Genotyping of clinically relevant human adenoviruses by array-in-well hybridization assay

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Abstract

A robust oligonucleotide array-in-well hybridization assay using novel up-converting phosphor reporter technology was applied for genotyping clinically relevant human adenovirus types. A total of 231 adenovirus-positive respiratory, ocular swab, stool and other specimens from 219 patients collected between April 2010 and April 2011 were included in the study. After a real-time PCR amplification targeting the adenovirus hexon gene, the array-in-well assay identified the presence of B03 (n = 122; 57.5% of patients), E04 (29; 13.7%), C02 (21; 9.9%), D37 (14; 6.6%), C01 (12; 5.7%), C05 (5; 2.4%), D19 (4; 1.9%), C06 (2; 0.9%), D08 (1; 0.5%), A31 (1; 0.5%) and F41 (1; 0.5%) genotypes among the clinical sample panel. The typing result was obtained for all specimens that could be amplified (n = 223; 97%), and specificity of the typing was confirmed by sequencing specimens representing each of the different genotypes. No hybridization signal was obtained in adenovirus-negative specimens or specimens with other viruses (n = 30). The array-in-well hybridization assay has great potential as a rapid and multiplex platform for the typing of clinically relevant human adenovirus genotypes in different specimen types.

Keywords: Adenovirus, genotype, oligonucleotide array-in-well, real-time PCR, up-converting phosphor
Original Submission: 9 February 2012; **Revised Submission:** 27 April 2012; **Accepted:** 17 May 2012
Editor: T. A. Zupanc
Article published online: 28 May 2012
Clin Microbiol Infect 2013; **19**: 551–557
10.1111/j.1469-0691.2012.03926.x

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Introduction

Human adenoviruses (hAdVs) belong to the genus Mastadenovirus of the family Adenoviridae. There are presently 57 different hAdV types grouped into seven species A to G [1]. Different types cause a wide range of acute and chronic diseases. Acute respiratory disease is predominantly caused by the hAdV types C01, C02, E04, C05 and C06; occasionally by types B03 and B07, whereas gastroenteritis is most commonly associated with the types F40 and F41 [2–4]. In all age groups, the hAdV infection can include asymptomatic infection or may provoke serious illnesses such as pneumonia (types B03, E04, B07, B14 and B21) or epidemic keratoconjunctivitis (types D08, D19 and D37) [5–8]. Types E04 and B07 have also caused severe outbreaks of respiratory illness among military recruits [9]. Human AdVs can cause especially severe disease among infants, young children, immunocompromised patients and transplant recipients [10–12] and hAdV infections occur worldwide as endemic, epidemic and sporadic infections. Therefore, genotype-specific analysis is important for epidemiological studies to provide information on the incidence and distribution of infections by individual hAdV types. Genotype identification is also necessary for the detection of new strains and for understanding hAdV pathogenesis.

Identification of serotypes is traditionally carried out by neutralization with type-specific antisera [13]. The serological detection methods are usually time-consuming, labour-intensive and do not permit a reliable detection of all hAdV serotypes. The availability of rapid diagnostic methods providing uncompromised analytical performance would allow more efficient screening and identification of the pathogen.

Today, genotyping by molecular methods such as PCR targeting the hexon or fibre gene, followed by amplicon sequencing or digestion of the amplicon with different restriction enzymes, is often preferred over serotyping [14]. Recently, PCR-based microsphere array and reverse line blot hybridization methods have been described for the detection and differentiation of hAdV genotypes [15,16]. Another approach to multiplex detection is the microarray analysis, which holds great potential for the detection and typing of pathogens. Microarray techniques are flexible, rapid and suitable for high throughput analysis. The sensitivity of PCR and the specificity of DNA hybridization can be combined when the microarray technology is used for the detection and typing of adenoviruses.

In our previous study [17], the feasibility of the up-converting phosphor (UCP) reporter technology and anti-Stokes photoluminescence detection with a new anti-Stokes photoluminescence imager was demonstrated in multi-analyte assays. UCPs are inorganic lanthanide-doped crystals that have a unique capability to convert infrared radiation to visible light via absorption of multiple photons. So, no autofluorescence is produced from any biological material, enabling sensitive assay [18]. We showed that real-time PCR and array-in-well hybridization test platform could be used for typing of hAdVs in standard 96-well microtitre plates.

In the present study, the array-in-well typing assay was expanded and technically improved. White plates and nano-sized (30 nm) up-converting nanoparticles (UCNPs) were used instead of transparent 96-well microtitre well plates and larger (>110 nm) UCP particles. The exposure time of the white plate is only 1 s, making the assay implementation more user-friendly [17]. New forward primers for real-time PCR were designed because a longer amplicon with more variation in the sequence was needed for the probe design. New oligonucleotide probes were designed to type clinically relevant members of hAdV groups A to F. The updated array-in-well test platform successfully identified hAdV genotypes from ocular swab, respiratory, stool and urine specimens.

Materials and Methods

HAdV prototypes and clinical specimens

The prototypes of hAdV genotypes C01, C02, B03, E04, C05, C06, B07, B11, B14, A18, A31, D36 and D37 were originally obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA). The prototypes C01, C02, B03, C05 and C06 were cultivated in the HeLa cell culture (human cervix carcinoma cell-line HeLa Ohio; Common Cold Unit, Salisbury, Wiltshire, UK) and prototypes E04, B07, B11, B14, A31, D36 and D37 were cultivated in the A549 cell culture (human lung carcinoma cell-line A549; American Type Culture Collection (ATCC), Rockville, MD) for the isolation of viral DNA. The recent isolates of hAdV genotypes D19 and B21 were kindly donated by HUSLAB, Helsinki, Finland. The HeLa and A549 cells were maintained in Eagle's minimal essential and Ham's F12 medium (both from Gibco, Invitrogen, Carlsbad, CA), respectively, at 37°C in 5% CO_2. Nasopharyngeal aspirate or swab (n = 86), ocular swab (n = 119), stool (n = 16), other types of respiratory specimens (n = 3), others (n = 5) and unknown (n = 2) specimens were selected from the processed clinical specimens sent to the Department of Virology, University of Turku, for hAdV detection between April 2010 and April 2011. All specimens had tested positive for the hAdV using time-resolved fluoroimmunoassay (16% of specimens) [19], single PCR [20] or multiplex PCR detection (Seeplex; Seegene, Seoul, Korea) (20%), or virus culture (64%). A total of 30 adenovirus-negative specimens including specimens with influenza A or B virus (n = 5), parainfluenza virus 1 or 3 (n = 2), coronavirus (n = 1), varicella zoster virus (n = 1), herpes simplex virus type 1 or 2 (n = 6), rhinovirus (n = 3), enterovirus (n = 1) or respiratory syncytial virus (n = 3) were also tested to evaluate the specificity of the assay (see Supplementary material, Data S1).

Synthesis, surface modification and conjugation of streptavidin to UCNPs

The nanocrystalline $NaYF_4$:Yb^{3+}, Er^{3+} materials (crystallite size 25 nm) were prepared with a modification of the method reported earlier [21]. A description of the synthesis and the surface modification of UCNPs can be found in the Supplementary material (Data S2) [29,30]. The procedures for coating UCNPs with streptavidin have been described earlier [22].

Hexon gene amplification and sequencing

Nucleic acids were extracted from the specimens by using the NucliSense easyMAG (BioMerieux, Boxtel, the Netherlands) or MagNA Pure 96 (Roche, Mannheim, Germany) automated extractors and the extracts were stored at −70°C. An asymmetric real-time PCR amplification reaction targeted to the hexon coding region was performed in a 25-μL reaction mixture containing 0.1 μM of each generic forward primer (ADHEX2F1, ADHEX2F2 and ADHEX2F3), 0.9 μM of 5′-end biotinylated reverse ADHEX1R primer and a 5-μL aliquot of extracted DNA (see Supplementary material Data S3). A positive control of type C02 DNA and a no template control were included in each PCR run. Reactions

with melting curve analysis were carried out using a Maxima™ SYBR Green qPCR Master Mix (Fermentas, St Leon-Rot, Germany) in a Rotor-Gene 6000 instrument (Corbett Research, Mortlake, Victoria, Australia). Thermal cycling consisted of initial denaturation and enzyme activation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 60 s, and extension at 72°C for 45 s, and final melting curve generation steps from 72 to 95°C with 1°C for 5 s increments. The PCR products were used as such in the hybridization assay [17] or, for sequencing, were purified (QuickClean, PCR Purification Kit; GenScript, Piscataway, NJ) and eluted with a 30 μL volume of nuclease-free sterile water. The amplicons were sequenced in the DNA Sequencing Service Laboratory of the Turku Centre for Biotechnology (Turku, Finland) using the ADHEX2F1, ADHEX2F2 or ADHEX2F3 primer.

Oligonucleotide microarrays

Oligonucleotide probes 1–16 for the hAdV genotypes C01, C02, B03, E04, C05, C06, B07, D08, B11/B14, D19, B21, A31, D36, D37 and F40/F41 were designed using published hexon sequences [23] (Data S3). The HPLC-purified oligonucleotides were obtained from http://www.Biomers.net (Ulm, Germany). To improve the probe availability for hybridization, the oligonucleotides were synthesized with three extra T/C nucleotides as spacers at their 5′ ends before the terminal amino-link C6 group. Probes g1, g2, g3, g4, g5, g6, g7 and g8 were used for the generic detection of hAdVs (Data S3). Any hAdV genotype was aimed to be identified by at least one generic probe and each target genotype by one or more specific probes in a unique pattern. Microarrays in white 96-well microtitre plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were prepared at the VTT Medical Biotechnology Centre (Turku, Finland).

Hybridization, imaging and image analysis

The hybridization assay, imaging the array and the image analysis were carried out as described earlier [17]. Briefly, 16 hAdV genotype-specific 5′-amino-modified probe spots were printed covalently as three replicates on the bottom of a white 96-well microtitre plate. The oligonucleotide probes were hybridized with the denatured biotinylated PCR products and the bound biotinylated products were detected with streptavidin-coated UCNPs (see Supplementary material, Data S4). The array-in-well assay was measured with an anti-Stokes photoluminescence imager [17]. The device was constructed for multiplexed testing with laser diode excitation and CCD image sensor, with a minor change in the optics: a Round Diffuser (RD-206-I-Y-A; Holo/Or., Rechovot, Israel) was added between the collimator and the excitation filter. The parameters used in this study were: 2× binning, 1 s exposure per well and 7 W laser power. Genotypes were called according to the pattern of fluorescent probe spots.

Results

Hexon gene amplification

The combination of one reverse primer and three forward primers specific to a part of the conserved hexon gene region allowed amplification of hAdV genotypes belonging to different species (A to F). Asymmetric real-time PCR amplified nucleic acids from all the prototypes used in the study. Human AdV DNA was amplified in 223 (97%) of the 231 clinical specimens that had originally tested positive for hAdV. The sensitivity of the real-time PCR was determined with type C02, which was detected at concentrations of below ten copies per reaction (data not shown). The plot of cycle threshold (C_t) values for the prototypes and clinical specimens is shown in Fig. 1. Adenovirus negative clinical specimens frequently showed non-specific late amplifications ($C_t > 30$) complicating hAdV detection by the PCR method alone.

Human AdV genotypes

Eleven different hAdV genotypes: C01, C02, B03, E04, C05, C06, D08, D19, A31, D37 and F41 were identified among the hAdV-positive clinical specimens. The microarray pro-

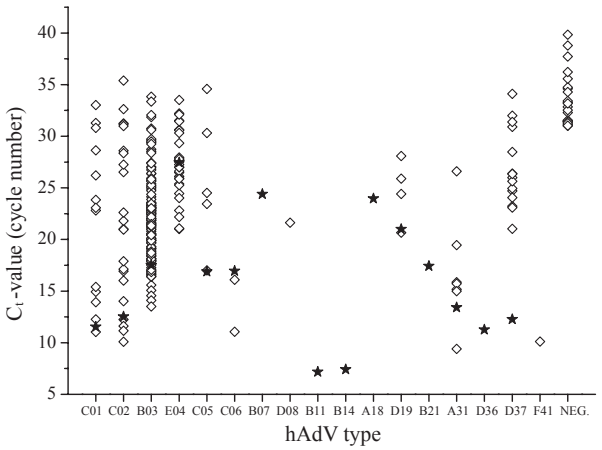

FIG. 1. Distribution of real-time PCR cycle threshold (C_t) values for the human adenovirus (hAdV)-typed specimens and unspecific amplification of hAdV-negative specimens or specimens with other viruses. DNA from prototypes (stars) was extracted from culture supernatants with varying level of infection and their C_t values do not reflect the assay sensitivity.

vided a clear hybridization pattern for each hAdV prototype and amplified clinical specimen (Fig. 2). Cross-reactions with other viruses or hAdV-negative specimens (n = 30) were not observed. A non-targeted prototype A18 showed strong hybridization signal with generic probes only. The array was not able to discriminate between the B11 and B14, because the targeted hexon sequence showed 98% sequence homology (five mismatches in the 270 bp region) between these two types. Randomly selected samples (n = 22) representing all detected genotypes were sequenced to assure the assay result. In all cases, the genotype revealed by the microarray was consistent with the sequencing result.

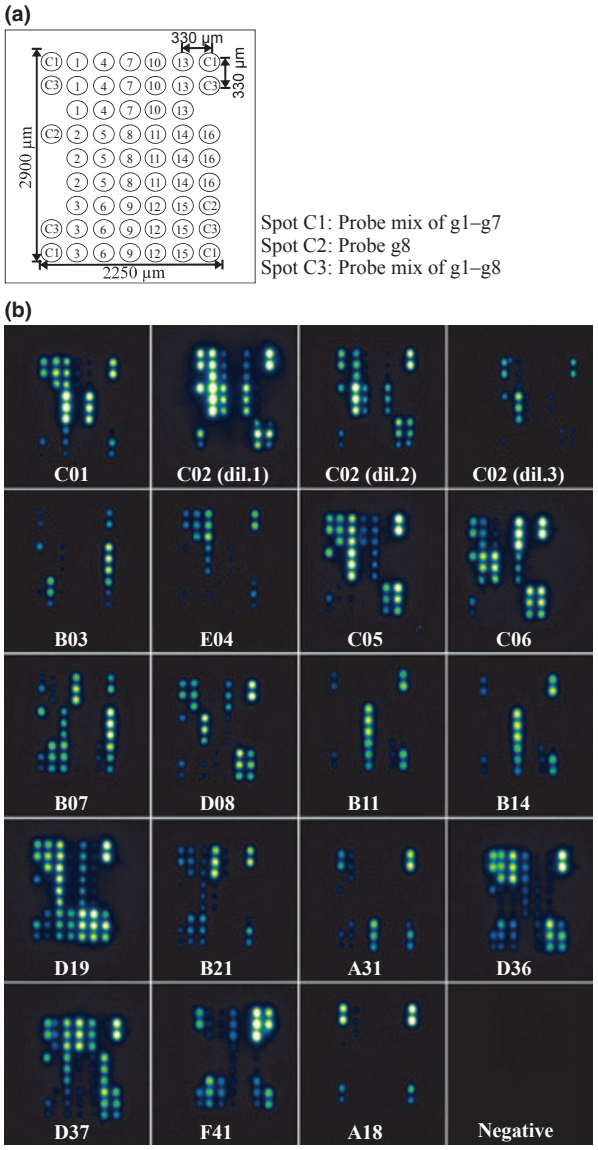

FIG. 2. (a) The array-in-well layout. The symbols C1, C2 and C3 denote generic control probe spots and spot numbers 1–16 correspond to the specific probes listed in the Supplementary material, Data S3. The probe number does not refer to the human adenovirus (hAdV) genotype. (b) Hybridization patterns of representative prototypes from hAdV genotypes C01, C02, B03, E04, C05, C06, B07, B11, B14, D19, B21, A31, D36, D37, clinical specimens D08 and F41 (genotypes were determined by sequencing), non-targeted hAdV prototype A18, and hAdV-negative specimen. The assignment to hAdV type in unknown specimens was based on the hybridization pattern in comparison to that of the known types. Genotype C02 dilution 1 (dil.1): 5×10^5 copies, dil.2: 500 copies, and dil.3: 5 copies per PCR, respectively.

Study population/age and gender distributions

Fever, cold, cough, pharyngitis and conjunctivitis were the most commonly reported clinical manifestations among the patients. The most prevalent hAdV genotypes were B03 (n = 122; 58% of all specimens), E04 (29; 14%), C02 (21; 10%) and D37 (14; 7%). In addition, several other hAdV genotypes were identified among the clinical sample panel: C01 (n = 12), C05 (n = 5), D19 (n = 4), C06 (n = 2), D08 (n = 1), A31 (n = 1) and F41 (n = 1). One female patient had subsequent infections with genotypes C02 and B03 6 months apart. The hAdV genotypes C01, C02, C05 and C06 were mainly detected in children under 4 years of age. The genotype A31 was detected in one immunocompromised female patient only. Ten co-infected specimens were detected among the sample panel. One boy had a co-infection of hAdV genotype C05 and coxackievirus 24 detected from stool. The other nine co-infections were detected in girls and boys (under the age of 14 years) from respiratory specimens: hAdV genotype C02 and rhinovirus; B03 and parainfluenza virus type 3; C01 and respiratory syncytial virus; C01 and influenza virus B; C01 and rhinovirus; C02 and influenza virus B; C01 and respiratory syncytial virus; C01 and metapneumovirus; C02 and metapneumovirus. The age range of the patients was from <1 to 67 years with a median age of 25 years. There were 100 (47%) male patients and 111 (53%) female patients in this study. Most hAdV infections were prevalent in adults: 125 (59%) patients were aged 18 or more and 73 (60%) of them were women. The hAdV genotype B03 was distributed throughout all age groups (Fig. 3).

Specimen types/seasonal distributions

The hAdV genotypes detected in ocular swabs were B03 (n = 79; 68%), E04 (18; 15%), D37 (15; 13%), D19 (4; 3%) and D08 (1; 1%). The hAdV genotypes detected in respiratory (NP in Fig. 4) specimens were B03 (n = 40; 47%), C02 (19; 22%), C01 (11; 13%), E04 (10; 12%), C05 (3; 4%) and C06 (2; 2%) (Fig. 4). Human AdV infections were prevalent throughout the year but peaked in September and October 2010 (Fig. 5).

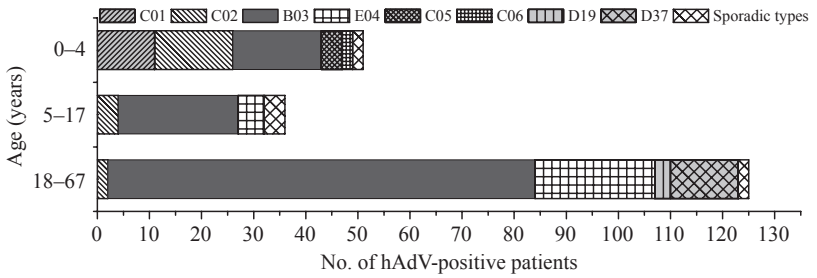

FIG. 3. Adenovirus genotypes detected in different age groups. Sporadic types in the age group 0–4 years: E04 and F41; 5–17 years: C01, C05, D19 and D37; 18–67 years: D08 and A31.

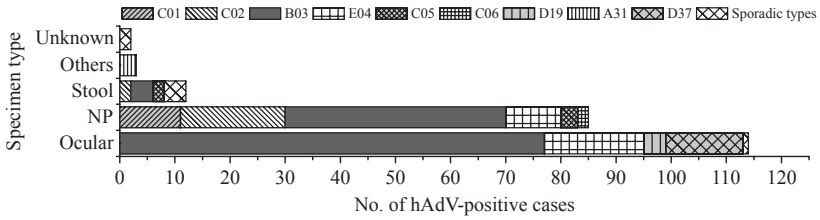

FIG. 4. Adenovirus genotypes detected in different specimen types (n = 216). NP = nasopharyngeal aspirate or swab and other respiratory specimens. Others include tissue, urine and colonoscopy specimens. Sporadic types in ocular swab specimens: D08; in stool specimens: C01, E04, A31 and F41; in unknown specimens: C02 and B03.

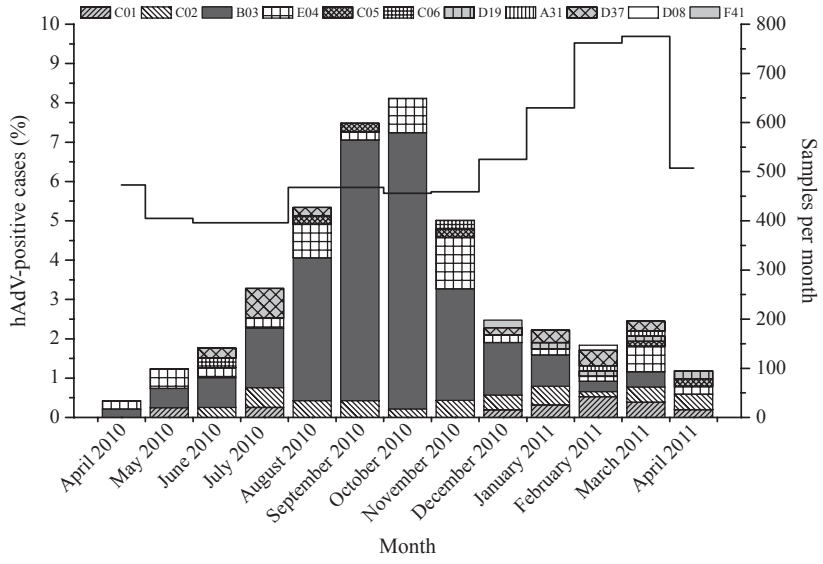

FIG. 5. Monthly distribution of detected adenovirus genotypes. The number of samples is presented in relation to the total number of specimens (positive and negative) analysed by PCR, multiplex PCR, antigen detection and virus culture during these months.

Discussion

The main purpose of this study was to demonstrate the capability of the array-in-well hybridization assay to identify the hAdV genotypes prevalent in patients in Finland during the study period. We used asymmetric real-time PCR to produce amplicons that were typed in the hybridization assay using UCNP-label and obtained a genotyping result in 97% of the specimens.

The developed array-in-well hybridization assay has broader type selection than many other published hAdV typing assays based on hybridization [16,24] except for the reverse line blot hybridization method [15]. Although not all hAdV prototypes were available for *in vitro* testing, *in silico* analyses gave a specific pattern for most of the targeted genotypes. Predictably, F40 would not be differentiable from F41 just as B14 was not differentiable from B11. Another weakness is that the PCR method alone is not sufficiently specific for hAdV detection. However, the assay has some attractive advantages compared with other assays. First, the hybridization assay is performed in 96-well microtitre plates allowing the use of common laboratory equipment, and the assay is easy to carry out. Second, microtitre plates enable

the analysis of 96 samples at one time. When necessary, it is also possible to add more probes to the 96-well microtitre plate, enabling the typing of additional hAdV genotypes in one assay.

During the study period, an outbreak of hAdV type B03 infections occurred in the autumn of 2010. Type B03 was commonly associated with conjunctivitis (n = 79; 68%), and respiratory diseases (40; 47%). A study conducted in 2010 [25] reported that restriction enzyme analysis identified three genetic variant types, including two newly described variants, Ad3a50 and Ad3a51. New variant types could be a reason for the high incidence of B03 infections in this study.

The predominant adenovirus genotypes differ between countries or regions and change over time. In 2006 and 2007, the hAdV type B14 emerged as a significant contributor to acute respiratory disease and severe pneumonias in the USA and at military recruit training centres across the USA [26]. The rarely identified B14 has also emerged in Europe [27,28]. In the present study, neither B14 nor B11, which are indistinguishable in the current assay, was detected among the clinical specimens.

In this study, a multiplex assay platform was generated to identify all common epidemic hAdV genotypes. The developed array-in-well hybridization assay with specific probes offers a rapid (1 day from sample preparation to imaging the hybridized microarray) tool for the genotyping of significant hAdV types involved in different clinical conditions.

Acknowledgements

Timo Valta MSc is thanked for the technical help with the anti-Stokes photoluminescence imager and Ms Tiina Ylinen is thanked for the technical assistance with the clinical specimens.

Transparency Declaration

This work was supported by the Graduate School of Advanced Diagnostic Technologies and Applications (DIANET), the Finnish Funding Agency for Technology and Innovation (TEKES), and the Jenny and Antti Wihuri Foundation. All authors declare no potential conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Specimens with other viruses and human adenovirus (hAdV) -negative specimens (n = 30).

Data S2. Synthesis and surface modification of up-converting nanoparticles (UCNPs).

Data S3. Oligonucleotide probes and PCR primers.

Data S4. Assay principle.

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References

1. King A, Lefkowitz E, Adams M, Carstens E, eds. *Virus taxonomy, ninth report of the international committee on taxonomy of viruses*. San Diego: Academic Press, 2011.
2. Guo L, Gonzalez R, Zhou H *et al.* Detection of three human adenovirus species in adults with acute respiratory infection in China. *Eur J Clin Microbiol Infect Dis* 2011; 31: 1051–1058.
3. Kolavic-Gray SA, Binn LN, Sanchez JL *et al.* Large epidemic of adenovirus type 4 infection among military trainees: epidemiological, clinical, and laboratory studies. *Clin Infect Dis* 2002; 35: 808–818.
4. Uhnoo I, Wadell G, Svensson L, Johansson ME. Importance of enteric adenoviruses 40 and 41 in acute gastroenteritis in infants and young children. *J Clin Microbiol* 1984; 20: 365–372.
5. Kaneko H, Iida T, Ishiko H *et al.* Analysis of the complete genome sequence of epidemic keratoconjunctivitis-related human adenovirus type 8, 19, 37 and a novel serotype. *J Gen Virol* 2009; 90: 1471–1476.
6. Selvaraju SB, Kovac M, Dickson LM, Kajon AE, Selvarangan R. Molecular epidemiology and clinical presentation of human adenovirus infections in Kansas City children. *J Clin Virol* 2011; 51: 126–131.
7. Tang L, Wang L, Tan X, Xu W. Adenovirus serotype 7 associated with a severe lower respiratory tract disease outbreak in infants in Shaanxi Province, China. *Virol J* 2011; 8: 23–30.
8. Tate JE, Bunning ML, Lott L *et al.* Outbreak of severe respiratory disease associated with emergent human adenovirus serotype 14 at a US air force training facility in 2007. *J Infect Dis* 2009; 199: 1419–1426.
9. Ludwig SL, Brundage JF, Kelley PW *et al.* Prevalence of antibodies to adenovirus serotypes 4 and 7 among unimmunized US Army trainees: results of a retrospective nationwide seroprevalence Survey. *J Infect Dis* 1998; 178: 1776–1778.
10. Madisch I, Wolfel R, Harste G, Pommer H, Heim A. Molecular identification of adenovirus sequences: a rapid scheme for early typing of human adenoviruses in diagnostic samples of immunocompetent and immunodeficient patients. *J Med Virol* 2006; 78: 1210–1217.
11. Munoz FM, Piedra PA, Demmler GJ. Disseminated adenovirus disease in immunocompromised and immunocompetent children. *Clin Infect Dis* 1998; 27: 1194–1200.
12. Walls T, Shankar AG, Shingadia D. Adenovirus: an increasingly important pathogen in paediatric bone marrow transplant patients. *Lancet Infect Dis* 2003; 3: 79–86.
13. Piedra PA, Poveda GA, Ramsey B, McCoy K, Hiatt PW. Incidence and prevalence of neutralizing antibodies to the common adenoviruses in children with cystic fibrosis: implication for gene therapy with adenovirus vectors. *Pediatrics* 1998; 101: 1013–1019.
14. McCarthy T, Lebeck MG, Capuano AW, Schnurr DP, Gray GC. Molecular typing of clinical adenovirus specimens by an algorithm

which permits detection of adenovirus coinfections and intermediate adenovirus strains. *J Clin Virol* 2009; 46: 80–84.
15. Cao Y, Kong F, Zhou F *et al.* Genotyping of human adenoviruses using a PCR-based reverse line blot hybridisation assay. *Pathology* 2011; 43: 488–494.
16. Washington C, Metzgar D, Hazbon MH *et al.* Multiplexed luminex xMAP assay for detection and identification of five adenovirus serotypes associated with epidemics of respiratory disease in adults. *J Clin Microbiol* 2010; 48: 2217–2222.
17. Ylihärsilä M, Valta T, Hattara L *et al.* Oligonucleotide array-in-well platform for detection and genotyping human adenoviruses by utilizing upconverting phosphor label technology. *Anal Chem* 2011; 83: 1456–1461.
18. Soukka T, Kuningas K, Rantanen T, Haaslahti V, Lövgren T. Photochemical characterization of up-converting inorganic lanthanide phosphors as potential labels. *J Fluoresc* 2005; 15: 513–528.
19. Hierholzer JC, Johansson KH, Anderson LJ, Tsou CJ, Halonen PE. Comparison of monoclonal time-resolved fluoroimmunoassay with monoclonal capture-biotinylated detector enzyme immunoassay for adenovirus antigen detection. *J Clin Microbiol* 1987; 25: 1662–1667.
20. Honkinen M, Lahti E, Österback R, Ruuskanen O, Waris M. Viruses and bacteria in sputum samples of children with community-acquired pneumonia. *Clin Microbiol Infect* 2011; 18: 300–307.
21. Wang F, Han Y, Lim CS *et al.* Simultaneous phase and size control of upconversion nanocrystals through lanthanide doping. *Nature* 2010; 463: 1061–1065.
22. Kuningas K, Rantanen T, Ukonaho T, Lövgren T, Soukka T. Homogeneous assay technology based on upconverting phosphors. *Anal Chem* 2005; 77: 7348–7355.
23. Ebner K, Pinsker W, Lion T. Comparative sequence analysis of the hexon gene in the entire spectrum of human adenovirus serotypes: phylogenetic, taxonomic, and clinical implications. *J Virol* 2005; 79: 12635–12642.
24. Lopez-Campos G, Coiras M, Sanchez-Merino JP *et al.* Oligonucleotide microarray design for detection and serotyping of human respiratory adenoviruses by using a virtual amplicon retrieval software. *J Virol Methods* 2007; 145: 127–136.
25. Landry ML, Lebeck MG, Capuano AW, McCarthy T, Gray GC. Adenovirus type 3 outbreak in Connecticut associated with a novel variant. *J Med Virol* 2009; 81: 1380–1384.
26. Metzgar D, Skochko G, Gibbins C, Hudson N, Lott L, Jones MS. Evaluation and validation of a real-time PCR assay for detection and quantitation of human adenovirus 14 from clinical samples. *PLoS One* 2009; 4: 7081–7087.
27. Carr M, Kajon A, Lu X *et al.* Deaths associated with human adenovirus-14p1 infections, Europe, 2009–2010. *Emerg Infect Dis* 2011; 17: 1402–1408.
28. O'Flanagan D, O'Donnell J, Domegan L *et al.* First reported cases of human adenovirus serotype 14p1 infection, Ireland, October 2009 to July 2010. *Euro Surveill* 2011; 16 Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19801. (last accessed 5 June 2012).
29. Abdul Jalil R, Zhang Y. Biocompatibility of silica coated NaYF(4) upconversion fluorescent nanocrystals. *Biomaterials* 2008; 29 (suppl): 4122–4128.
30. Rantanen T, Järvenpää ML, Vuojola J, Arppe R, Kuningas K, Soukka T. Upconverting phosphors in a dual-parameter LRET-based hybridization assay. *Analyst* 2009; 134 (suppl): 1713–1716.