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Comparison of different approaches to quantitative adenovirus detection in stool specimens of hematopoietic stem cell transplant recipients

K. Kosulin[a], S. Dworzak[a], A. Lawitschka[b], S. Matthes-Leodolter[b], T. Lion[a,c,*]

[a] Children's Cancer Research Institute, Zimmermannplatz 8, 1090 Vienna, Austria
[b] St. Anna Children's Hospital, Kinderspitalgasse 6, 1090 Vienna, Austria
[c] Department of Pediatrics, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria

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ABSTRACT

Background: Adenoviruses almost invariably proliferate in the gastrointestinal tract prior to dissemination, and critical threshold concentrations in stool correlate with the risk of viremia. Monitoring of adenovirus loads in stool may therefore be important for timely initiation of treatment in order to prevent invasive infection.
Objectives: Comparison of a manual DNA extraction kit in combination with a validated in-house PCR assay with automated extraction on the NucliSENS-EasyMAG device coupled with the Adenovirus R-gene kit (bioMérieux) for quantitative adenovirus analysis in stool samples.
Study design: Stool specimens spiked with adenovirus concentrations in a range from 10E2-10E11 copies/g and 32 adenovirus-positive clinical stool specimens from pediatric stem cell transplant recipients were tested along with appropriate negative controls.
Results: Quantitative analysis of viral load in adenovirus-positive stool specimens revealed a median difference of 0.5 logs (range 0.1–2.2) between the detection systems tested and a difference of 0.3 logs (range 0.0–1.7) when the comparison was restricted to the PCR assays only. Spiking experiments showed a detection limit of 10^2–10^3 adenovirus copies/g stool revealing a somewhat higher sensitivity offered by the automated extraction. The dynamic range of accurate quantitative analysis by both systems investigated was between 10^3 and 10^8 virus copies/g.
Conclusions: The differences in quantitative analysis of adenovirus copy numbers between the systems tested were primarily attributable to the DNA extraction method used, while the qPCR assays revealed a high level of concordance. Both systems showed adequate performance for detection and monitoring of adenoviral load in stool specimens.

1. Background

Human adenoviruses (HAdVs) represent a family of genetically diverse pathogens, which are divided into seven species, termed A-G, and a steadily increasing number of currently 70 published types [1–3]. In immunocompromised individuals, the virus can result in invasive infections which are associated with high mortality rates, particularly in children undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT) [4–7]. We and others have shown that the initiation of treatment upon first HAdV detection in blood maybe too late for successful therapy [4,9,10]. However, the onset of invasive adenoviral infections in pediatric transplant recipients is generally preceded by appearance of the virus in feces, and HAdV copy numbers in serial specimens exceeding the threshold of 10^6 copies per gram of stool were shown to correlate with a high risk of invasive infection [4,8,9]. The monitoring of HAdV loads in stool during the post-transplant period provides a basis for the initiation of pre-emptive antiviral treatment to prevent progression to invasive infection and disseminated disease.

Technical challenges of molecular HAdV monitoring in stool specimens include inhibitors affecting the efficiency of PCR-based analysis, and extremely high viral loads (up to 10^{11} viral copies/g)

Abbreviations: HAdV, human adenovirus; allo-HSCT, allogeneic hematopoietic stem cell transplantation; PhHV, phocine herpesvirus; IC2, internal control (Adenovirus R-gene PCR Kit bioMérieux).

* Corresponding author at: Children's Cancer Research Institute, Zimmermannplatz 10, A-1090 Vienna, Austria.
E-mail address: thomas.lion@ccri.at (T. Lion).

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presenting a high risk of carry-over contamination [3,4]. Various manual and automated methods for DNA extraction from stool specimens have been described [11–16]. Automated extraction could provide advantages with regard to uniform performance and possibly decreased probability of contamination. A number of in-house PCR tests for quantitative detection of human adenoviruses have been reported, covering a variable spectrum of HAdV types [11,14,15,17–19]. Of the commercially available PCR-based tests for HAdV detection, only one (Adenovirus R-gene kit, bioMérieux) reportedly covers all types of the virus known to date [3]. The variety of currently used methods for extraction of viral DNA from stool and for qPCR-based analysis of human adenoviruses poses clinically relevant questions regarding the comparability of results. Since specific thresholds of HAdV load in stool appear to correlate with the risk of potentially life-threatening infections in allo-HSCT recipients, it is pertinent to assess the comparability of different approaches to quantitative virus analysis. We have therefore tested a validated assay used for routine HAdV testing in our center based on manual DNA extraction in combination with an in-house PCR test [11,20] in a head-to-head comparison to automated DNA extraction and the Adenovirus R-Gene PCR assay (bioMérieux). We have compared the complete detection systems in accordance with the way they had been validated in order to assess their equal or unequal adequacy for routine clinical application. The main focus of the comparison was HAdV analysis in stool specimens, and the approaches tested were compared for the occurrence of carry-over-contamination, detection limits, amplification efficiency for different HAdV types, and dynamic range of quantitative analysis.

2. Study design

2.1. Patients and specimens

In total, 143 stool samples and select peripheral blood samples derived from routine diagnostic investigation in patients undergoing allo-HSCT at St. Anna Childrensí Hospital, Vienna, Austria, were used for HAdV analysis. Written informed consent was obtained from all patients or their legal representatives for diagnostic testing including viral analyses, and for the use of residual sample material for related research applications at the associated Childrenís Cancer Research Institute (CCRI), Vienna, Austria. In addition to fresh clinical samples, the specimens tested included 41 DNA preparations from HAdV-positive stool samples extracted with the QIAamp DNA Stool Mini Kit (Qiagen) and archived at −80 °C. For spiking experiments with defined virus copy numbers, HAdV-negative clinical samples including eight different stool and two blood specimens were used. Liquid suspensions of stool specimens were prepared prior to DNA extraction to ensure homogeneous sample distribution.

2.2. Viral preparations

Human adenovirus types C2, B3 and A12 (all strains derived from ATCC) were expanded in A549 cells cultivated in DMEM with 5% fetal calf serum and 1% penicillin/streptomycin (Life technologies). The HAdV copy numbers were quantified by the in-house DNA extraction and real-time PCR assays [20] against quantified plasmids containing the adenoviral hexon gene in a pBluescript II KS/SK(+) vector. For spiking experiments addressing carry-over contamination, which required total virus copy numbers exceeding 10^{10}, a stool specimen displaying 10^{12} (HAdV-C2) copies/g was used. For testing of the dynamic range of HAdV quantification by PCR, quantified control plasmids were employed.

2.3. Spiking experiments

Defined HAdV copy numbers were spiked into 200 μl virus-free stool suspensions or peripheral blood (PB) samples for further analysis. For the testing of detection limits, spikes containing 10^2 and 10^3 copies/g of stool were analyzed in ten replicates, and spikes with 10^4 copies/g in duplicates. All analyses with PB samples were performed in pentaplicates at 5×10^2 and 10^3 virus copies/ml. To assess the dynamic range of quantitative analysis, HAdV types A12, B3 and C2 were spiked into stool samples at concentrations ranging from 10^3 to 10^8 (HAdV-A12, B3) and 10^3 to 10^9 (HAdV-C2). For the testing of higher virus titers of up to 10^{11} copies/g stool, plasmids were used for spiking experiments as indicated above.

2.4. Automated and manual DNA extraction

Automated extraction of DNA from stool and blood samples was performed on the Nuclisens easy MAG instrument (bioMérieux) by using the appropriate protocols and reagents specified in the manufacturerís manual. Sample volumes of 200 μl were used for individual DNA extractions, and an internal control (IC2) supplied with the kit was analyzed in parallel for surveillance of the extraction process and the presence of amplification inhibitors. The standard elution volumes on the Nuclisens easy MAG instrument were 50 μl in all instances. Manual DNA extractions were performed with the QIAmp Fast DNA stool Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. The sample size of individual stool suspensions for this extraction procedure was also 200 μl. Manual DNA extraction from PB samples was performed with the QIAmp blood mini Kit (Qiagen GmbH) according to the recommendations of the manufacturer. For the manually processed samples, phocine herpesvirus (PhHV), which had been added at defined copy numbers into each specimen prior to DNA extraction, was used as internal control, and the elution volume of purified DNA was 240 μl in all instances.

2.5. Real-time PCR analysis

The Adenovirus R-gene PCR Kit (bioMérieux) was used according to the manufacturer's instructions. The amplification reactions were performed in a total volume of 25 μl including ten μl of extracted DNA and 15 μl PCR master mix. In addition, quantification standards supplied in the Kit representing 50, 500, 5000, and 50000 virus copies were included in each run. The in-house PCR assay was performed with 6 μl DNA template and 20 μl master mix including dUTP and uracil *N*-glycosylase [UNG] under cycling conditions described previously [20,21]. Internal controls comprising either IC2 for automated extraction or PhHV for manual extractions were included in each assay according to the manufacturer's instructions or as described earlier [21]. The run-to-run variability for the extraction efficiency was in the range of 3.3 ct-values for PhHV analyses, and less than 3 delta ct-values for the IC2 and the IC2W0 (negative control) for the automated Nuclisens easy MAG extraction. The ABI Prism Fast 7500 instrument (Life Technologies) was used for all PCR analyses.

2.6. Statistics

Bland-Altman analyses with 95% limits of agreement were employed for comparison of the methods tested, and the Spearman correlation coefficient was calculated using the GraphPad Prism 5 software.