Detection of small amounts of human adenoviruses in stools: comparison of a new immuno real-time PCR assay with classical tools

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Abstract

The detection of low virus concentrations in biological matrices, especially stool samples, is facing significant limitations as far as common diagnostic methods (enzyme-linked-immunosorbent assay (ELISA) or quantitative real-time PCR (qPCR)) are considered. Here the development of a new immuno real-time PCR (iPCR) is described and its performance in the detection of human adenoviruses (HAdVs) in spiked stools is compared with those of ELISA and qPCR assays. For the iPCR, detection of the sandwich formed by the complexation of capture antibody-antigen-detection antibody was performed by qPCR thanks to the substitution of peroxydase by a chimeric DNA. This modification increased the detection sensitivity 200-fold compared to ELISA. The direct qPCR results revealed that only 0.3–9.5% of the spiked HAdV were detectable, resulting from important losses of DNA occurring at the extraction step. This step was not necessary in the iPCR workflow, avoiding this drawback. The losses of viral particles occurred at the elution step from the stool only. The recovery rate of the iPCR was thus better and ranged between 21 and 54%. As a result, iPCR enabled the detection of lower virus concentrations in stool samples compared to those detected by ELISA and qPCR. The iPCR could be considered as a 'hyper sensitive ELISA' for early detection of HAdV infections, especially in the case of immunocompromised patients after haematopoietic stem cell transplant.

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Introduction

Human adenoviruses (HAdVs) are non-enveloped, icosahedral, double-stranded DNA viruses ranging in size between 80 and 110 nm in diameter. Currently, all described human types are dispatched into 54 types representing seven species (A-G), which belong to the genus *Mastadenovirus* in the *Adenoviridae* family [1]. They are commonly responsible for a wide range of respiratory, gastrointestinal or ophthalmic illnesses. All HAdV types are excreted in very high numbers in faeces of infected people, regardless of the initial infection site [2]. Some HAdVs can establish long-lasting but indiscernible infections. As a result, the infected person sheds viruses unknowingly, and can then serve as a source of infection for other individuals [3]. After recovery from illness, HAdVs, especially the members of the HAdV-C species, may maintain latent persistent infections in the tonsils, the adenoids and other lymphoid tissues [4]. Some types (e.g., 1, 2, 3 and 5) are continuously present in the population. Most people have thus been exposed (primary infection) and at least 90% of the human population are positive to one or more HAdV antibodies [5,6], even though no acute disease has been declared. On the contrary, HAdV infections (primary infections or reactivation of latent viruses) in immunocompromised people tend to become invasive, and the fatality rate may be as high as 50% [7]. After haematopoietic stem cell transplantation, the immune system is weakened

and many viruses are identified as a major risk for patients [8,9]. HAdVs have therefore emerged as life-threatening agents in severely immunocompromised patients, such as haematopoietic stem cell recipients [10].

The main methods used to detect HAdVs in clinical samples (blood and stool) are antigen detection assays, such as enzyme-linked-immunosorbent assay (ELISA), immunofluorescence or immunochromatography tests, or molecular biology assays such as quantitative real-time PCR (qPCR). Immuno-detection methods are of particular interest because they yield results quickly, despite their low sensitivity. They are generally used for screening HAdVs in stool samples or respiratory fluids from infected children, because these kinds of samples are heavily loaded with HAdV particles in the case of infection. The qPCR is considered to be a very sensitive, robust and fast technique. Recent European guidelines for diagnostis and treatment of adenovirus infection in stem cell transplantation (ECIL-4) recommend qPCR for monitoring HAdVs of high-risk patients [11], as previously suggested [12]. Using such a tool, an interest in screening HAdVs in stool samples instead of blood has been highlighted, because the HAdV viral load in stool samples precedes the presence of HAdVs in blood [13,14].

The qPCR workflow requires a preliminary viral DNA isolation. This extraction and purification of nucleic acids is a critical step in the molecular detection of viruses from complex samples such as stools [15], even though most of the extraction kits are well adapted for use in a clinical setting. At this stage, DNA losses can occur, resulting in an under-estimation of HAdV concentration. Moreover, the presence of potential PCR inhibitors found in stool specimens (as lipids, polysaccharides and bile salts) can also perturb the qPCR reaction and lead to false-negative results [16].

An alternative method, named immuno-PCR has been developed during the last decades [17] and is considered to be a promising ultrasensitive diagnostic tool despite its still scarce routine application [18]. This technique combines the specificity of the antibodies with the amplification power and the sensitivity of (q)PCR, without a nucleic acid extraction step. In this context, the aim of this study was (i) to develop and optimize an immuno real-time PCR (iPCR) to detect HAdV particles in stool samples and (ii) to compare the performance of this newly developed assay with the existing routinely used methods, sandwich ELISA and qPCR.

Methods

Adenoviruses

Multiplication of HAdV-2 (Health Protection Agency culture collection NCPV#213) and HAdV-41 (American Type Culture Collection VR-930) was performed by infection of human embryonic kidney cells 293A (R705-07; Life Technologies, Halle, Belgium) as previously described [19]. The concentrations of viral stocks were estimated by a most probable number assay, and the concentration expressed in most probable number of cytopathogenic units per millilitre (MPNCU/mL).

Stool samples and spiking experiments

Stool samples from a 3-year-old child not infected by HAdVs (detection performed with ELISA, qPCR and iPCR) were used for spiking experiments. Stool samples were spiked within a range of 10^1–10^8 MPNCU/450 mg of stool of HAdV-2 and HAdV-41 using a syringe and mixed with a Potter-Elvehjem homogenizer. Elution of viruses was performed by adding 2 mL phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4,2H_2O$; pH = 7.4) and four 2-mm-diameter glass beads. Stools were dissolved in PBS by vortexing for 1 h at 500 rpm at room temperature (RT). Samples were then centrifuged at 3000 *g* for 60 min at 4°C. The collected supernatant was used to perform analysis using the three different detection methods.

Antibodies

The antibody isotypes 8C4 and 1E11 (Hytest, 3AV13) were used as capture and detection antibodies, respectively. The detection antibody 1E11 was biotinylated. Both antibodies were directed against genus-specific hexon antigen. The targeted *Mastadenovirus* genus includes all the human adenovirus types, among others. The specificity of both antibodies was validated through the sandwich ELISA procedure using the human types HAdV-1, -2, -3 -6, -19 and -41, belonging to four distinct human adenovirus species (data not shown).

Sandwich ELISA

Step 1. Fifty microlitres of 8C4 capture antibody at a final concentration of 44 mg/mL in coating buffer (4.3 mg/L NaHCO₃; 5.3 mg/L Na_2CO_3; pH 9.4) were placed in wells of a polypropylene 96-well PCR plate (4346907; Life Technologies) and incubated at 37°C for 1 h. Non-binding antibodies were removed by five washings with 200 μL of PBS-T (PBS, 0.1% Tween-20).

Step 2. One hundred and fifty microlitres of casein 1% (solution in PBS) were placed in wells and incubated at 37°C for 15 min, followed by five washings with PBS-T.

Step 3. Fifty microlitres of sample (or dilutions) were placed in wells (dilutions were performed in casein 1%) and incubated at RT for 1 h, followed by five washings with PBS-T.

Step 4. Fifty microlitres of biotinylated 1E11 detection antibody (final concentration, 11 mg/mL in casein 1%) were

placed in wells and incubated for 1 h at RT. Non-binding antibodies were eliminated by three washings with PBS-T. Three additional washings were performed with PBS containing 1% of casein.

Step 5. Fifty microlitres of streptavidin-conjugated horseradish peroxidase (HRP) (PO397; Dakodiagnostics) at a final concentration of 1/7500 (diluted in casein 1%) were added to wells and incubated for 30 min in the dark at RT, followed by five washings with PBS-T.

Step 6. Quantification of antigens was indirectly measured by activity of HRP. Fifty microlitres of 3,3′,5,5′-tetramethylbenzidine (1-Step™ Ultra TMB-Elisa, Fisher Scientific, Tournai, Belgium) were placed in wells and incubated in dark for 15 min at RT. The reaction was stopped by the addition of 50 μL 1 M H_2SO_4, and the absorbance was read at 450 nm with a Synergy 2 multi-mode microplate reader (Biotek, Bad Friedrichshall, Germany).

Immuno real-time PCR

Steps 1, 2, 3 and 4 were the same as previously described for sandwich ELISA. For steps 5 and 6, the peroxidase was substituted by a 5′-end biotinylated DNA reporter to form a streptavidin-DNA reporter complex, which was then quantified by qPCR (Fig. 1). The DNA reporter design and its detection system were previously described [20]. This DNA molecule is a combination of two DNA fragments from eukaryotic and prokaryotic plasmids.

Step 5. Fifty microlitres of streptavidin-DNA reporter complex (diluted in casein 1%) were placed in wells and incubated for 15 min at RT, followed by five washings with WB and ten washings with DNase/RNase free water.

Step 6. DNA reporter quantification was performed using Mesa Green qPCR MasterMix Plus Low Rox (Eurogentec, Liège, Belgium) with the following final concentrations in 50 μL final volume: 1X MasterMix, 0.3 μM forward and reverse primers, on a 7500 Fast Real-Time PCR System (Life Technologies). Thermal cycling conditions were: initial 5 min at 95°C for activation of Meteor*Taq* polymerase, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, and a final dissociation step. Primer specificity was controlled by the presence of a single peak in the melting curves.

Direct qPCR

DNA was extracted from 140 μL of viral stocks using the QIAamp viral RNA mini kit (Qiagen, Venlo, the Netherlands). From the supernatant of stool samples, viral DNA was extracted by the QIAamp viral RNA mini kit and QIAamp DNA stool mini kit (Qiagen). The quantification of the adenovirus DNA was performed by a previously developed qPCR system using degenerated primers and a TaqMan probe [21]. The upstream and downstream primer sequences were 5′-CWT ACA TGC ACA TCK CSG G-3′ and 5′-CRC GGG CRA AYT GCA CCA G-3′, respectively. The sequence (5′–3′) of the Ad(ACDEF) probe was 6FAM-CCG GGC TCA GGT ACT CCG AGG CGT CCT-BHQ1. The detection of qPCR inhibitors in DNA extract from stools was performed by adding the equivalent of 10^5 copies/reaction of HAdV-2 DNA in extracted DNA from a non-spiked stool sample. The detected concentrations in a stool sample (C_{stool}) were compared with concentrations obtained for the same HAdV-2 DNA concentration in water ($C_{pur\ DNA}$). The potential inhibition was expressed as a percentage and calculated as follows: $100 - [(C_{stool} \times 100)/C_{pur\ DNA}]$.

Definitions of detection and quantification limits

For qPCR, the limit of detection (LOD) was measured as the concentration of DNA that gives a signal significantly different from the negative control.

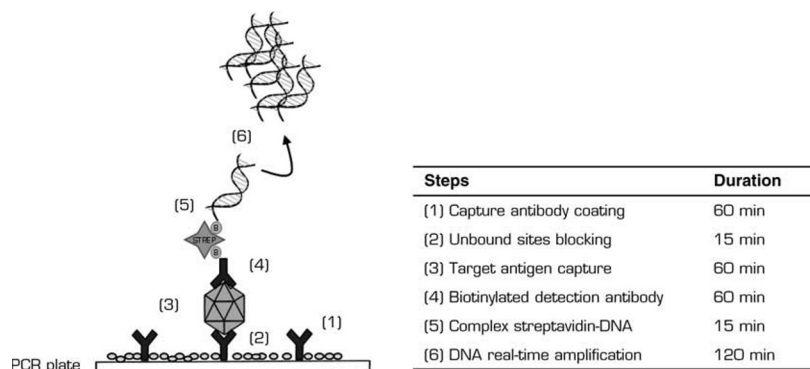For sandwich ELISA, the LOD was defined as the value of the negative control + two standard deviations. The limit of

Steps	Duration
(1) Capture antibody coating	60 min
(2) Unbound sites blocking	15 min
(3) Target antigen capture	60 min
(4) Biotinylated detection antibody	60 min
(5) Complex streptavidin-DNA	15 min
(6) DNA real-time amplification	120 min

FIG. 1. Schematical representation of the iPCR procedure and duration of each step. The general setup of iPCR is similar to that of antigen detection through ELISA. The DNA reporter is amplified by real-time PCR for signal generation. The duration of the whole iPCR workflow is about 6 h, including the required time for all the washing steps.

quantification (LOQ) was defined as the minimum value of the linear dynamic range observed on the standard curve.

For iPCR, the LOD was defined as the lower concentration giving a difference of 2.2 C_t (corresponding to 2 SD) compared with that given for the negative control [20]. The LOQ was defined as the lowest level of DNA detected that provided an acceptable level of precision (i.e. 3/3 replicates amplified with a C_t standard deviation inferior to 0.8 from the mean C_t).

Results

Sensitivities and performances of sandwich ELISA, real-time PCR and iPCR

In order to define the LOD, LOQ and the dynamic range of the different techniques, a range of concentrations between 10 and 10^8 viruses/reaction of both HAdV types (HAdV-41 and HAdV-2) in suspension in sterile milliQ water were analysed by ELISA, qPCR and iPCR. The results are shown in the Table 1. Standard curves obtained for both HAdV-2 and HAdV-41 using the iPCR assay are presented in Fig. 2.

Detection of HAdV in spiked stool samples

For the qPCR analysis, the recovery rate ranged from 0.3 to 9.5% (Fig. 3) and seemed to be proportional to the viral concentration (i.e. recovery rate decreased as the added concentration decreased). Due to this low recovery rate, it was estimated that the lowest detectable virus concentration in a stool sample was about 10^6 particles per gram of stool (Table 1). Similar results were obtained using the QIAamp DNA stool kit (Qiagen) for the DNA extraction (yields of detection ranging from 0.2 to 5%).

Using sandwich ELISA, the losses of viral signals were less important than the ones occurring during the qPCR assay. Considering that all values below this threshold were negative,

the lower detected concentration of HAdV-2 was 2.5×10^5 MPNCU/reaction, corresponding to an added concentration of 5.0×10^5 MPNCU/reaction. For HAdV-41, the lower positive concentration was 1.2×10^5 MPNCU/reaction, corresponding to an added concentration of 2.1×10^5 MPNCU/reaction (Fig. 3). Thus the recovery rate ranged from 13 to 54% (Fig. 3), but the LOQ (7.3×10^4 MPNCU/reaction) is considered here to be the restrictive factor. As a consequence, the lowest detectable virus concentration was superior to 10^7 particles per gram of stool (Table 1).

Using iPCR, recovery rate ranged from 21 to 59% (Figs 2 and 3). A better detection of HAdV-2 was observed, with a yield ranging from 41 to 59%. Recovery rates for HAdV-41 ranged from 21 to 36%. Additionally, the better detection limit of iPCR allowed the quantification of lower concentrations of HAdVs in stool samples, as detected by direct qPCR. Thus using iPCR, positive results were obtained for virus concentrations superior or equal to 5.10^4 particles per gram of stool sample (Table 1).

Discussion

Today qPCR-based assays have been established as a standard diagnostic tool for detection of HAdVs in any clinical material from immunocompromised patients due to their technical benefits (high sensitivity and specificity, and rapid and quantitative detection). In this study, a new diagnostic tool, based on a combination of immuno-detection with a successive qPCR, is proposed for the detection of HAdVs, especially in stool samples where detection is known to be often tricky. The usefulness of the iPCR assay has already been demonstrated for several viral antigens (e.g. noroviruses and rotaviruses), with detection of low concentrations in stool samples [22,23].

TABLE 1. Characteristics of the three detection methods: ELISA, iPCR and qPCR

|	ELISA sandwich		qPCR		iPCR	
	HAdV-2	HAdV-41	HAdV-2	HAdV-41	HAdV-2	HAdV-41
Limit of detection (MPNCU or genome copies/reaction)	1.2×10^4	2×10^4	2×10^1	2×10^1	5×10^1	9×10^1
Limit of quantification (MPNCU or genome copies/reaction)	9.7×10^4	7.3×10^4	2×10^1	2×10^1	1.3×10^2	1.9×10^2
Dynamic range (MPNCU or genome copies/reaction)	$9.7 \times 10^4 – 2.5 \times 10^7$	$7.3 \times 10^4 – 9 \times 10^6$	$2 \times 10^1 – 4 \times 10^7$	$2 \times 10^1 – 1.5 \times 10^7$	$1.3 \times 10^2 – 1.6 \times 10^4$	$1.9 \times 10^2 – 5 \times 10^4$
Lowest detectable concentration in stools (MPNCU or genome copies/g of stool)	4.4×10^7	1.9×10^7	1.2×10^6	4.5×10^6	7×10^4	5.3×10^4
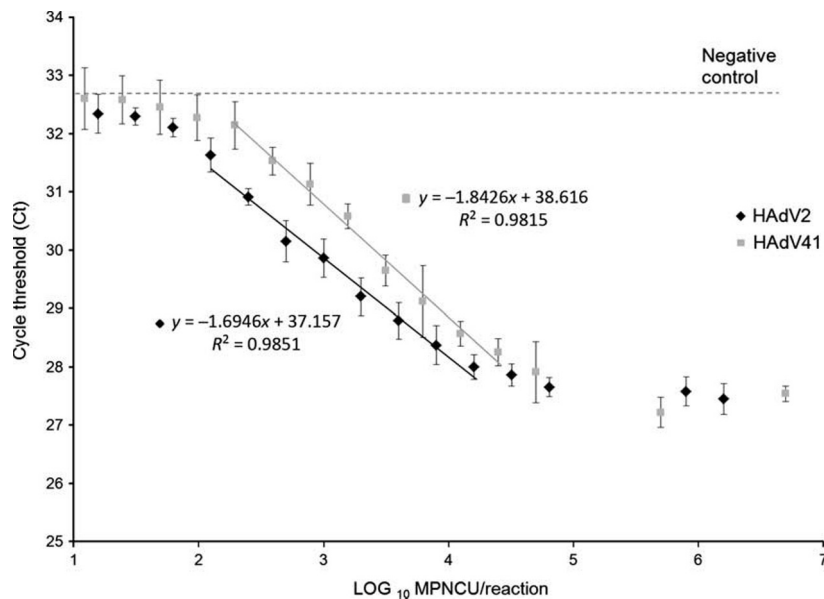
FIG. 2. Standard curves obtained by the optimized iPCR for HAdV-2 and HAdV-41 strains. The mean C_t for each dilution was determined from 10 replicates.

For detection by immuno-detection tools, viral particles must be released beforehand from stool samples by an elution step (transfer of viruses in the aqueous phase by mechanical dispersing of the stool in PBS), which can be a major cause of virus loss. Also, in order to reduce the concentration of interfering components within the eluate, stool samples were clarified by centrifugation before the analysis [22]. However, in complex matrices such as stools, a large concentration of interfering components could persist in the eluate, even with a preliminary clarification step, leading to an impact on recovery rates as observed in the present study.

The iPCR procedure developed here displays an LOD of 50 purified viral particles per reaction, indicating that its sensitivity is about 200-fold higher than that of the sandwich ELISA assay (10^4 purified viral particles). These findings are consistent with previous reports on iPCR, which describe a sensitivity improvement between 100- and 1000-fold compared to ELISA [18,22]. In stool samples, iPCR and sandwich ELISA exhibit similar recovery rates, which are considered as high. This can be easily explained by the identical first step of antigen-antibody detection in both methodological approaches. The unrecovered viruses are either sequestered in stools [22] or not detected due to interference occurring at the immuno-detection stage. Some compounds, such as lipids or some proteins, are known to hinder the antigen-antibody reaction by modifying and/or neutralizing fixation sites of antigens and/or antibodies [24,25].

A drawback of iPCR is a high background noise, mainly due to non-specific binding of chimeric DNA on the walls of wells [26]. To avoid this phenomenon, chimeric DNA could be coupled with a detection antibody before the addition to wells [26,27]. The background noise may thus be reduced, resulting in improved detection sensitivity.

According to our results, iPCR sensitivity is comparable to that of qPCR using purified viral suspensions. However, iPCR does not require a DNA extraction and purification step. This is an asset in the analysis of complex clinical samples, such as stools. Our results show that a maximum of 9.5% of spiked HAdVs in stool samples were detectable using direct qPCR, while the detection rate of iPCR can reach 59%. Important DNA losses occurring during the nucleic acids extraction step can certainly explain these findings. A study shows that only 0.1% of spiked HAdV-2 in stool samples is detected by qPCR, regardless of the extraction kit used [15]. The results of the latter study highlighted a considerable loss of DNA equal to or higher than 3 $\log_{10}$ using three different extraction kits, including the QIAamp viral RNA mini kit. The amplification reaction can also be hindered by some interfering components. Two studies on extraction and detection of coronaviruses in stools demonstrated that PCR inhibitors present in nucleic acid extracts could interfere with the performance of the qPCR [28,29]. In the present work, low PCR inhibition was observed (<5%), indicating that the poor HAdV detection using qPCR was principally due to the low DNA extraction efficiency.

In conclusion, the two major drawbacks of qPCR, loss of viral DNA occurring at the extraction step and the PCR inhibition, when used for the analysis of stool samples can be avoided by using iPCR. Indeed, the numerous washing steps occurring in the iPCR workflow allow eliminating a large part of the qPCR inhibitors [18], and a viral DNA
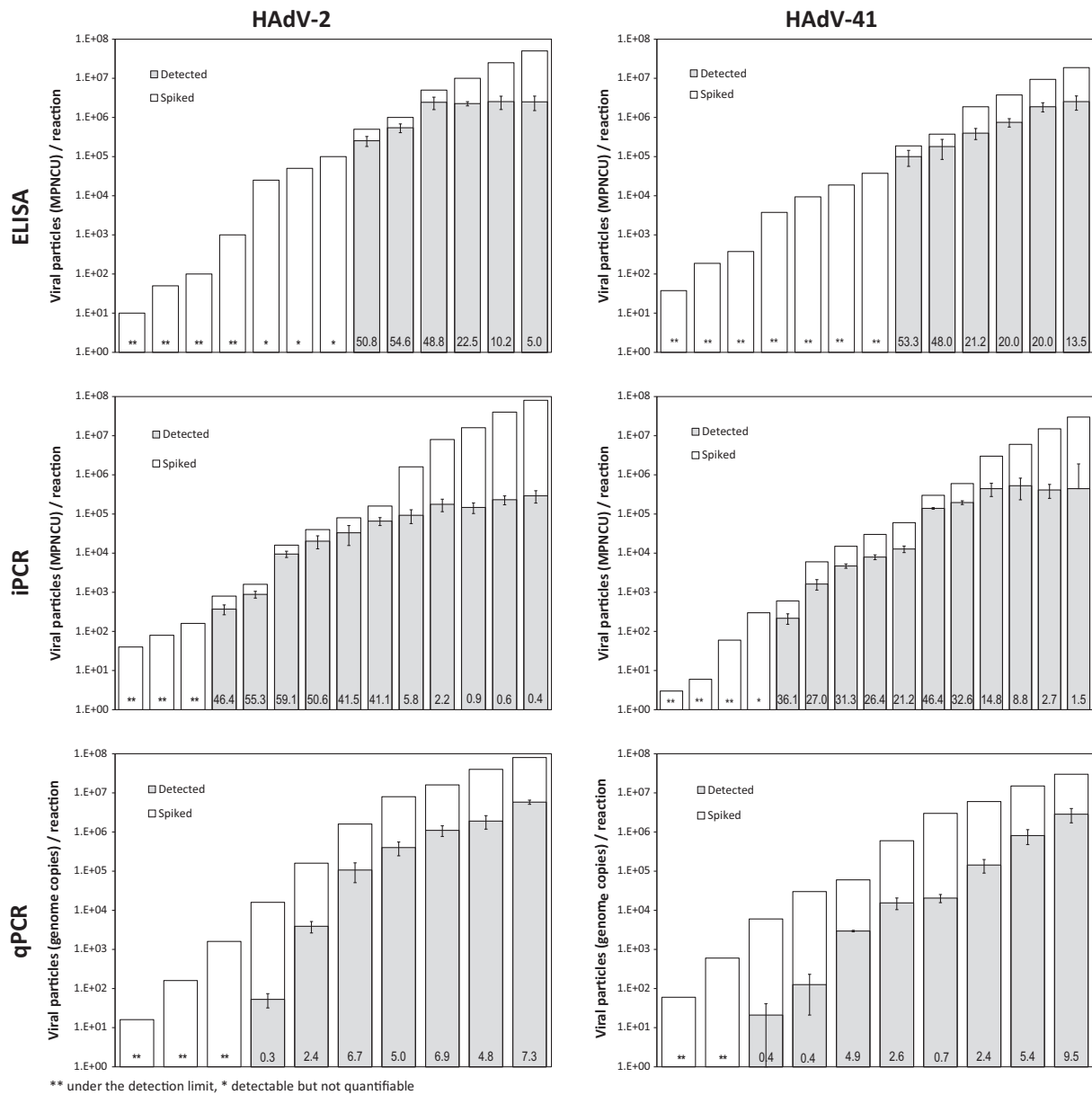
FIG. 3. Efficiency of HAdV-2 and HAdV-41 detection in spiked stools by ELISA, iPCR and qPCR (*n* = 9). The values in the histograms correspond to the recovery rate (in %) of each assay for all the virus concentrations tested. *detectable but not quantifiable, **under the detection limit.

purification step is not needed. The developed technique is powerful and allows detection of small amounts of HAdV particles in stool samples. To our knowledge this is the first description of iPCR for HAdVs. It improves our ability to decrease the detection limit of HAdVs in complex biological matrices and thus avoid the underestimation of viral concentrations. Its ability can be particularly interesting for the early detection of HAdVs in immuno-compromised patients. An early detection of HAdVs in stools should allow the rapid implementation of treatments using antivirals or

adoptive immunotherapy to prevent the dissemination of the infection.

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Transparency Declaration

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