JCM Accepts, published online ahead of print on 21 April 2010
J. Clin. Microbiol. doi:10.1128/JCM.00029-10

A Multiplexed Luminex xMAP Assay for Detection and Identification of Five Adenovirus Serotypes Associated with Respiratory Disease Epidemics in Adults.

Cicely Washington [1*], David Metzgar [2], Manzour Hernando Hazbón[1], Leonard Binn[1], Arthur Lyons[1], Carl Coward[1] and Robert Kuschner[1]

[1] Division of Viral Diseases (DVD), Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland, 20910, USA

[2] Department of Respiratory Diseases Research, Naval Health Research Center (NHRC), San Diego, California 92152, USA

Running title: Multiplex Luminex- based Adenovirus Diagnostic Assay

Corresponding author information

* 503 Robert Grant Ave, Silver Spring MD, 20910

Phone: (301)319-9824

Fax: (301)319-9661

E-mail: Cicely.Washington@us.army.mil

Abstract

Several serotypes of human adenovirus (HAdV) cause acute respiratory disease (ARD) among healthy adults, sometimes generating broad outbreaks with high attack rates and occasional fatalities. Timely serotype identification provides valuable epidemiological information and significantly contributes to prevention (vaccination) strategies. The prevalence of specific serotypes causing ARD varies geographically. HAdV-3, HAdV-4, HAdV-7, HAdV-14, and HAdV-21 are the serotypes most commonly found in adult populations in the Western Hemisphere. Unfortunately, conventional serotype identification is a tedious process which can take a week or longer. For this reason, new molecular methods for serotype identification are needed. Commercially available rapid antigen and polymerase chain reaction (PCR) assays for the detection of HAdV are universal but do not distinguish between the different serotypes.

We describe the development of a sensitive and specific multiplex assay capable of identifying serotypes 3, 4, 7, 14 and 21. Two sets of primers were used for nonspecific (universal) PCR amplification and serotype-specific probes coupled to Luminex tags were used for target specific extension (TSE). PCR and TSE primers were designed using known hexon gene sequences of HAdV. The TSE products of HAdV-3, HAdV-4, HAdV-7, HAdV-14, and HAdV-21 were correctly identified using the Luminex xMAP® fluid microsphere-based array system. No cross reactivity with other respiratory pathogens or other HAdV serotypes was observed. This multiplexed assay can be expanded to include more serotypes and will allow broad and rapid detection and identification of adenoviral serotypes in a high-throughput environment.

Introduction

Human adenoviruses (HAdVs) cause a wide range of disease in humans, including upper and lower respiratory illness, urinary tract infections, conjunctivitis, and gastroenteritis. There are 51 different serotypes based on type-specific serum neutralization classified into six species (A, B, C, D, E, and F) on the basis of hemagglutination, oncogenic, and phylogenetic properties (13, 24, 28, 31).

The most common serotypes known to cause respiratory illness in the adult population are 3, 4, 7, 11, 14 and 21 (21, 24, 25, 37). All of these can cause locally severe outbreaks with high attack rates. These types of outbreaks are rarely reported in civilian populations, but are essentially continuous at military training camps, particularly with serotype 4 (HAdV-4) and, to a lesser extent, HAdV-7 (10). Some recent studies have suggested that specific serotypes cause more severe disease, especially in immunocompromised patients (8, 13, 14, 24, 28, 32). The military previously established universal vaccination of new recruits for HAdV-4 and 7, which reduced adenovirus-induced ARD by 95%-99% (20). Production of these vaccines ceased in 1996, but similar replacement vaccines are currently in the final stages of licensure. In 2006 and thereafter HAdV-14 and HAdV-21 produced extensive outbreaks of ARD in military recruits (20).

It is vital to be able to identify the serotype and changes in the serotype over time in order to evaluate viral virulence, vaccine efficacy, and the potential impact of antiviral use. Identification of serotypes was traditionally done by virus isolation in cell culture, followed by neutralization tests, antibody studies, and/or antigen detection by immunofluorescence (3, 16, 19, 23). These techniques are time consuming and labor intensive (11, 34). Another technique that has been

63 used is whole genome restriction endonuclease analysis, which relies on large-scale viral culture
64 to generate the full genomic substrate (2, 4, 5).
65
66 More recently, PCR-based detection and discrimination methods have been developed (1, 21, 35,
67 36). These techniques are faster and can also detect co-infections when used in a multiplex assay
68 (20, 21, 33, 35, 36) thus reducing cost, labor and sample volume needed for analysis. Current
69 PCR assays identify the 6 subgenera (A to F) or up to 3 serotypes per reaction (1, 21, 33, 35, 36).
70 Real time PCR also exists for generic detection (but not discrimination) of all 51 serotypes (6, 7,
71 12, 21, 33), and sequence analysis of the genomic region coding for the seven hypervariable
72 loops of the hexon (the primary antigenic determinant) can identify and discriminate all 51
73 serotypes with a single assay (27), albeit a relatively time-consuming and complex one.
74
75 Luminex has designed an xMAP® system that in theory can detect up to 100 pathogens in a
76 single sample by coupling bioassays with digital signal processing in real time. The platform is a
77 suspension array where specific capture moieties are covalently coupled to the surfaces of
78 internally dyed microspheres (22). The diversity of these microspheres increases the number of
79 targets that can be identified in a single sample approximately 20-fold over traditional real-time
80 PCR.
81
82 In this study we designed and tested a Luminex-based assay capable of detecting and identifying
83 HAdV-3, HAdV-4, HAdV-7, HAdV-14, and HAdV-21 in a single reaction. The assay was tested
84 and the results indicate its potential as a diagnostic tool.
85

Materials and Methods

Sample collection and initial identification. Clinical specimens were collected by the Naval Health Research Center under IRB approved protocol (NHRC.1999.0002) with support from the Armed Forces Health Surveillance Center (AFHSC)/Division of Global Emerging Infections Surveillance and Response System (GEIS) under work unit 60805. Inclusion criteria for consented subjects enrolled through the NHRC febrile respiratory illness surveillance system included military recruits reporting for medical care with respiratory symptoms and a fever of ≥38°C, provider-diagnosed pneumonia, or both. Specimens were oropharyngeal swabs suspended in Viral Transport Medium (VTM), (Copan Diagnostics Inc., Murrieta, CA) and subsequently frozen at -80°C and transported on dry ice for testing. The presence of HAdV serotypes 3, 4, 7, 14 and 21 was initially identified in 78 of these specimens at NHRC using a modified colorimetric microneutralization assay (19), PCR, or both methods, as described previously (20).

Viral strains and isolates: HAdV isolates used in this study were part of the strain bank from the DVD, Division of Viral Disease at WRAIR, and were grown using A549 cells as previously described (15, 26). The following HAdV strains were used: HAdV-1 (Adenoid 71), HAdV-2 (Adenoid 6), HAdV-3 (GB), HAdV-4 (RI-67), HAdV-5 (Adenoid 75), HAdV-6 (Tonsil 99), HAdV-7a (S-1058), HAdV-7 (Gomen), HAdV-9 (Hick), HAdV-11 (Slobitski), HAdV-144 (DeWit), HAdV-16 (CH76), HAdV-17, HAdV-21 (strain #128), HAdV-31 (1315/63), HAdV-34 (Compton), HAdV-35 (Holden), and HAdV-40 (Dugan). Cultures from strains GB, RI-67, S-1058, Gomen, DeWit and strain #128 HAdV were titrated in tube cultures for 21 days and titers were expressed in 50% tissue culture infective dose ($TCID_{50}$).

Other common respiratory pathogens were obtained using the NATrol™ Respiratory Validation Panel (Zeptometrix, Buffalo, NY). This panel includes Corona OC43, Corona SARS, Influenza A H1N1 and H3N2, Influenza B, Parainfluenza 2 and 3, Adenovirus 7a, Metapneumovirus, Respiratory Syncytial Virus (RSV) A and B, Enterovirus and Rhinovirus strains.

DNA extraction. Oropharyngeal swabs and cultured isolates were processed under BSL-2 conditions at the DVD, WRAIR. DNA was isolated using the MinElute® Virus Spin kit (Qiagen, Valencia, CA) according to the manufacturers' recommendations. The sample and elution volumes were 200 µl. Sample extracts were stored at -70 ºC.

PCR Primer and TSE Primer Design.

For PCR and TSE primer design, eight hexon gene sequences from the serotypes of interest (GenBank accession numbers AY599834, AY599836, AY599837, AY594255, AF065066, AY495969, AY803294 and AY008279) were ClustalW aligned by DNAStar®, Lasergene 8.0 software and used. A primer pair previously described was used to amplify a 605 base pair (bp) long region of the hexon gene (27). A set of serotype-specific TSE primers were designed to bind serotypes 3, 4, 14 and 21. In the case of serotype 7, a separate PCR primer pair was designed with its corresponding TSE primer (Table 1).

The description of the primers including the position, sequence and amplicon size are described in Table 1. The same alignment used to design the PCR primers was used to design the TSE primers. TSE primers were chosen to have a melting temperature between 50 ºC and 56 ºC.

PCR Amplification

The multiplex PCR reaction was performed using the Multiplex PCR kit (Qiagen, Valencia, CA). The reaction contained 12.5 µl of 2×Master Mix Buffer, primer (2 pmol of each), (Sigma Genosys, The Woodlands, TX) (a total of 4 primers), sample (5 µL) and water (6.7 µL) to produce a final volume of 25 µl. The resulting mixture was then thermo cycled in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) with an initial denaturation cycle at 95 ºC for 15min, followed by 35 cycles at 94ºC for 30 sec, 52ºC for 1.5min, 72ºC for 1 min and a final incubation at 72ºC 10 min.

The resulting PCR product was then treated with shrimp alkaline phosphatase (3.125 µL) (USB, Cleveland, OH) and exonuclease (2.5 µL) at 37ºC for 30 min followed by 99ºC for 30 sec to remove the remaining dNTPs and primers.

Target Specific Primer Elongation

The reaction contained 10× Qiagen PCR Buffer (2 µL), 50 mM $MgCl_2$ (0.5 µL), Tsp polymerase (0.15 µL of 5U/µl) (Invitrogen, Carlsbad, CA), dATP (0.1 µL of 1mM) (Invitrogen), dGTP (0.1 µL of 1mM) (Invitrogen), dTTP (0.1 µL of 1mM) (Invitrogen), biotin-dCTP (0.25 µL of 4mM) (Invitrogen), each TSE primer (0.125µL of 1µM) (a total of 5 TSE primers, (Sigma)), treated PCR product(5µL) and of water (11.2 µL) to produce a final volume of 20µl. The resulting mixture was then thermo cycled for an initial cycle at 95ºC for 2 min, followed by 40 cycles at 94ºC for 30 sec, 55ºC for 1 min, 74ºC for 2 min).

Hybridization and Luminex Analysis

Biotinylated TSE products were hybridized to a fluid micro-bead array in wells of a 96- well plate and detected using a streptavidin-phycoerythrin conjugate. The microsphere mix consists of 5 microspheres, each containing a different fluorescent dye mix and each coupled to a unique

anti-tag oligonucleotide sequence complementary to the oligonucleotide tag sequence incorporated into the 5 TSE primers. TSE product (12.5 µL) and H_2O (12.5 µL) were mixed with microsphere mix (25 µL, 2500 microspheres per set) and incubated (at 96ºC for 2 min, then 37ºC for 30 min). After hybridization the plate was centrifuged (at 2,250 × g for 3 min) and the supernatant removed. Strepavidin-phycoerythrin (2 µg/ml) in 1X Tm (0.1M Tris-HCL, pH 8.0, 0.2M NaCl, 0.08% Triton X-100) was added to each well. The plate was incubated (at 37ºC for 15 min) in the dark. Hybridized microspheres were then analyzed on the Luminex 200 at 37ºC. TSE products bound to the microspheres were detected with a streptavidin-phycoerythrin conjugate, and signals produced for each bead were analyzed by the Luminex 200 and expressed as the Mean Fluorescence Intensity, MFI (18). Any signal that was greater than 3 times the highest background MFI signal for a given bead set was considered a positive call.

Results

In this study we describe the design and evaluation of a multiplexed assay capable of detecting and identifying HAdVs-3, 4, 7, 14, and 21. The original assay design consisted of an amplification of a single region from the hexon gene. This region was selected because the sequences where the PCR primers hybridize are conserved amongst different serotypes of HAdVs, but the areas in between them vary between serotypes, allowing a selective binding for TSE primers designed to specifically bind each serotype. With the exception of serotype 7, this approach was successful. Several TSE primers designed to identify serotype 7 showed cross reactivity with serotype 3 (data not shown). In order to circumvent this, the final assay design uses a combination of two PCR primer pairs, one that amplifies the target sequences for

serotypes 3, 4, 14, and 21 and the second for serotype 7 (Table 1). With this assay design, we observed TSE signals only in the presence of the corresponding serotype, without cross-reactivity between TSEs. The assay was further tested with a combination of two HAdV serotypes in a single reaction. The assay was able to identify the two serotypes present in the reaction (Table 2).

Analytical specificity and sensitivity.

The specificity of the multiplexed assay was examined by testing in triplicate 30 different pathogens (including the common respiratory pathogens found on the NATrol™ Respiratory Validation Panel, (Zeptometrix, Buffalo, NY) and 18 HAdV serotypes; coronavirus OC43, coronavirus SARS, influenza A H1N1 and H3N2, influenza B, parainfluenza 2 and 3, adenovirus 7a, metapneumovirus, respiratory syncytial virus A and B, enterovirus, and rhinovirus). In addition, isolates of the following adenovirus serotypes were tested : HAdV-1 (Adenoid 71), HAdV-2 (Adenoid 6), HAdV-3 (GB), HAdV-4 (RI-67), HAdV-5 (Adenoid 75), HAdV-6 (Tonsil 99), HAdV-7a (S-1058), HAdV-7 (Gomen), HAdV-9 (Hick), HAdV-11 (Slobitski), HAdV-14 (DeWit), HAdV-16 (CH76), HAdV-17, HAdV-21 (strain #128), HAdV-31 (1315/63), HAdV-34 (Compton), HAdV-35 (Holden), and HAdV-40 (Dugan), as shown in Table 2. All our reference adenoviruses were prepared in A549 cells and were harvested when the cell cultures developed ≥75% cytopathic effect. After harvesting, virus preparations were titrated in tube cultures for 21 days and titers were expressed in 50% tissue culture infective dose [$TCID_{50}$]. All samples were extracted following the protocol above. In order to confirm the presence of HAdV in the extracted cultured isolates Adenovirus r-gene™ (Argene, North Massapequa, NY), an universal adenovirus PCR based kit, was used according to the manufacturer's protocol. The assay was able to detect and identify HAdV-3, HAdV-4, HAdV-7, HAdV-7a, HAdV-14, and HAdV-21

199 (Table 2). To further test the performance of the assay the presence of multiple targets were
200 tested in combination (Table 2). The ability to detect mixed HAdV infections is valuable since
201 they do occur naturally and are difficult to detect by many methods (30).
202 Having determined the assay specificity, the limit of detection for each of the detected serotypes
203 was determined. This was achieved by testing in duplicate 5 serial dilutions (10-fold each) of
204 cultured isolates of serotypes 3, 4, 7, 14, and 21. The virus isolates were prepared in A549 cells.
205 They were harvested when the cell cultures developed 75% or more cytopathic effects (CPE).
206 After harvesting the virus preparations, they were titrated in tube cultures for 21 days and titers
207 were expressed in 50% tissue culture infective dose [$TCID_{50}$] (16, 26). The lowest dilutions in
208 which the TSEs were able to detect the presence of the viruses are shown in Table 2.
209 **Evaluation of Clinical Specimens.**
210 In order to evaluate the assay's performance using clinical specimens, we tested 104 respiratory
211 samples previously determined to be positive for HAdVs of the target serotypes. These samples
212 were tested blindly in triplicate. The reproducibility for TSE primers HAdV-3, HAdV-4,
213 HAdV7, HAdV14, and HAdV21 were 99%, 93%, 94%, 94% and 96% agreement respectively.
214 The results obtained are shown in Table 3. A total of five discrepancies between the Luminex
215 results and the original results were observed, equal to 5% of the total tested. One positive
216 HAdV-3 sample and three positive HAdV-21 samples were negative by our assay, while a
217 specimen previously identified as HAdV-negative tested positive for HAdV-4 with our assay.
218 These five discrepant specimens were tested by the Luminex RVP kit (17), and HAdV was
219 detected in all but one of these specimens (the exception being a specimen that was positive for
220 HAdV-21 by the PCR/microneutralization assay). Two samples identified as co-infections by the
221 custom Luminex assay were initially identified as single infections by the

PCR/microneutralization assays (Table 3). Both samples were HAdV-4 positive. One was also positive for HAdV-7 and the other for HAdV-14 (see Supplemental Table 1).

Discussion

Adenovirus is a common cause of ARD in military recruit populations. There are 5 major serotypes that affect US military training camps. Using Luminex technology, we have developed a sensitive and specific multiplexed PCR assay that can detect and identify the 5 relevant serotypes, HAdV-3, HAdV-4, HAdV-7, HAdV-14, and HAdV-21 . The assay targets the hexon gene because the conserved nature of specific structural regions among all serotypes allows design of universal primers, while the diversity of intervening hypervariable regions can be used to distinguish serotypes with differential probes. One hundred and four clinical throat swab samples previously identified as positive or negative for serotypes HAdV-3, HAdV-4, HAdV-7, HAdV-11, HAdV-14, and HAdV-21 were tested. The assay exhibits high sensitivity and specificity in both analytical and clinical specimens. When challenged with various other common respiratory viruses and adenovirus serotypes, no cross reactivity was detected. With the reintroduction of the vaccine program in the military recruit population there are fears that different serotypes may emerge as the dominant agents of ARD outbreaks. The ability to quickly identify shifts in serotype dominance enables a more informed assessment of the vaccine's efficacy. Past studies have suggested that there is an increase in levels of neutralizing antibodies in the serum against HAdV-3 and HAdV-14 after HAdV-7 immunization (20, 29). Analysis of the vaccine's efficacy against both homotypic and heterotypic HAdVs will certainly require an

244 efficient and robust method for measuring the impact of the vaccine on the rates of disease
245 associated with specific serotypes.
246 With multiplexed molecular assays there are concerns of decreased sensitivity and specificity
247 related to the multiplicity of primers, probes, and the possible combinations thereof. Primers and
248 probes for each target may interfere with one another by forming dimers and or by nonspecific
249 partial binding to target sequences. This can be minimized by optimizing primer and probe
250 design, most importantly by appropriate use of sequence conservation and variability among the
251 targets. This assay was designed to minimize the number of oligonucleotides in the reaction
252 through the use of broadly targeted primers designed to amplify multiple targets, paired with
253 highly specific probes that identify individual serotypes. Based on the 104 clinical samples
254 tested, the sensitivity for the different serotypes tested was 90%, 100%, 100%, 100% and 85%
255 for HAdV-3, HAdV-4, HAdV-7, HAdV-14 and HAdV-21 respectively (Table 3). It is possible
256 that a new design for the HAdV-21 TSE primer could increase the level of sensitivity further.
257 The results showed five discrepant results with the original calls obtained at NHRC. Three of the
258 discrepant results were cases in which HAdV-21 was not detected by the Luminex assay. We
259 observed that the TSE primer for serotype 21 showed the lowest sensitivity ($4.6x10^5$ $TCID_{50}$).
260 This is the likely source of these false negatives. The Luminex RVP kit was used to test those
261 samples and HAdV was detected in two of the three (Supplemental Table 1), suggesting that one
262 could be a real negative, while the others were confirmed as false negatives. The apparent false
263 positive HAdV-4 result, detected as HAdV by both the novel Luminex test described here and
264 the Luminex RVP kit (Supplemental Table 1) is most likely real. All of the negatives were taken
265 from US military recruit populations, in which HAdV-4 is essentially endemic. This discrepancy
266 is likely a false negative on the part of the original PCR/microneutralization tests.

The new assay was capable of detecting co-infections of multiple adenovirus serotypes in clinical specimens as well as in artificial mixtures of cultured isolates (Table 2 and Table 3). In our study, two throat swab specimens were found to be co-infected, one with HAdV-4 and HAdV-14 and the other with HAdV-4 and HAdV-7. Previously, these were determined to be HAdV-14 and HAdV-7 by PCR testing. Resolution of these discrepant results would require confirmatory testing with additional molecular tests. Previous work by Vora et al (30) saw a high rate of co-infections of HAdV-4 and HAdV-7, and observation of them in the sample set tested here is not surprising since they are the 2 most common ARD-associated serotypes in recruit populations (30). Co-infections are harder to detect because one serotype, usually the one with a higher titer, often dominates detection assays - especially in the case of single-pass comparative immunological methods. Co-infections are of interest because they provide the opportunity for adenovirus strains to recombine and possibly form new variants. Our assay has the ability to detect co-infections in a single test.

The results in this study indicate that the assay described has potential as a diagnostic tool. It is simple, the cost is relatively low, and it provides for the identification of up to 5 HAdV serotypes in a single reaction. The assay can be expanded to include more serotypes as needed. For example, pediatric and immunocompromised patients tend to experience infections of Group C HAdV serotypes. Current efforts are underway to include Group C serotypes, HAdV-1, HAdV-2, HAdV-5, and HAdV-6.

Acknowledgments

This work is supported by the Global Emerging Infections Surveillance (GEIS) and Response System, a Division of the Armed Forces Health Surveillance Center under WRAIR protocol #1566. The opinions or assertions contained herein are the private views of the author, and are

not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

References

1. **Adhikary, A. K., T. Inada, U. Banik, J. Numaga, and N. Okabe.** 2004. Identification of subgenus C adenoviruses by fiber-based multiplex PCR. J Clin Microbiol **42:**670-3.
2. **Adrian, T., G. Wadell, J. C. Hierholzer, and R. Wigand.** 1986. DNA restriction analysis of adenovirus prototypes 1 to 41. Arch Virol **91:**277-90.
3. **Akalu, A., W. Seidel, H. Liebermann, U. Bauer, and L. Dohner.** 1998. Rapid identification of subgenera of human adenovirus by serological and PCR assays. J Virol Methods **71:**187-96.
4. **Allard, A., B. Albinsson, and G. Wadell.** 2001. Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis. J Clin Microbiol **39:**498-505.
5. **Blasiole, D. A., D. Metzgar, L. T. Daum, M. A. Ryan, J. Wu, C. Wills, C. T. Le, N. E. Freed, C. J. Hansen, G. C. Gray, and K. L. Russell.** 2004. Molecular analysis of adenovirus isolates from vaccinated and unvaccinated young adults. J Clin Microbiol **42:**1686-93.
6. **Chmielewicz, B., A. Nitsche, B. Schweiger, and H. Ellerbrok.** 2005. Development of a PCR-based assay for detection, quantification, and genotyping of human adenoviruses. Clin Chem **51:**1365-73.
7. **Damen, M., R. Minnaar, P. Glasius, A. van der Ham, G. Koen, P. Wertheim, and M. Beld.** 2008. Real-time PCR with an internal control for detection of all known human adenovirus serotypes. J Clin Microbiol **46:**3997-4003.
8. **Ebner, K., M. Rauch, S. Preuner, and T. Lion.** 2006. Typing of human adenoviruses in specimens from immunosuppressed patients by PCR-fragment length analysis and real-time quantitative PCR. J Clin Microbiol **44:**2808-15.
9. **Elder, B. L., S. A. Hansen, J. A. Kellogg, M. F.J., and Z. R.J.** 1997. Cumitech 31, Verification and Validation of Procedures in the Clinical Microbiology Laboratory. ASM Press, Washington, DC.
10. **Gaydos, C. A., and J. C. Gaydos.** 1995. Adenovirus vaccines in the U.S. military. Mil Med **160:**300-4.
11. **Gerna, G., E. Cattaneo, M. G. Revello, and M. Battaglia.** 1982. Grouping of human adenoviruses by early antigen reactivity. J Infect Dis **145:**678-82.
12. **Gu, Z., S. W. Belzer, C. S. Gibson, M. J. Bankowski, and R. T. Hayden.** 2003. Multiplexed, real-time PCR for quantitative detection of human adenovirus. J Clin Microbiol **41:**4636-41.
13. **Hierholzer, J.** 1995. Adenoviruses, p. 169-188. *In* E. H. Lennette, D. A. Lennette, and E. T. Lennette (ed.), Diagnostics procedures for viral, rickettsial and chlamydial infections, 7th ed. American Public Health Association, Washington, DC.
14. **Hierholzer, J. C.** 1992. Adenoviruses in the immunocompromised host. Clin Microbiol Rev **5:**262-74.
15. **Houng, H. S., S. Clavio, K. Graham, R. Kuschner, W. Sun, K. L. Russell, and L. N. Binn.** 2006. Emergence of a new human adenovirus type 4 (Ad4) genotype: identification of a novel inverted terminal repeated (ITR) sequence from majority of Ad4 isolates from US military recruits. J Clin Virol **35:**381-7.

16. **Kolavic-Gray, S. A., L. N. Binn, J. L. Sanchez, S. B. Cersovsky, C. S. Polyak, F. Mitchell-Raymundo, L. V. Asher, D. W. Vaughn, B. H. Feighner, and B. L. Innis.** 2002. Large epidemic of adenovirus type 4 infection among military trainees: epidemiological, clinical, and laboratory studies. Clin Infect Dis **35:**808-18.
17. **Krunic, N., T. D. Yager, D. Himsworth, F. Merante, S. Yaghoubian, and R. Janeczko.** 2007. xTAG RVP assay: analytical and clinical performance. J Clin Virol **40 Suppl 1:**S39-46.
18. **Mahony, J., S. Chong, F. Merante, S. Yaghoubian, T. Sinha, C. Lisle, and R. Janeczko.** 2007. Development of a respiratory virus panel test for detection of twenty human respiratory viruses by use of multiplex PCR and a fluid microbead-based assay. J Clin Microbiol **45:**2965-70.
19. **Malasig, M. D., P. R. Goswami, L. K. Crawford-Miksza, D. P. Schnurr, and G. C. Gray.** 2001. Simplified microneutralization test for serotyping adenovirus isolates. J Clin Microbiol **39:**2984-6.
20. **Metzgar, D., M. Osuna, A. E. Kajon, A. W. Hawksworth, M. Irvine, and K. L. Russell.** 2007. Abrupt emergence of diverse species B adenoviruses at US military recruit training centers. J Infect Dis **196:**1465-73.
21. **Metzgar, D., M. Osuna, S. Yingst, M. Rakha, K. Earhart, D. Elyan, H. Esmat, M. D. Saad, A. Kajon, J. Wu, G. C. Gray, M. A. Ryan, and K. L. Russell.** 2005. PCR analysis of egyptian respiratory adenovirus isolates, including identification of species, serotypes, and coinfections. J Clin Microbiol **43:**5743-52.
22. **Pabbaraju, K., K. Tokaryk, S. Wong, and J. Fox.** 2008. Comparison of the Luminex xTAG Respiratory Viral Panel with In-House Nucleic Acid Amplification Tests for Diagnosis of Respiratory Virus Infections. Journal Of Clinical Microbiology **46:**3056-3062.
23. **Raty, R., M. Kleemola, K. Melen, M. Stenvik, and I. Julkunen.** 1999. Efficacy of PCR and other diagnostic methods for the detection of respiratory adenoviral infections. J Med Virol **59:**66-72.
24. **Rubin, B. A.** 1993. Clinical picture and epidemiology of adenovirus infections (a review). Acta Microbiol Hung **40:**303-23.
25. **Ryan, M. A., G. C. Gray, B. Smith, J. A. McKeehan, A. W. Hawksworth, and M. D. Malasig.** 2002. Large epidemic of respiratory illness due to adenovirus types 7 and 3 in healthy young adults. Clin Infect Dis **34:**577-82.
26. **Sanchez, J. L., L. N. Binn, B. L. Innis, R. D. Reynolds, T. Lee, F. Mitchell-Raymundo, S. C. Craig, J. P. Marquez, G. A. Shepherd, C. S. Polyak, J. Conolly, and K. F. Kohlhase.** 2001. Epidemic of adenovirus-induced respiratory illness among US military recruits: epidemiologic and immunologic risk factors in healthy, young adults. J Med Virol **65:**710-8.
27. **Sarantis, H., G. Johnson, M. Brown, M. Petric, and R. Tellier.** 2004. Comprehensive detection and serotyping of human adenoviruses by PCR and sequencing. J Clin Microbiol **42:**3963-9.
28. **Schmitz, H., R. Wigand, and W. Heinrich.** 1983. Worldwide epidemiology of human adenovirus infections. Am J Epidemiol **117:**455-66.
29. **van der, V., and A. Prins.** 1960. Studies of the significance of the recall phenomenon in the antibody response to adenovirus vaccine and infection. J Immunol **84:**562-8.

30. **Vora, G. J., B. Lin, K. Gratwick, C. Meador, C. Hansen, C. Tibbetts, D. A. Stenger, M. Irvine, D. Seto, A. Purkayastha, N. E. Freed, M. G. Gibson, K. Russell, and D. Metzgar.** 2006. Co-infections of adenovirus species in previously vaccinated patients. Emerg Infect Dis **12:**921-30.
31. **Wadell, G., M. L. Hammarskjold, G. Winberg, T. M. Varsanyi, and G. Sundell.** 1980. Genetic variability of adenoviruses. Ann N Y Acad Sci **354:**16-42.
32. **Walls, T., A. G. Shankar, and D. Shingadia.** 2003. Adenovirus: an increasingly important pathogen in paediatric bone marrow transplant patients. Lancet Infect Dis **3:**79-86.
33. **Wong, S., K. Pabbaraju, X. L. Pang, B. E. Lee, and J. D. Fox.** 2008. Detection of a broad range of human adenoviruses in respiratory tract samples using a sensitive multiplex real-time PCR assay. J Med Virol **80:**856-65.
34. **Wood, S. R., I. R. Sharp, E. O. Caul, I. Paul, A. S. Bailey, M. Hawkins, S. Pugh, J. Treharne, and S. Stevenson.** 1997. Rapid detection and serotyping of adenovirus by direct immunofluorescence. J Med Virol **51:**198-201.
35. **Xu, W., and D. D. Erdman.** 2001. Type-specific identification of human adenovirus 3, 7, and 21 by a multiplex PCR assay. J Med Virol **64:**537-42.
36. **Xu, W., M. C. McDonough, and D. D. Erdman.** 2000. Species-specific identification of human adenoviruses by a multiplex PCR assay. J Clin Microbiol **38:**4114-20.
37. **Zhu, Z., Y. Zhang, S. Xu, P. Yu, X. Tian, L. Wang, Z. Liu, L. Tang, N. Mao, Y. Ji, C. Li, Z. Yang, S. Wang, J. Wang, D. Li, and W. Xu.** 2009. Outbreak of acute respiratory disease in China caused by B2 species of adenovirus type 11. J Clin Microbiol **47:**697-703.

406

407 **Table 1.** Coordinates and sequences of the amplification and TSE primers used in this study.

PCR primers (Forward/Reverse):	TSE primer:
HVR7' F[a,b]: 1003-CTGATGTACTACAACAGCACTGGCAACATGGG-1033 **HVR7 R**[a,b]: 1575-CGGTGGTGGTTAAATGGATTCACATTGTCC-1604 Amplicon size 601 bp.	**HAdV-3**[c]: 2616-GTTAAAACCGATGACACTAATGG-2638 **HAdV-4**[d]: 19382-GGTGTGGGATTGACAGACACTTAC-19405 **HAdV-14**[c]: 19544-CCAAGCTTGGAAAGATGTAAATC-19566 **HAdV-21**[b]: 1299-GGGTGCAGATTGGAAAGAGC-1318
HAdV-7 F: 383-CGCCCAATACATCTCAGTGG-402 **HAdV-7 R**: 595-ACTCCAACTTGAGGCTCTGG-614 Amplicon size 253 bp.	**HAdV-7**[f]: 399-GTGGATAGTTACAACGGGAGAAG-421

408 [a] Primer from Sarantis et al (2004); Nucleotide numbering based on hexon gene of HAdV accession numbers

409 AY008279[b], AY599834[c], AY599837[d], AY803294[e] and AY594255[f].

Table 2. Assay specificity and sensitivity. Assay specificity was assessed by testing the pathogens indicated. Assay sensitivity was assessed by determining the lowest dilution detected out of serial dilutions done for each reference HAdVs strain tested (see the text for details). The corresponding $TCID_{50}$ of the lowest dilution detected is indicated. The co-infections were not tested by a commercial method since they do not distinguish serotypes.

ND* samples were not tested on the Luminex RVP kit but rather tested and detected by `the Argene, Adenovirus r-gene™

Viral pathogen	TSE signal/ Lowest $TCID_{50}$ detected by the assay					Luminex RVP Assay call
	HAdV-3	HAdV-4	HAdV-7	HAdV-14	HAdV-21	
HAdV-3	**+ / $5x10^3$**	-	-	-	-	Adenovirus
HAdV-4	-	**+ / $9.5x10^3$**	-	-	-	Adenovirus
HAdV-7	-	-	**+ / $1.1x10^4$**	-	-	Adenovirus
HAdV-7a	-	-	**+ / ND**	-	-	Adenovirus
HAdV-14	-	-	-	**+ / $5x10^3$**	-	Adenovirus
HAdV-21	-	-	-	-	**+ / $4.6x10^5$**	Adenovirus
HAdV 3 + 7	**+**	-	**+**	-	-	N/A
HAdV 4 + 14+ 21	-	**+**	-	**+**	**+**	N/A
HAdV 3+ 4+ 7+ 14 + 21	**+**	**+**	**+**	**+**	**+**	N/A
HAdV 1, 2, 5, 6, 9, 11, 16, 17, 31, 34, 35 and 40	-	-	-	-	-	ND*
Influenza A H1N1	-	-	-	-	-	Influenza A, H1
Influenza A H3N2	-	-	-	-	-	Influenza A, H3
Influenza B	-	-	-	-	-	Influenza B
Corona OC43	-	-	-	-	-	Corona OC43
Corona SARS	-	-	-	-	-	Corona SARS
Parainfluenza 2	-	-	-	-	-	Para 2
Parainfluenza 3	-	-	-	-	-	Para 3
RSV A	-	-	-	-	-	RSV A
RSV B	-	-	-	-	-	RSV B
Metapneumovirus	-	-	-	-	-	Metapneumovirus
Enterovirus	-	-	-	-	-	Entero/Rhinovirus
Rhinovirus	-	-	-	-	-	Entero/Rhinovirus
Blank	-	-	-	-	-	-

Table 3. Comparison of the results obtained at NHRC by PCR/microneutralization with the Luminex HAdV assay. W+ and W-, positive and negative respectively by the multiplexed Luminex HAdV assay; N+ and N- positive and negative by PCR/microneutralization. Sensitivity, specificity, were based on "true positives and "true negative", defined as specimens giving positive or negative results according to the PCR/microneutralization assay. The sensitivity and specificity were calculated according to Cumitech 31.(9)

Serotype	W+ N+	W- N+	W+ N-	W- N-	Sensitivity	Specificity
HAdV-3	9	1	0	94	90%	100%
HAdV-4	19	0	2 [a, b]	83	100%	98%
HAdV-7	10[a]	0	0	94	100%	100%
HAdV-14	19[b]	0	0	85	100%	100%
HAdV-21	17	3	0	84	85%	100%

[a]One HAdV-7 sample by was positive for both HAdV-4 and HAdV-7

[b] One HAdV-14 sample by was positive for both HAdV-14 and HAdV-4.