Rapid Detection and Identification of Human Adenovirus Species by Adenoplex, a Multiplex PCR-Enzyme Hybridization Assay

Karen Pehler-Harrington,¹ Marilyn Khanna,¹ Chris R. Waters,¹ and Kelly J. Henrickson^{1,2*}

Prodesse, Inc., Waukesha,¹ and Department of Pediatrics, Medical College of Wisconsin, Milwaukee,² Wisconsin

Received 19 November 2003/Returned for modification 29 December 2003/Accepted 8 May 2004

Human adenoviruses (AdV) have been implicated in a wide variety of diseases and are ubiquitous in populations worldwide. These agents are of concern particularly in immunocompromised patients, children, and military recruits, resulting in severe disease or death. Clinical diagnosis of AdV is usually achieved through routine viral cell culture, which can take weeks for results. Immunofluorescence and enzyme-linked immunosorbent assay-based techniques are more timely but lack sensitivity. The ability to distinguish between the six different AdV species (A to F) is diagnostically relevant, as infections with specific AdV species are often associated with unique clinical outcomes and epidemiological features. Therefore, we developed a multiplex PCR-enzyme hybridization assay, the Adenoplex, using primers to the fiber gene that can simultaneously detect all six AdV species A through F in a single test. The limit of detection (LOD) based on the viral 50% tissue culture infective dose/ml for AdV A, B, C, D, E, and F was 10^{-2} , 10^{-1} , 10^{-1} , 10^{-2} , 10^{-1} , and 10^{-2} , respectively. Similarly, the LOD for the six DNA controls ranged from 10^2 to 10^3 copies/ml. Twelve common respiratory pathogens were tested with the Adenoplex, and no cross-reactivity was observed. We also validated our assay using clinical specimens spiked with different concentrations of AdV strains of each species type and tested by multiplex PCR and culture. The results demonstrated an overall sensitivity and specificity of Adenoplex of 100%. This assay can be completed in as few as 5 h and provides a rapid, specific, and sensitive method to detect and subtype AdV species A through F.

Human adenoviruses (AdV) cause a variety of diseases and are prevalent throughout the world. Common clinical manifestations resulting from AdV infection include pneumonia, cystitis, conjunctivitis, diarrhea, hepatitis, myocarditis, and encephalitis (10). In the general population, AdV often cause mild or self-limiting disease; however, severe disseminated disease can occur in immunocompromised individuals and can be fatal. Pediatric bone marrow transplant patients are at increased risk for AdV infection and have high mortality rates (22). Discontinuation of vaccinating U.S. military trainees against AdV has resulted in a resurgence of respiratory disease epidemics in this population, leading to increased AdV morbidity (12, 15).

There are currently 51 recognized AdV serotypes that have been grouped into six different species (formerly subgenera), A to F, based on their physiochemical, biological, and genetic properties (3, 21). Identification of AdV species types can be particularly useful to clinicians, as specific species can cause infections with unique clinical outcomes and epidemiological features. For example, the ability to distinguish between group D AdV, which cause severe and highly contagious keratoconjunctivitis, and group B and E AdV, which cause mild ocular infections, may be of great clinical importance. Also, species typing may help identify disease from asymptomatic chronic infection and also nosocomial infections.

The “gold standard” of diagnosis for AdV infection has been viral culture. Although culture provides a sensitive method for diagnosis, it can take as long as 3 weeks to achieve results.

Immunofluorescence and other immunodiagnostic assays using direct antigen detection are more rapid than culture, but they lack sensitivity. Recently, PCR-based assays have been employed in clinical practice for the detection of AdV and have proven to be comparable or better than the classic methods (1, 4, 5, 8, 11, 13, 17, 18, 23). However, many of these assays do not allow for AdV species typing in a rapid and efficient manner. Thus, we developed a multiplex PCR-enzyme hybridization assay, the Adenoplex, for rapid, simultaneous detection and identification of AdV species in a single test.

MATERIALS AND METHODS

Virus and bacteria stocks. AdV strains (serotypes 1 through 9, 11, 12, 16, 19, 21, 30, 31, 34, 35, 37, 40, 41, 48, and 49), human parainfluenza viruses 1, 2, and 3, influenza viruses A and B, respiratory syncytial viruses A and B, *Bordetella pertussis*, *Chlamydia pneumoniae*, *Legionella micdadei*, *Legionella pneumophila*, and *Mycoplasma pneumoniae* strains used in this study were obtained from the American Type Culture Collection (Manassas, Va.).

Specimens. A total of 150 nasopharyngeal (NP) swab specimens, which were previously determined to be AdV negative, were used in our study. We spiked 20 each ($n = 120$) with AdV A, B, C, D, E, and F and left 30 unspiked as negative controls. Fifty percent of the specimens were spiked at 10^3 50% tissue culture infective doses (TCID₅₀)/ml, and the other 50% were spiked at 10^2 TCID₅₀/ml. A total sample volume of 2.0 ml was split equally for blind testing by either culturing (City of Milwaukee Health Department, Milwaukee, Wis.) or by the Adenoplex assay. For discrepant analysis, frozen DNA from the specimens that were culture negative and Adenoplex positive was sent to another commercial laboratory for testing using their AdV PCR-based assay.

Virus culture and DFA testing. A volume of 1.0 ml of clinical specimen was split into two 0.5-ml aliquots, and each aliquot was inoculated onto two cell types. Specimens spiked with species A to E were inoculated onto HEp2 and Caco2 cells, and specimens spiked with species F were inoculated onto RMK-1 and Caco2 cells. Cells were cultured for 4 to 14 days and observed for cytopathic effect (CPE). Cultures that did not have adequate CPE development for quantification of virus after 14 days were then fixed with methanol, washed, and stained using the Light Diagnostics AdV direct immunofluorescence assay

* Corresponding author. Mailing address: Department of Pediatrics, MACC Fund Research Center, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Phone: (414) 456-4122. Fax: (414) 456-6539. E-mail: kellyj@mcw.edu.

TABLE 1. Primers and probes for species-specific AdV detection by Adenoplex assay

AdV species	Oligonucleotide sequence, 5'-3' (forward, probe, and reverse)
A	TGC ATT TAG TGT TTG ATG AA ATG CCT AAT GTG AGT GCT TAC CCT AGA CCA AA ATA GGT TTA GAT GTA TCT CCC TGT AA
B	TCT TCC CAA CTC TGG TAC TAT GAA GAT GAA AGG GGC ACC TCA AGC CCA GAC GGA GT CCT GGG TTT ATA AAG GGG TG
C	TCA TAT CAT GGG TAA CAG ACA T TTC TTA GGT GTT ATA TTC CAC ACG GTT TCC TG CCC ATG TAG GCG TGG ACT TC
D	CTC CGG GTG GAA GAT GAC T ACC CCT ATG GCT ACG GCG GCG GAA TCA GAA TA ATT GGG TCA GCC AGT TTG AGT
E	TTG GCT CAG GTT TAG GAC TCA GT AGC GCC CTG GCA GTA CAG TTA GCC TCT CCA CT CTG TTT AGG GTA ATC TTT ATA TTC CCT
F	AAC ATG CTC ATC CAA ATC TCG CCT A ACA TCA CCT TCA GTG CGT CTA CAA CGA GAT TTC AGT TAT GTA GCA AAA TAC AGC

(Chemicon International Inc., Temecula, Calif.) and quantified by using direct fluorescent antibody (DFA).

Recombinant DNA controls. DNA controls were generated using genomic DNA isolated from specific serotypes belonging to the six different AdV species. Genomic DNA was amplified using species-specific primer pairs to yield PCR products for cloning. PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen, San Diego, Calif.). Briefly, PCR products were ligated into the pCR4-TOPO plasmid vector under standard conditions. Transformation of TOP10 competent cells with the plasmid and insert was carried out according to the manufacturer's instructions. Clones were verified for the specific PCR insert by restriction enzyme digestion as well as PCR-enzyme hybridization assay. DNA was quantified on a spectrophotometer to obtain the copy number and frozen at -70°C .

Adenoplex assay. Species-specific oligonucleotide primers and probes were designed from conserved regions of the fiber gene of the six respective AdV species A to F available from GenBank (Table 1). The assay methodology was as previously described (9). Briefly, DNA was extracted from 400 μl of clinical specimen or DNA control using the Clontech Nucleospin tissue kit (BD Biosciences, Palo Alto, Calif.). Purified DNA was amplified by PCR using a Supermix containing the six pairs of primers and 2.5 U of *Taq* polymerase (Applied Biosystems, Foster City, Calif.). The PCR parameters were 95°C for 10 min, two cycles at 95°C for 1 min, 55°C for 30 s, and 72°C for 45 s, and 38 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s, and the reactions were then held at 72°C for 7 min. Following amplification, PCR products were purified using a Clontech Nucleospin extraction kit (BD-Biosciences). Hybridization solutions containing horseradish peroxidase-labeled probes corresponding to each AdV species (A to F) were added to 96-well streptavidin-coated microtiter plates, and the purified PCR products were then added to each well. These oligonucleotide probes represented highly conserved regions of the previously mentioned fiber gene. Following hybridization, plates were washed and tetramethyl benzidine substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to each well. After 10 min, the reaction was stopped by adding 1 N H_2SO_4 , and the optical density (OD) of each well was measured at 450 nm on a spectrophotometer. The positive cutoff value was calculated as four times higher than the negative control value and greater than or equal to an OD value of 0.400.

Analytical sensitivity. Serial dilutions of both recombinant DNA controls and whole virus were used to determine the assay's limit of detection (LOD). Ten-fold serial dilutions of DNA controls were made from 10^6 to 10 copies/ml in M4 (viral transport medium; Microtest, Snellville, Ga.) and tested. AdV stocks were diluted 10-fold from 10^3 to 10^{-3} TCID₅₀/ml in M4 and tested with the Adenoplex.

Analytical specificity. AdV strains were diluted to 10^2 TCID₅₀/ml in M4. All other viral strains, with the exception of influenza B virus, were diluted to 10^4 TCID₅₀/ml. Influenza B virus and the bacterial strains tested were directly diluted 1:1,000 with M4. All dilutions were run through the Adenoplex to evaluate the specificity of the assay.

TABLE 2. Specificity of the Adenoplex assay tested against various AdV serotypes and other respiratory viruses and bacteria for cross-reactivity

Microorganism	OD ₄₅₀ with probe for AdV species:					
	A	B	C	D	E	F
AdV serotype 12 ^a	>3.0	0	0	0	0	0
AdV serotype 31	>3.0	0	0	0	0	0
AdV serotype 3	0	2.8	0	0	0	0
AdV serotype 7	0	>3.0	0	0	0	0
AdV serotype 11	0	>3.0	0	0	0	0
AdV serotype 16	0	>3.0	0	0	0	0
AdV serotype 21	0	>3.0	0	0	0	0
AdV serotype 34	0	>3.0	0	0	0	0
AdV serotype 35	0	>3.0	0	0	0	0
AdV serotype 2	0	0	>3.0	0	0	0
AdV serotype 5	0	0	>3.0	0	0	0
AdV serotype 6	0	0	>3.0	0	0	0
AdV serotype 8	0	0	0	1.3	0	0
AdV serotype 9	0	0	0	>3.0	0	0
AdV serotype 19	0	0	0	2.4	0	0
AdV serotype 30	0	0	0	>3.0	0	0
AdV serotype 37	0	0	0	>3.0	0	0
AdV serotype 48	0	0	0	>3.0	0	0
AdV serotype 49	0	0	0	>3.0	0	0
AdV serotype 4	0	0	0	0	>3.0	0
AdV serotype 40	0	0	0	0	0	>3.0
AdV serotype 41	0	0	0	0	0	>3.0
Parainfluenza virus I ^b	0	0	0	0	0	0
Parainfluenza virus II ^b	0	0	0	0	0	0
Parainfluenza virus III ^b	0	0	0	0	0	0
Influenza virus A ^b	0	0	0	0	0	0
Influenza virus B ^c	0	0	0	0	0	0
RSV A ^{b,d}	0	0	0	0	0	0
RSV B ^{b,d}	0	0	0	0	0	0
<i>Bordetella pertussis</i> ^c	0	0	0	0	0	0
<i>Chlamydia pneumoniae</i> ^b	0	0	0	0	0	0
<i>Legionella micdadei</i> ^c	0	0	0	0	0	0
<i>Legionella pneumophila</i> ^c	0	0	0	0	0	0
<i>Mycoplasma pneumoniae</i> ^c	0	0	0	0	0	0

^a Tested at 10^2 TCID₅₀/ml.

^b Tested at 10^4 TCID₅₀/ml.

^c Tested at a 10^{-3} dilution.

^d RSV, respiratory syncytial virus.

RESULTS

Analytical sensitivity and specificity. The specificity of the Adenoplex assay was tested using 23 AdV serotypes representing all six species, as well as other viruses and bacteria that cause similar respiratory symptoms (Table 2). Our assay was able to detect and specifically identify the species type for each of the AdV serotypes tested. Also, no cross-reactivity with other respiratory viruses or bacteria was observed.

The analytical sensitivity of the Adenoplex was tested using serial dilutions of recombinant DNA controls and whole virus. As shown in Table 3, the LOD for the DNA controls AdV A, B, D, E, and F was 100 copies/ml and for AdV C it was 1,000 copies/ml. AdV species A, B, C, D, E, and F had LODs in terms of the TCID₅₀/ml value equal to 10^{-2} , 10^{-1} , 10^{-1} , 10^{-2} , 10^{-1} , and 10^{-2} , respectively, when serial dilutions of virus stocks were tested.

Detection of dual infection by Adenoplex. Samples containing various combinations of AdV representative of the six different species were evaluated with the Adenoplex assay. Clinical specimens were spiked with either medium (1,000

TABLE 3. Analytical sensitivity of the Adenoplex assay as determined by the LOD^a

AdV species	DNA controls		Whole virus	
	DNA copies/ml	OD ₄₅₀	TCID ₅₀ /ml	OD ₄₅₀
A	10 ²	0.96	10 ⁻²	2.51
B	10 ²	2.49	10 ⁻¹	0.71
C	10 ³	2.30	10 ⁻¹	0.70
D	10 ²	1.61	10 ⁻²	0.58
E	10 ²	1.44	10 ⁻¹	0.52
F	10 ²	1.60	10 ⁻²	0.69

^a LOD was defined as the lowest dilution that gave an A₄₅₀ of >4 times the negative control and was greater than 0.400.

TCID₅₀/ml) and low (100 TCID₅₀/ml) concentrations or equal (100 TCID₅₀/ml) concentrations of the stock viruses. The results shown in Table 4 indicate that dual AdV species infections were accurately detected in each sample tested. In specimens spiked with uneven concentrations of species D and E or of species E and F, slight inhibition of detection of one of the viruses was observed. The OD values for E and F samples that were spiked at the lower concentration (100 TCID₅₀/ml) were slightly lower (2.8 and 2.0) than the >3.0 values for species D and E samples spiked at the higher concentration (1,000 TCID₅₀/ml). This slight inhibition was only observed in dual infections, because when each of these AdV species was tested individually at either concentration (100 or 1,000 TCID₅₀/ml) the OD values were >3.0 (data not shown). Furthermore, it is unlikely this slight inhibition would affect the detection of dual AdV infection in clinical specimens. No inhibition in detecting either species was observed when virus concentrations were equal.

Detection and identification of adenovirus species A through F in clinical specimens by Adenoplex. We compared the Adenoplex assay to the diagnostic gold standard of cell culture (CPE) and DFA testing to evaluate our assay's ability to detect and distinguish between the different AdV species. Our multiplex PCR assay precisely detected AdV in all 120 spiked nasopharyngeal swab specimens and did not detect AdV in the 30 unspiked specimens, as shown in Table 5. CPE alone was not sufficiently sensitive to detect AdV species A, B, and F (sensitivity, 75%). DFA performed on infected cells at day 14 detected 27 of the 30 CPE-negative samples, yielding a final culture sensitivity of 97.5%. In specimens spiked with group F AdV, 3 out of the 20 spiked specimens were positive by PCR analysis but negative by culture and DFA. Discrepant

TABLE 4. Detection of dual AdV infections in NP specimens

AdV species dual spike	Spike concn (TCID ₅₀ /ml)	EHA ^a A ₄₅₀ value
B + C	B, 1,000; C, 100	B, >3.0; C, >3.0
C + D	C, 1,000; D, 100	C, >3.0; D, >3.0
D + E	D, 1,000; E, 100	D, >3.0; E, 2.8
E + F	E, 1,000; F, 100	E, >3.0; F, 2.0
A + B	A, 100; B, 100	A, >3.0; B, >3.0
B + C	B, 100; C, 100	B, >3.0; C, >3.0
E + F	E, 100; F, 100	E, >3.0; F, >3.0
F + A	F, 100; A, 100	F, >3.0; A, >3.0

^a EHA, enzyme hybridization assay.

TABLE 5. Comparison of Adenoplex (multiplex PCR), tissue culture, and DFA in the detection of AdV species A to F in spiked NP specimens

Adenoplex result	Culture CPE		DFA	
	Positive ^a	Negative	Positive	Negative
AdV A ^b				
Positive	20	0	20	0
Negative	0	5	0	5
AdV B ^b				
Positive	10	10	20	0
Negative	0	5	0	5
AdV C ^b				
Positive	20	0	ND ^c	ND
Negative	0	5	ND	ND
AdV D ^b				
Positive	20	0	ND	ND
Negative	0	5	ND	ND
AdV E ^b				
Positive	20	0	ND	ND
Negative	0	5	ND	ND
AdV F ^c				
Positive	0	20	17	3 ^d
Negative	0	5	0	5

^a Fifty percent ($n = 10$) were spiked with 10² TCID₅₀/ml (low positive), and 50% ($n = 10$), were spiked with 10³ TCID₅₀/ml (medium positive).

^b Grown on Caco2 and HEp2 cells.

^c Grown on RMK-1 and Caco2 cells.

^d Discrepant analysis using commercial PCR (ViroMed Labs) determined samples to be true positives.

^e ND, not done.

analysis on these samples was performed at another reference laboratory using a different PCR-based assay. All three samples were confirmed as true positives by the other laboratory's assay. Compared to culture or DFA and PCR, the Adenoplex specificity and sensitivity were 100%, with a 95% confidence interval of 97 to 100% ($n = 150$).

DISCUSSION

Human AdV are responsible for causing a variety of diseases affecting all organ systems. The 51 known AdV serotypes are grouped into six different species and, generally, the AdV serotypes within a species have similar tropisms, pathogenicity, latency, and occurrence (14). Species F AdV (serotypes 40 and 41) are the second leading cause of gastroenteritis in young children, and group A AdV (serotype 31) have also been implicated in gastroenteritis in infants. AdV types belonging to species C (serotypes 1, 2, and 5) cause acute respiratory disease in children, and types in groups B (serotype 7) and E (serotype 4) are leading causes of acute respiratory disease in military trainees. Highly contagious keratoconjunctivitis is primarily caused by AdV types belonging to species D (serotypes 8, 19, and 37). For immunocompromised individuals, especially bone marrow transplant recipients, species B (serotypes 11, 34, and 35) and C (serotypes 1, 2, and 5) AdV can cause severe and sometimes fatal disease. It is unknown how many different serotypes or species can infect someone at the same time

during acute infection. We have observed dual infections (species A and C) in clinical specimens (unpublished data). Thus, an assay that is able to detect as well as differentiate between AdV species groups such as the Adenoplex can have important clinical relevance in diagnosis and for epidemiological purposes.

PCR methods of AdV detection offer a more sensitive and rapid alternative for diagnosis than traditional culture or immunofluorescence (1, 18, 20). Our data demonstrated that the Adenoplex assay was more sensitive (100%) and had equivalent specificity (100%) compared to viral culture. Three of the 20 clinical specimens that were spiked with species F AdV were positive by the Adenoplex but negative by culture and DFA. These three samples were spiked at the low concentration of 100 TCID₅₀/ml. Discrepant analysis using a different PCR assay (with the hexon gene as the target) performed at another reference laboratory confirmed that the three specimens were indeed true positives. These results demonstrated that detection of AdV by PCR is more sensitive than culture and DFA. Furthermore, the Adenoplex achieved its sensitivity in 5 h, while culture and DFA took up to 14 days. Some clinical specimens may have even lower quantities of AdV (e.g., immunocompromised patients), and the Adenoplex's lower LOD compared to culture may result in larger sensitivity differences in clinical practice.

The Adenoplex assay has advantages over several of the PCR-based assays recently described in the literature. The Adenoplex primer pairs were designed to highly conserved areas of the fiber gene. Xu et al. found that when they used hexon gene primers from another recently described species-specific multiplex assay they had poor amplification of serotypes 11, 21, 34, and 35 of species B, yet their assay using fiber gene primers amplified all species B serotypes equally well (23). The Adenoplex accurately detected these four serotypes as well as all of the other serotypes tested. The fiber gene may have areas more highly conserved among AdV serotypes. However, additional clinical testing and sequencing would need to be done to determine this. The most effective diagnostic test for AdV is one that is easy and quick to perform. Several other PCR-based assays use restriction enzyme digestion (8, 14) and gel electrophoresis (19, 23) methods for detection. These techniques are time-consuming and limit the scope of detection. In contrast, the Adenoplex detection method of enzyme hybridization is rapid, provides increased sensitivity, and gives objective results (reported as OD readings). Amplified PCR products extracted from samples with low titers of whole organisms or recombinant controls give low, but definitely positive, OD values, whereas no bands are observed by gel electrophoresis (unpublished observations). Nested PCR assays have been shown to detect AdV with good sensitivity (1, 18) but involve a two-step PCR procedure increasing the time to complete the assay and chance of carryover contamination. The Adenoplex's sensitivity (at 100 and <1,000 copies of DNA/ml) is comparable to or better than that of the nested PCR assays, which detected between 400 and 2,500 copies/ml (1) and 640 copies/ml (18), respectively. In addition, the Adenoplex is a single-step PCR and can be completed in less time than a two-step nested PCR assay. Recent interest has been shown for real-time PCR assays, which have advantages over conventional PCR in terms of time and de-

creased risk of false-positive results due to contamination. However, the sensitivity of the Adenoplex was two times greater than that of a recently published real-time assay (13). Furthermore, the Adenoplex is able to differentiate between AdV species, whereas the real-time assay could only generically detect AdV.

Understanding the epidemiology and natural history of AdV infections in humans requires a rapid, sensitive, and specific diagnostic test. Proper management and treatment of AdV infections are equally dependent on effective methods of diagnosis. Same-day, rapid-turnaround PCR assays may provide cost-effective reporting that improves patient care and treatment. Recently it has been reported that detection of AdV in serum or blood may be a precursor of severe or disseminated disease. Timely and sensitive detection of AdV may serve as a basis for preemptive antiviral treatment (6, 16). Earlier AdV detection in pediatric transplant recipients by using PCR, compared with detection by culture, may result in reduced mortality (17). PCR may be the only means by which rapid and reliable diagnosis of AdV keratoconjunctivitis can be achieved, leading to a reduction in the overall cost of diagnosis (7). Finally, PCR is able to facilitate accurate identification of viruses that cause specific diseases. In the case of viral myocarditis, AdV, not enteroviruses as previously thought, were found to be the leading cause of disease (2).

In summary, we report here the development of a sensitive and specific multiplex PCR assay, the Adenoplex, for the detection of all human AdV serotypes as well as for identification of species types. This rapid and definitive method of diagnosis offers a powerful alternative approach to AdV detection and may improve clinical management, decrease hospitalization, and lessen medical costs for patients.

ACKNOWLEDGMENTS

We thank Gerald Sedmak at the City of Milwaukee Health Department for his expertise and assistance in culturing the different AdV species and Pam Douglass, Tracy Enslow, Kristine Schraufnagel, and Katie Wilson for their technical assistance.

REFERENCES

- Avellon, A., P. Perez, J. C. Aguilar, R. Ortiz de Lajarazu, and J. E. Echevarria. 2001. Rapid and sensitive diagnosis of human adenovirus infections by a generic polymerase chain reaction. *J. Virol. Methods* **92**:113–120.
- Bowles, N. E., J. Ni, D. L. Kearney, M. Pauschinger, H. P. Schultheiss, R. McCarthy, J. Hare, J. T. Bricker, K. R. Bowles, and J. A. Towbin. 2003. Detection of viruses in myocardial tissues by polymerase chain reaction: evidence of adenovirus as a common cause of myocarditis in children and adults. *J. Am. Coll. Cardiol.* **42**:466–472.
- De Jong, J. C., A. G. Wermenbol, M. W. Verweij-Uijterwaal, K. W. Slaterus, P. Wertheim-Van Dillen, G. J. Van Doornum, S. H. Khoo, and J. C. Hierholzer. 1999. Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *J. Clin. Microbiol.* **37**:3940–3945.
- Echavarria, M., J. L. Sanchez, S. A. Kolavic-Gray, C. S. Polyak, F. Mitchell-Raymundo, B. L. Innis, D. Vaughn, R. Reynolds, and L. N. Binn. 2003. Rapid detection of adenovirus in throat swab specimens by PCR during respiratory disease outbreaks among military recruits. *J. Clin. Microbiol.* **41**:810–812.
- Echavarria, M., M. Forman, J. Ticehurst, J. S. Dumler, and P. Charache. 1998. Method for detection of adenovirus in urine of healthy and human immunodeficiency virus-infected individuals. *J. Clin. Microbiol.* **36**:3323–3326.
- Echavarria, M., M. Forman, M. J. D. van Tol, J. M. Vossen, P. Charache, and A. C. Kroes. 2001. Prediction of severe disseminated adenovirus infection by serum PCR. *Lancet* **358**:384–385.
- Elnifro, E. M., R. J. Cooper, P. E. Klapper, A. S. Bailey, and A. B. Tullo. 1999. Diagnosis of viral and chlamydial keratoconjunctivitis: which laboratory test? *Br. J. Ophthalmol.* **83**:622–627.

8. Elnifro, E. M., R. J. Cooper, P. E. Klapper, and A. S. Bailey. 2000. PCR and restriction endonuclease analysis for rapid identification of human adenovirus subgenera. *J. Clin. Microbiol.* **38**:2055–2061.
9. Fan, J., and K. J. Henrickson. 1998. Rapid simultaneous diagnosis of RSV A, B, influenza A, B, human parainfluenza virus type 1, 2, and 3 infection by multiplex quantitative RT-PCR enzyme hybridization (Hexaplex™) assay. *Clin. Infect. Dis.* **26**:1397.
10. Foy, H. 1997. Adenoviruses, p. 119–138. *In* A. S. Evans and R. A. Kaslow (ed.), *Viral infections in humans*, 4th ed. Plenum Medical Book Company, New York, N.Y.
11. Fujimoto, T., M. Chikahira, T. Kase, S. Morikawa, T. Okafuji, Y. Yokota, and O. Nishio. 2000. Single-tube multiplex PCR for rapid and sensitive diagnosis of subgenus B and other subgenera adenoviruses in clinical samples. *Microbiol. Immunol.* **44**:821–826.
12. Gray, G. C., P. R. Goswami, M. D. Malasig, A. W. Hawksworth, D. H. Trump, M. A. Ryan, and D. P. Schnurr. 2000. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. *Clin. Infect. Dis.* **31**:663–670.
13. Heim, A., C. Ebnet, G. Harste, and P. Pring-Akerblom. 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. *J. Med. Virol.* **70**:228–239.
14. Kidd, A. H., M. Jonsson, D. Garwicz, A. E. Kajon, A. G. Wermenbol, M. W. Verweij, and J. C. de Jong. 1996. Rapid subgenus identification of human adenovirus isolates by general PCR. *J. Clin. Microbiol.* **34**:622–627.
15. 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**:808–818.
16. Lion, T., R. Baumgartinger, F. Watzinger, S. Matthes-Martin, S., M. Suda, S. Preuner, B. Futterknecht, A. Lawitschka, C. Peters, U. Potschger, and H. Gadner. 2003. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. *Blood* **102**:1114–1120.
17. McLaughlin, G. E., S. Delis, L. Kashimawo, G. P. Cantwell, N. Mittal, R. E. Cirocco, P. Ruiz, T. Kato, and A. G. Tzakis. 2003. Adenovirus infection in pediatric liver and intestinal transplant recipients: utility of DNA detection by PCR. *Am. J. Transplant.* **3**:224–228.
18. Mitchell, S., H. J. O'Neill, G. M. Ong, S. Christie, P. Duprex, D. E. Wyatt, C. McCaughey, V. J. Armstrong, S. Feeney, L. Metwally, and P. V. Coyle. 2003. Clinical assessment of a generic DNA amplification assay for the identification of respiratory adenovirus infections. *J. Clin. Virol.* **26**:331–338.
19. Pring-Åkerblom, P., F. E. J. Trijssenaar, T. Adrian, and H. Hoyer. 1999. Multiplex polymerase chain reaction for subgenus-specific detection of human adenovirus in clinical samples. *J. Med. Virol.* **58**:87–92.
20. Rätty, 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.
21. Wadell, G. 1984. Molecular epidemiology of adenoviruses. *Curr. Top. Microbiol. Immunol.* **110**:191–220.
22. 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.
23. 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**:4114–4120.