....A..C. .C........ T...GGG... ........G.
HADV-5   (C)    .......... .......... .......... .......... .......... ..........
HADV-7   (B1)   .T........ ...C.....T ..A..A..C. .C........ TA..GGG... ..A.......
HADV-12  (A)    .T........ A..C...... .....G..C. .C.......C ....GGA... ..........
HADV-16  (B1)   .T........ ...C.....T ..A..A..C. .C........ TA..GGG... ..........
HADV-17  (D)    .T........ .......... ........C. .......... ....GGC... ........G.
HADV-21  (B1)   .T........ ...C...... .....A..C. .C.......A T...GGG... ........G.
HADV-34  (B2)   .T........ ...C...... .....A..C. .C.......A T..TGGA..T ..........
HADV-40  (F)    .C........ ...C.....T ........T. .C........ ....GGG... .....C....
HADV-41  (F)    .C........ A......... ........T. .......... ....GGG... .....C....
HADV-48  (D)    .T........ .......... ........C. .......... ....GGC... ........G.
consensus       .T........ (=AP)                                       ..........

                ....|....| ....|....|   melting temperature (°C)
                18981                  AQ2    AP    AQ1
HADV-2   (C)    ACCCCACGGT GGCACCTACG   62.5   71.9  61.2
HADV-3   (B1)   .......A.. ...G..C..C   54.6   71.3  54.4
HADV-4   (E)    .......... ...G.....C   60.5   76.1  54.6
HADV-5   (C)    .......... ...G......   62.5   71.9  61.2
HADV-7   (B1)   .......A.. ...G..C..C   54.6   76.1  54.4
HADV-12  (A)    .......C.. ...T..C..C   62.5   68.9  67.5
HADV-16  (B1)   .......A.. ...G..C..C   54.6   76.1  61.1
HADV-17  (D)    .......... ...C..G..C   50.6   79.0  57.8
HADV-21  (B1)   .......C.. ...G..C..C   60.4   76.1  64.9
HADV-34  (B2)   .T.....C.. A..G..G..C   60.4   76.1  54.6
HADV-40  (F)    .......C.. ...T..C..C   57.3   76.1  63.8
HADV-41  (F)    .T.....T.. ...T..G..C   57.3   73.1  51.4
HADV-48  (D)    .......... ...C..G..C   50.6   79.0  57.1
consensus       .......C.. ... (=AQ1)
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Fig. 1. Multiple alignment of partial hexon sequences of various HAdV-types and the consensus sequences of the TaqMan PCR primers (AQ1 and AQ2) and the probe AP. Melting temperatures were calculated for the binding of AQ1, AQ2, and AP to each sequence considering the mismatches between the consensus sequence and each HAdV sequence. Base numbering according to the HAdV-2 sequence. Accession numbers are HAdV-2 (NC 001405), HAdV-3 (X76549), HAdV-4 (AF06062), HAdV-5 (NC 00146), HAdV-7 (Z48571), HAdV-12 (AF065065), HAdV-16 (X74662), HAdV-17 (AF108105), HAdV-21 (AY008279), HAdV-34 (AB052911), HAdV-40 (L19443), and HAdV-41 (M21163).

TABLE I. Interassay Variability of HAdV TaqMan PCR Determined in Different Runs on Different Days

Genome equivalents	Proportion positive	Crossing point (mean)	SD %	Calculated concentration (mean)	SD %
1.5×10^8	10/10	15.47	4.30	1.91×10^8	15.66
1.5×10^7	10/10	19.27	4.09	1.56×10^7	13.88
1.5×10^6	10/10	22.96	3.07	1.39×10^6	15.58
1.5×10^5	10/10	26.59	2.27	1.26×10^5	11.25
1.5×10^4	10/10	29.93	2.03	1.47×10^4	32.73
1.5×10^3	10/10	33.37	1.77	1.49×10^3	28.13
1.5×10^2	10/10	37.23	2.03	1.22×10^2	35.13
1.5×10^1	10/10	39.63	2.97	2.43×10^1	49.95
1.5×10^0	4/10	>41	nd*	nd*	nd*
1.5×10^{-1}	0/10	nd*	nd*	nd*	nd*

*nd, not determined.

same range as for HAdV-2, and quantitation of all viruses using the HAdV-2 plasmid standard varied less than 1 log (Table II). Slopes (CP vs. log $TCID_{50}$ concentration) were also similar, indicating almost equally efficient amplification of different HAdV types (Table II).

Evaluation of HAdV TaqMan PCR in Comparison to Conventional PCR

Two hundred thirty-four clinical specimens [58 EDTA-blood, 60 serum and plasma, 21 throat washes, 5 combined nasopharynx swabs, 17 eye swabs, 26 cerebrospinal fluid, 22 fecal samples, 12 bronchoalveolar lavage and tracheal aspirates, and 13 other materials (as pericardial, pleural, and peritoneal fluids, urine), and biopsies from lymph nodes and bowel] were tested for HAdV DNA both by TaqMan PCR and an established conventional diagnostic PCR protocol. Specimens had been collected either from patients who were admitted to the Hannover Medical School clinical centre with suspected adenovirus infection or were sent in from all over Germany to the national adenovirus laboratory for diagnostic purposes. After extraction of DNA, samples were divided into aliquots that were used for TaqMan PCR, conventional PCR, and in case of positive samples also for identification of HAdV species by multiplex PCR. In 218 samples, both PCRs had a concordant result (38 positive samples and 180 negative samples). Sixteen samples had divergent results between both assays. In 15 of these samples real-time PCR was positive with high CP values (CP <37, which equals approximately <150 copies HAdV DNA/run), whereas conventional PCR was negative, indicating a higher sensitivity of TaqMan PCR compared to the conventional PCR protocol. Only one sample (EDTA-blood) was positive in the conventional PCR protocol (faint band on the agarose gel) but negative by real-time PCR. This result was confirmed by multiplex PCR and the virus turned out to be species HAdV-D. Of the 57 other EDTA blood samples, 54 had concordant results (4 positive and 50 negative). Concentrations detected by TaqMan PCR were low in these 4 cases (3.3×10^2 to 8.4×10^2 copies/run equivalent to 6.6×10^3 to 1.6×10^4 copies/ml blood) and bands of conventional PCR amplicons were faint; thus direct sequencing was not successful. In one of these four samples, multiplex PCR was positive, indicating a species HAdV-D. In 3 EDTA blood samples, only the TaqMan PCR gave a positive result indicating low copy viremia (1×10^3, 1.1×10^3, and 2.2×10^3 copies/ml blood) and conventional PCR and multiplex PCR were negative. Sixty serum and plasma samples were tested, 57 had a concordant result (2 positive and 55 negative), and 3 were positive only with the TaqMan assay. One of the concordantly positive samples had a virus load of 4.5×10^3 copies/run (about 9.0×10^4 copies/ml blood); this sample originated from an immunosuppressed bone marrow transplanted child ("patient A") described in detail later. All other positive serum and plasma samples had low virus concentrations (3.6×10^2, 3.8×10^2, and 4.8×10^2 copies/ml serum), including the other serum sample concordantly positive in both assays (originating from an immunocompetent male infant suffering from conjunctivitis and myocarditis). Twenty-four of 26 CSF samples had concordant results in both PCR protocols (1 positive with 1.6×10^4 copies/ml CSF, 23 negative). Two CSF samples were only positive by TaqMan PCR; however virus loads were low (8.1×10^2 and 2.5×10^3 copies/ml CSF). These patients suffered from aseptic meningitis and meningo-encephalitis, and no other infectious agent was identified in these samples (HSV, VZV, CMV, and enterovirus tested by PCR; bacterial cultures were all negative). All 21 throat wash samples had concordant results (8 positive and 13 negative). Virus loads in concordant throat wash samples were about 3.3×10^2 to 3.2×10^5 copies/run. Because the quality of throat wash samples varies widely, quantitation is not feasible and results were not calculated as copies/ml. In five of these samples typing was achieved by nucleic acid sequencing (HAdV-3, HAdV-4, and HAdV-5 in three samples). All nasopharynx swabs had concordant results (1 positive and 4 negative). The positive sample had a high virus DNA load ($>1.5 \times 10^8$ copies/run), and was typed as HAdV-3 by nucleic acid sequencing. Absolute quantitation of HAdV copies in swabs is not a sensible strategy as the material is not homogenous and varies widely. Fourteen of 17 eye swabs had a concordant result (9 positive and

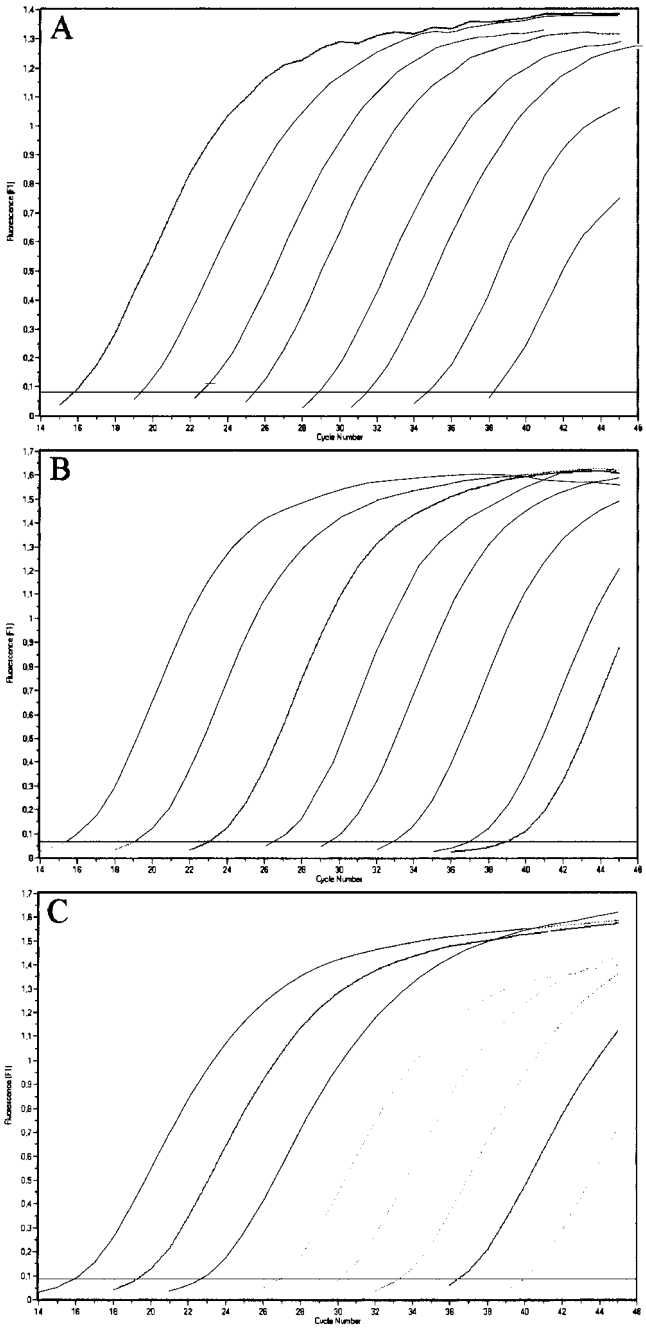

Fig. 2. Comparison of amplification plots of 10-fold serial dilutions of (**A**) the quantitation standard plasmid DNA (HAdV-2 sequence, 1.5×10^8 copies/run, approximately equivalent to 3×10^9 copies/ml of a virus stock considering the DNA extraction procedure used for virus stocks) and (**B**) DNA extracted from a HAdV-2 virus stock with HAdV-2 (4.3×10^8 $TCID_{50}$/ml, approximately equivalent to 4.3×10^9 copies/ml considering the usual particle/$TCID_{50}$ ratio) and (**C**) DNA extracted from a HAdV-3 virus stock (5×10^8 $TCID_{50}$/ml, approximately equivalent to 5×10^9 copies/ml). Horizontal line indicates threshold of fluorescence.

5 negative). In three samples with a high virus load (about 6.1 × 104, 1.1 × 106, and 5.5 × 106 copies/run), HAdV-37 was typed by nucleic acid sequencing. In another sample with a high virus DNA concentration (1.2×10^5 copies/run), identification of the HAdV type by BLAST and FASTA alignments of the nucleic acid sequence (Genbank #AJ 344624) as a homologous sequence was not in the database. However, by multiple alignments and clustering, the sequence was identified as a species HAdV-D sequence. This result was confirmed by multiplex PCR. In two conjunctival swab samples with lower virus DNA concentrations (about 3.7×10^4 and 1.8×10^2 copies/run), HAdV-7 was found, and in two other samples with concordant positive results typing failed. Three of 17 eye swab samples were primarily positive by TaqMan PCR only (3.1×10^2, 1.5×10^2, and 3.4×10^1 copies/run); however retesting with another generic primer pair in a conventional PCR protocol [Xu et al., 2000] gave positive results and nucleic acid sequencing of the amplicons demonstrated HAdV-7. Twenty-two fecal samples were tested in parallel by TaqMan PCR and conventional PCR giving identical results in 21 samples (10 positive and 11 negative). Quantitative values were not calculated per g fecal sample as feces is not a homogenous material. Three of the positive samples had very high virus loads with crossing points <15, indicating virus loads $>1.5 \times 10^8$ copies/run and HAdV-40, HAdV-41 and HAdV-1 (sample of immunosuppressed patient "A") were identified by nucleic acid sequencing. HAdV-41 was also found in another sample with a high virus load of 7.6×10^5 copies/run, whereas virus loads in two faecal samples with HAdV-7 sequences were lower (1.2×10^4 and 2.6×10^4 copies/run). In four fecal samples with concordantly positive results but lower virus loads (4.2×10^2 to 2.6×10^4 copies/run), we were unable to type the virus. One fecal sample had low HAdV DNA loads (8.6×10^1 copies/run) by TaqMan PCR and was negative by conventional PCR. Ten of 12 bronchoalveolar lavage samples had concordant results (2 positive and 8 negative) but virus loads were low in positive samples ($<1 \times 10^3$ copies/run); one of these samples contained HAdV-D. Two other bronchoalveolar lavage samples were only positive by TaqMan PCR, and HAdV DNA concentrations were very low. Eleven of 13 samples from various other sites were concordantly negative by both PCR assays. A lymph node biopsy was positive by both assays with a DNA concentration of 1.7×10^3 copies/run and identified as HAdV-D by multiplex PCR. Moreover, a peritoneal effusion sample was only positive by TaqMan PCR with a low HAdV DNA concentration.

Frequency and DNA Load of HAdV Viremias in Immunosuppressed Bone Marrow Transplant Recipients

HAdV viremia was investigated comparatively with EDTA blood samples of in three groups of patients: adult bone marrow recipients (421 samples of 93 patients), pediatric bone marrow recipients (117 samples of 27 patients), and healthy blood donors as a control group (306 samples of 306 donors). For a direct comparison of the frequency of HAdV viremias in immunosuppressed adult bone marrow transplant recipients with the control group (5 of 306 blood donor samples positive,

TABLE II. Quantitation of Various HAdV Stock Virus Solutions Representing the Six Species of Human Adenoviruses*

Type	Species	Sensitivity ($TCID_{50}$/ml)	Quantitation (copies/$TCID_{50}$)	Amplification efficiency (slope)
2	C	4.3×10^1	38.8	1.93 (−3.50)
3	B (B1)	5.0×10^1	30.8	1.95 (−3.46)
4	E	6.3×10^1	10.8	1.87 (−3.68)
5	C	1.5×10^0	20.8	1.84 (−3.78)
7	B (B1)	6.3×10^0	17.2	2.0 (−3.23)
11	B (B2)	7.5×10^0	13.0	1.98 (−3.36)
21	B (B1)	6.3×10^1	12.0	1.98 (−3.43)
31	A	6.3×10^0	138.0	1.98 (−3.46)
34	B (B2)	7.5×10^0	90.0	1.98 (−3.65)
41	F	3.2×10^1	11.4	1.98 (−3.82)
48	D	2.7×10^0	20.4	1.93 (−3.49)

*Approximate sensitivity ($TCID_{50}$/ml) was determined by serial dilution of the HAdV stock virus. Quantitation of each sample was performed using the HAdV-2 plasmid standard, and a ratio of the copy number determined by PCR per $TCID_{50}$ was calculated. Amplification efficiency (x-fold increase of DNA/PCR cycle) was calculated using the slope value as described in Material and Methods.

1.6%), only the first sample obtained of each patient was evaluated, with 8 of 93 samples being positive (8.6%, $P = 0.0030$, Fisher's exact test, two-tailed). Twenty-eight of 93 adult patients were HAdV positive in at least one sample (with 1 to 12 samples/patient available). If all samples of adult bone marrow recipients (40 of 421 samples positive, 9.5%) were compared with the blood donor samples, an even more clear difference was found ($P < 0.0001$, Fisher's exact test). However, the latter analysis of the data may be biased as there may have been more samples available of bone marrow transplant recipients who had clinical complications than from those who did well. HAdV viremia was also observed more frequently in pediatric bone marrow recipients compared to the adult blood donor control group; 4 of 27 first samples were positive (14.8%, $P = 0.0033$, Fisher's exact test). Eight of 27 pediatric patients were HAdV positive in at least one sample (with 1 to 14 samples/patient available adding up to 117 samples). Pediatric samples were significantly more often positive (21 of a total of 117, 18.8%) compared to the adult blood donor control group ($P < 0.0001$), and compared to all samples of adult bone marrow transplant recipients ($P = 0.0137$, Fisher's exact test).

The highest viremia observed in a healthy, nonimmunosuppressed blood donor of the control group was 1.7×10^3 copies/ml blood (Fig. 3). In adult bone marrow transplant recipients the median virus load was 2.3×10^3 copies/ml blood (range 3×10^2 to 5.8×10^5, mean $5{,}0 \times 10^4$, SD $= 1.4 \times 10^5$). Considering only the first sample of each patient, the median virus load was 2.0×10^3 copies/ml blood (range 6.5×10^2 to 4.6×10^4, mean 7.9×10^3, SD $= 1.5 \times 10^4$). Virus loads of pediatric bone marrow transplant recipients (median 1.7×10^5 copies/ml blood, range 4×10^2 to 1.1×10^{10}, mean 1.2×10^9, SD $= 3.9 \times 10^9$) were significantly higher compared to blood donors ($P = 0.009$, Mann-Whitney test) and adult bone marrow recipients ($P < 0.001$). Two pediatric (A and B) and four adult bone marrow transplant recipients (C, D, E, F) presented with high virus loads at least one magnitude higher than observed in the control group ($>2 \times 10^4$ copies/ml blood). Patient A was a 5-year-old male child who had been liver transplanted and bone marrow transplanted. On day 41 after bone marrow transplantation, an EDTA blood sample was HAdV positive (5.8×10^3 copies/run) and a serum sample obtained 1 day later was also positive (Fig. 4). At that time, leukocyte counts were normal but the patient suffered from diarrhea and a fecal sample was also positive for HAdV DNA. In the following days, HAdV DNA loads increased as depicted in Figure 4, leukocyte counts dropped, and fever, cholestasis, and an

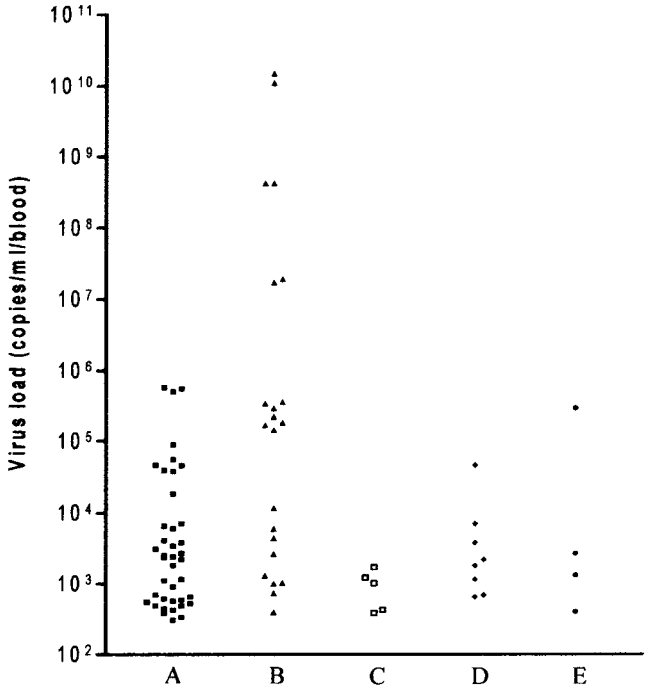

Fig. 3. HAdV DNA loads of immunosuppressed bone marrow transplant recipients compared to a control group of healthy blood donors: (**A**) all samples of adult patients, (**B**) all samples of paediatric patients, (**C**) control group of healthy blood donors, (**D**) only first samples of adult patients, and(**E**) only first samples of paediatric patients.

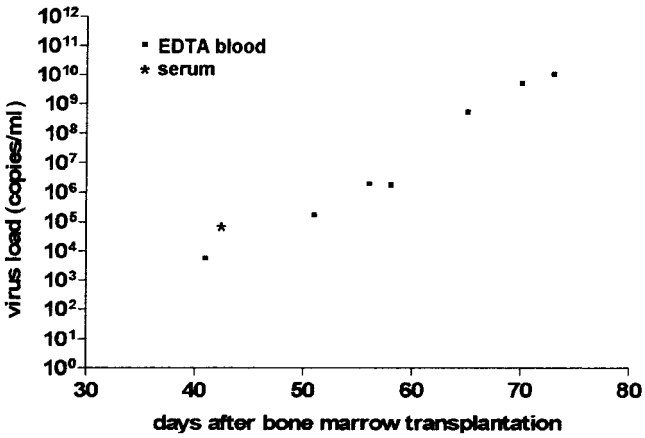

Fig. 4. Time course of HAdV DNA loads of patient "A."

atypical pneumonia developed. HAdV DNA was identified as HAdV-1 by conventional PCR and nucleic acid sequencing from a blood sample and a fecal sample. Patient A died on day 75 post bone marrow transplantation of respiratory insufficiency and circulatory failure. Patient B was a 15-months-old female child who underwent bone marrow transplantation for hemophagocytic lymphohistiocytosis. Six consecutive positive blood samples were obtained with a peak virus load of 3.6×10^5 copies/ml when the patient suffered from fever and diarrhoea. HAdV was also detected in stool samples and typed as HAdV-2. Virus loads decreased subsequently and the patient recovered. The four other patients with comparatively high HAdV loads did not present with any symptoms of localised or disseminated HAdV disease but in adult patient C high virus loads were associated with increasing the immunosuppressive therapy. The first three blood sample obtained over a period of 17 days were HAdV negative or had low virus loads (2.3×10^3 copies/ml blood). Meanwhile, immunosuppressive therapy was increased by adding prednisolone because of a graft vs. host disease of the skin. Six days later, the virus load had increased to 5.0×10^5 copies/ml and two other samples obtained during the following 2 weeks had also high virus loads (5.8×10^5 and 5.5×10^5 copies/ml). From adult patient D, eight samples were received over a period of 28 days, only the fourth sample was HAdV positive with a virus load of 3.9×10^4 copies/ml. A similar pattern was observed in adult patient E; six samples were obtained over a period of 3 weeks with only the last sample positive (4.5×10^4 copies/ml). Adult patient F (nine samples available over a period of 28 days) was occasionally positive (samples 3 and 6 with low virus loads of 9.5×10^2 and 8.3×10^2 copies/ml) and sample 9 with a high virus load, 5.5×10^4 copies/ml.

DISCUSSION

Various PCR protocols for the "generic" detection of HAdV DNA are sensitive and rapid procedures for the diagnosis of HAdV infections [Echavarria et al., 1998; Allard et al., 1992; Hierholzer et al., 1993; Pring-Akerblom and Adrian, 1994]. However, all these protocols include labour intensive and time consuming steps for the detection of PCR amplicons, as for example ethidium bromide-stained gel electrophoresis or hybridisation procedures requiring open handling of PCR products that bears a high risk of template carry over contamination. In contrast, real-time detection of the amplicon using the "TaqMan" principle during thermal cycling avoids these drawbacks [Holland et al., 1991]. However, the "generic" detection of HAdV DNA had not yet been achieved by TaqMan PCR, probably because of the high genetic diversity of the 51 HAdV types [Wadell et al., 1996, 1999]. So far, a real-time protocol for the detection of only a single HAdV type had been published in detail [Ma et al., 2001]. In the present study, real-time detection of HAdV DNA of all 51 HAdV prototypes was achieved by a strategy of constructing consensus sequences that balanced mismatches with various so far sequenced HAdV types achieving similar melting temperatures (Fig. 1) and setting reaction conditions accordingly. Using this approach, HAdV DNA of various types (including HAdV-1, -3, -4, -5, -7, -34, -37, -40, -41, and various viruses of the species HAdV-D) was detected directly in clinical samples (without virus isolation). Simple and rapid detection of HAdV DNA including types 4 and 7 as achieved by TaqMan PCR should also be helpful in potentially life threatening cases of acute respiratory distress in young adults, especially military recruits [CDC, 2001]. A panel of 234 clinical samples was used to determine the sensitivity and specificity of real-time PCR in comparison to an established conventional PCR protocol. Real-time PCR detected HAdV DNA in 53 of 54 positive clinical samples, whereas the conventional PCR protocol detected only 38 of 54 positive samples. All samples positive in real-time PCR but negative in conventional PCR had HAdV DNA concentrations $<10^3$ copies/run, indicating a slightly lower sensitivity of the conventional one step PCR protocol with the same number of cycles (40) as used in real time PCR [Wadell et al., 1999; Allard et al., 2001]. The sensitivity of the conventional PCR can be enhanced by performing an additional nested PCR amplification [Allard et al., 2001]. However, we were afraid of cross-contamination problems, which are inherent to nested PCR protocols, and therefore did not perform nested PCR.

In general, cross-contamination is a serious risk for all sensitive assays that may detect as few as a single copy of target DNA. Contamination by amplicon carry-over seems to be almost impossible by the TaqMan principle itself, which avoids open handling of PCR products. Furthermore, UNG treatment was undertaken and guidelines for avoiding contamination were followed [Kwok and Higuchi, 1989]. However, cross-contamination during DNA extraction and PCR set-up cannot be excluded completely, considering the highly positive feces and eye swab samples containing $>10^8$ copies HAdV DNA template/run. Therefore, a negative control (water) was extracted with each batch of clinical

samples, this extraction control and two additional negative controls (water and hDNA) were amplified with each run. DNA extraction from feces and eye swab samples was performed in separate batches than from blood, plasma, and serum samples. In a single batch of DNA extraction from fecal samples, the extraction control was false positive, as were four of seven fecal samples. Three of these samples and the extraction control had low HAdV concentrations of up to 5×10^2 copies/run, whereas the fourth fecal sample had a very high HAdV concentration ($>1.5 \times 10^8$ copies/run). Nucleic acid sequencing demonstrated identical HAdV-41 sequences in these samples and the extraction control. DNA was extracted from the original fecal samples a second time (only positive the high virus load sample) and run together with the original DNA extraction samples (results as before), demonstrating a cross-contamination event during the DNA extraction procedure. In future, automated DNA extraction may minimise the risk of sample cross contamination.

Quantitative generic detection of HAdV DNA is not yet possible, and thus the virus load dynamics of human adenovirus infections are not known precisely. In this study, it was demonstrated that very high HAdV DNA concentrations were detected in highly contagious adenovirus diseases, for example, epidemic keratoconjunctivitis caused by HAdV-37 and diarrhea caused by HAdV-40 and -41, whereas less severe and contagious eye infections as PCF caused by HAdV-7 were associated with lower HAdV DNA concentrations in eye swabs. True quantitation of HAdV loads by quantitative PCR is feasible for clinical samples as blood, plasma, serum, and CSF, which are homogenous and can be sampled in a highly reproducible manner. Quantitation of HAdV DNA in blood samples is an interesting point considering the fact that HAdV DNA was detected in blood samples of healthy persons with a widely varying frequency. Using a nested PCR method very sensitive for HAdV-C (but less sensitive for HAdV-B2), HAdV DNA was detected in 1 of 73 healthy persons (about 1.4%) [Flomenberg et al., 1997], which is about the same frequency as that observed by TaqMan PCR in this study (1.6%), whereas in a previous study 13 of 17 lymphocyte samples were positive [Horvath et al., 1986]. This study used a Southern blot hybridisation protocol for HAdV-C DNA but the high incidence of HAdV DNA may have been caused by insufficient stringency [Horvath et al., 1986]. In general, HAdV DNA detection in blood samples of healthy persons is related to a latent or persistent infection of lymphoid cells, which can also be persistently infected by HAdV in vitro [Andiman and Miller, 1982; Chu et al., 1992; Flomenberg et al., 1996]. Therefore, the clinical significance of qualitative HAdV DNA detection in blood is difficult to interpret in immunosuppressed individuals who are at risk of a life-threatening disseminated HAdV infection. HAdV DNA quantitation demonstrated now that HAdV DNA loads in healthy immunocompetent individuals are low ($\leq 1.7 \times 10^3$ copies/ml blood), whereas HAdV DNA was observed with significantly higher virus loads in immunosuppressed patients. Six patients had virus loads at least 10-fold higher than the highest HAdV DNA concentration observed in healthy controls. Although not a primary goal of the study, it was investigated whether high HAdV loads were associated with disseminated adenovirus disease, as in other virus infections of immunosuppressed patients, for example, CMV, high DNA loads are associated with disease [Zaia et al., 1997; Ferreira-Gonzalez et al., 1999; Emery et al., 2000]. Symptoms suggestive for a disseminated or localized HAdV infection were observed only in two of these six patients with high virus loads (patient A and B, both children) but not in any other patients. Patient A had also a fatal outcome after presenting with extremely high virus loads ($>1 \times 10^{10}$ copies/ml blood). Similar cases of fatal disseminated HAdV infection associated with very high virus loads were reported recently but the method used for HAdV quantitation was not described in detail [Lankester et al., 2002]. Virus loads of patient A were so high that direct complement activation by HAdV-1 may be suspected as cause of multi-organ failure and septic shock [Cichon et al., 2001]. However, it is not completely clear whether HAdV was the only cause of the fatal outcome because patient A survived high and increasing HAdV loads for at least 3 weeks and suffered also from a generalised candida albicans infection. Therefore, further studies are required on HAdV loads in disseminated HAdV disease and the prognostic significance of high HAdV loads. Previously, the detection of HAdV from different body sites (e.g., in feces, throat washing, urine, and blood) was proposed for the diagnosis of disseminated HAdV disease in immunodeficient patients but this approach seems to be quite awkward [Carrigan 1997; Howard et al., 1999]. More recently, qualitative detection of HAdV DNA in serum was proposed as an indicator of fatal disseminated HAdV infections [Echavarria et al., 2001]. Although patient A was HAdV DNA positive in serum (9×10^4 copies/ml) and suffered from a disseminated disease with a fatal outcome, our data does not support the high predictive value of qualitative HAdV DNA detection in serum in general. For example, two patients were HAdV DNA positive in serum with low copy numbers and did not suffer from any HAdV disease.

There is no antiviral therapy for disseminated HAdV infections, although in vitro activity against HAdV was demonstrated with various antiviral agents. Cidofovir and ribavirin are used occasionally in life-threatening HAdV infections in immunosuppressed patients [Cassano, 1991; Sabroe et al., 1995; Ribaud et al., 1999; Arav-Boger et al., 2000; Miyamura et al., 2000; Bordigoni et al., 2001, Legrand et al., 2001;] but controlled clinical trials have nit been carried out. Moreover, antiviral effects have not been demonstrated in a clinical setting as it should be possible in future by following virus loads with quantitative PCR. Therefore, the quantitative TaqMan PCR for HAdV holds promise of value in clinical studies on the antiviral therapy of life-threatening HAdV infections.

In conclusion, TaqMan PCR protocol permits rapid and quantitative detection of HAdV DNA in clinical samples. Detection of high virus loads in blood holds promise for a simplified and earlier diagnosis of disseminated adenovirus disease in immunosuppressed patients.

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