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Molecular Typing of Clinical Adenovirus Specimens by an Algorithm which Permits Detection of Adenovirus Coinfections and Intermediate Adenovirus Strains

Troy McCarthy¹, Mark G. Lebeck¹, Ana W. Capuano¹, David P. Schnurr², and Gregory C. Gray¹

¹ Center for Emerging Infectious Diseases, Department of Epidemiology, University of Iowa College of Public Health, Iowa City, Iowa, USA

² CA Department of Public Health, Richmond, California

Abstract

Background—Epidemiological data suggest that clinical outcomes of human adenovirus (HAdV) infection may be influenced by virus serotype, coinfection with multiple strains, or infection with novel intermediate strains. In this report, we propose a clinical algorithm for detecting HAdV coinfection and intermediate strains.

Study Design—We PCR amplified and sequenced subregions of the hexon and fiber genes of 342 HAdV positive clinical specimens obtained from 14 surveillance laboratories. Sequences were then compared with those from 52 HAdV prototypic strains. HAdV positive specimens that showed nucleotide sequence identity with a corresponding prototype strain were designated as being of that strain. When hexon and fiber gene sequences disagreed, or sequence identity was low, the specimens were further characterized by viral culture, plaque purification, repeat PCR with sequencing, and genome restriction enzyme digest analysis.

Results—Of the 342 HAdV-positive clinical specimens, 328 (95.9%) were single HAdV strain infections, 12 (3.5%) were coinfections, and 2 (0.6%) had intermediate strains. Coinfected specimens and intermediate HAdV strains considered together were more likely to be associated with severe illness compared to other HAdV-positive specimens (OR=3.8; 95% CI = 1.2–11.9).

Conclusions—The majority of severe cases of HAdV illness cases occurred among immunocompromised patients. The analytic algorithm we describe here can be used to screen clinical specimens for evidence of HAdV coinfection and novel intermediate HAdV strains. This algorithm may be especially useful in investigating HAdV outbreaks and clusters of unusually severe HAdV disease.

Keywords

(MeSH): Adenoviridae; molecular epidemiology; recombinant viruses; genetic recombination; coinfection

Corresponding author: Troy McCarthy, Center for Emerging Infectious Diseases, Department of Epidemiology, College of Public Health University of Iowa, 2501 Crosspark RD. B158 MTF, Coralville, IA 52241, Tel:319 335-4983, Fax:319 335-4984, troy-mccarthy@uiowa.edu.

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Human adenoviruses (HAdVs) are classified by species (A-G), serotype (1–52), and genome restriction type in increasing order of uniqueness^{1, 2}. The clinical manifestations of HAdV infections, which can range from asymptomatic to multisystem failure and death, have been clearly documented to be associated with virus serotype^{3–5} and immunocompetence of the host. Novel recombinant HAdVs (intermediate strains) have also been associated with outbreaks and more severe disease. For example, HAdV19a, a novel genome type of HAdV19, caused outbreaks of keratoconjunctivitis in Europe and North America beginning in the early 1970s, and was later found to have acquired the fiber gene region of HAdV37, which likely contributed to its new ability to infect the eye^{6, 7}. HAdV7h, an intermediate strain of HAdV7 hexon and HAdV3 E3 and fiber gene regions has been associated with epidemics of severe respiratory disease in South America^{8–10}. Infections with multiple HAdV strains (coinfections) have caused vaccine breakthrough disease¹¹ and been associated with severe and fatal infections among transplant patients^{12, 13}. In spite of these consequences, specimens with evidence of HAdV are seldom typed and rarely examined for coinfections or intermediate strains. Typing HAdVs by the classical methods of serum neutralization or hemagglutination inhibition is no longer practical because of the tedious and time consuming nature of these methods and the diminishing availability of type-specific antisera. However, new sequence-based typing strategies have been shown to correlate well with virus serotype^{14–18}. As HAdV coinfections and novel intermediate strains can be particularly difficult to identify by classical serotyping methods they have rarely detected.

In a previous report, we demonstrated the value of PCR and sequence-based typing of HAdVs¹⁹ and suggested that methods to detect novel viruses should be included in HAdV surveillance programs. In this report, we present a strategy for using gene sequence typing to identify HAdV coinfection and intermediate HAdV strains.

MATERIALS AND METHODS

Specimen selection

As a pilot study to refine our molecular typing techniques, we sorted 2224 HAdV-positive clinical specimens from the National Adenovirus Surveillance Program²⁰ by their original hexon gene sequence type¹⁹ and selected, via random-number, 6 specimens from each of 16 different types (n=96). Specimens were then sorted by patient, and only the first clinical isolate per patient was included in the sample. This yielded 82 pilot study specimens. An additional sample of 277 sequentially collected HAdV-positive specimens received from the 14 collaborating laboratories during the period January 4, 2007 through March 7, 2007 were evaluated after the pilot study work.

DNA extraction, PCR, and sequencing

Total DNA was extracted using the QIAamp® DNA Blood Mini Kit (QIAGEN, Chatsworth, CA). In the pilot study, we evaluated 3 previously published HAdV molecular typing methods. The first method, described by Lu and Erdman¹⁴, uses PCR amplification and sequencing of hypervariable regions 1 through 6 (HVR1-6) of the hexon gene. PCR products for HVR1-6 ranging in size from 764 to 896 bp were obtained with these primers. A similar second method with PCR product sizes from 605 to 632 bp reported by Sarantis¹⁷ is based on sequencing hexon hypervariable region 7 (HVR7). Both methods were shown to identify the 52 recognized HAdVs by sequence identity with the respective prototype strains. A third molecular method described by Xu et al.¹⁸, uses a multiplex PCR to target the HAdV fiber gene, was also adapted for study. The fiber gene PCR generated species-specific product sizes were A, 1444–1537 bp; B, 670–772 bp; C, 1988–2000 bp; D, 1205–1221 bp; E, 967 bp; and F, 541–586 bp. However, this method had not been used to identify HAdV serotype. Therefore, we modified the method,

by employing sequencing of the fiber gene amplicons and compared sequences to those from the corresponding fiber gene region of the 52 prototype strains. Because the fiber gene amplicons for species A and C were too large for convenient sequencing, we developed primers that yielded smaller amplicons for sequencing (5'-GGCATGCTTGCGCTGAAAATGGGCA-3', Species CFwd; 5'-GATGGRKCGGGDGTGKTCCA-3', Species CRev; and 5'-TTAARCACARRGTKAGTTTTGCATC-3', Species ARev). These primers yielded smaller fiber amplicons for sequencing. In the pilot study, our goal was to compare sequence typing results from the three methods.

Amplicons were sequenced in both directions using a 3730 xl DNA Analyzer (Applied BioSystems, Inc., Foster City, CA). The forward and reverse nucleotide sequences were edited and aligned to form consensus sequences which were compared to our library of HAdV prototype strain sequences and the sequences available from GenBank ²¹. BioEdit software (Ibis Therapeutics, Carlsbad, CA) ²² was used for sequence alignments and comparisons. Genotype (formally serotype) was determined separately for the HVR1-6, HVR7, and fiber genes based on nucleotide sequence identity scores obtained with the respective prototype strains. For HVR1-6 and HVR7 sequence regions $\geq 97.5\%$ identity with corresponding prototype strain¹⁵ were designated that type. Due to the varied sizes of fiber region PCR products $\geq 95\%$ identity was the criteria for genotype determination. Specimens with typing disagreements between the hexon and fiber regions and specimens that did not meet the identity criteria were further evaluated by viral culture, plaque purification, repeat PCR and sequencing, and if warranted, restriction enzyme analysis (REA).

Restriction enzyme analysis

Nucleic acid concentrations were analyzed using the NanoDropTM spectrophotometer (NanoDrop Technologies, Wilmington, DE). Digestions were performed for each endonuclease *Bam* HI, *Bgl* II, *Hind* III, and *Sma* I. The DNA fragments were separated by electrophoresis on 1.0% agarose (Research Products International Corporation, Mt. Prospect, IL) horizontal slab gels and the gels stained with ethidium bromide as previously described ²³. Gel banding patterns were analyzed with Quantity One version 4.3.1 software (Bio-Rad, Hercules, CA) with Gel Doc transillumination. Whole genomic restriction patterns were compared with those of the HAdV prototypic strains ²⁴. All specimens still thought to be intermediate strains were further analyzed by plaque purification.

Plaque purification

Samples that were classified as containing a possible intermediate strain were purified by modified plaque purification ²⁵. Briefly, A549 cells were seeded onto 24-well culture dishes with Eagle's Minimal Essential Medium (EMEM) (Gibco/Invitrogen, Carlsbad, California) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco/Invitrogen). Serially diluted virus was used to infect each well. Plates were incubated for 4 hours at 37°C with 5% CO₂ before the A549 cells were overlaid with an agarose solution. Plates were incubated until CPE was evident. An additional overlay agarose containing of 0.01% Finter's neutral red dye was added to each well and incubated overnight. A sterile pipette tip was used to remove an agarose plug directly over each plaque. Virus was eluted from the agarose plugs by placing them into separate microcentrifuge tubes containing 1 ml EMEM and rocking the tube gently overnight at 4°C. The resultant supernatant was then used to infect A549 cells for analysis of individual plaques. The hexon and fiber genes of the purified viruses were then sequenced as described above.

Detection of coinfections and intermediate strains

We developed an algorithm (Fig. 1) to examine a clinical specimen for evidence of HAdV coinfection or the presence of an intermediate HAdV strain. After the initial analyses (step A), when the hexon and fiber gene sequence typing results indicated different HAdV strains, the fiber gene¹⁸ was reexamined with species-specific primer sets in separate PCR reactions (step B). These new fiber gene amplicons were then sequenced and compared with sequence data from HAdV prototypic strains. If the fiber species-specific primer sets indicated two different HAdV types the specimen was considered to have evidence of coinfection. Problematic specimens were plaque purified. The purified virus was analyzed (step A) to determine if the sample represented an intermediate HAdV strain or coinfecting sample. Purified virus was also studied with REA. These band patterns were compared to published band patterns²³ and those from our REA digests of the 52 prototypic HAdV strains. If the banding patterns confirmed the presence of two different HAdV strain types, the sample was classified as having evidence of HAdV coinfection.

Statistical analysis

DNA sequence and clinical specimen surveillance questionnaire data were merged. Using a Fisher's exact test, statistical comparisons were made of the patients' demographic risk factors and disease severity for specimens showing evidence of routine HAdV infection, coinfection, or the presence of novel intermediate strains. Odds for ordinal disease severity (death, ICU, hospitalization, and others) were obtained using a proportional odds model and SAS software version 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

Of the 86 pilot study specimens, 2 (2.1%) were classified as having evidence of HAdV coinfections and 2 (2.1%) were judged to contain intermediate HAdV strains. The HVR1-6, HVR7, and fiber gene sequences of the 2 intermediate strains indicated HAdV11/HAdV11/HAdV35 and HAdV34/HAdV34/HAdV11 recombination (Table 1).

Fourteen of the 277 selected sequential specimens were duplicates and therefore discarded. Seven (2.5%) of 277 specimens could not be typed by fiber gene sequencing. These samples had positive results with multiplex fiber PCR and individual species specific PCR, but sequencing of their fiber gene products were inconclusive. This could be due to a coinfection with other HAdVs of the same species. Of the remaining 256 specimens, 10 additional coinfections were identified (Table 1).

Considering both the pilot and sequential specimens, information regarding the severity of HAdV illness was available for 218 of the specimens (mostly children under 7 years old and transplant patients). Significantly higher disease severity (ordinal classification: death, ICU, hospitalization and others) was found among specimens with intermediate HAdV strains or evidence of HAdV coinfection compared to specimens with evidence of routine HAdV infection (OR=3.8; 95% CI = 1.2–11.9) (Table 2). However, most of the severity was explained by the immunocompromized condition of such patients. After adjusting for bone marrow transplant, odds for higher severity among intermediate strain/coinfection become not significant (OR=2.8; 95% CI = 0.9–9.1). All the patients with intermediate HAdV strain infection were hospitalized. Two of the patients coinfecting with multiple HAdV strains died. Among these deaths, one patient had received a bone marrow transplant and the other had received a liver transplant. HAdV infection was not reported as the primary cause of death in either case.

DISCUSSION

Most clinical virology laboratories detect HAdVs by immunoassay, culture, or broadly reactive PCR assays. Typing is seldom performed. When performed, classical HAdV typing involves tedious type-specific antiserum-mediated neutralization or hemagglutination inhibition methods (available now in only a few laboratories). This is time consuming, and it may be ineffective in distinguishing HAdV coinfections and intermediate strains. In contrast, sequencing the hexon and fiber genes can readily identify HAdV coinfections and recombinations occurring between the hexon and fiber regions of the HAdV genome.

Kroes et al. (2007)¹² and Zheng et al. (2008)²⁶ reported multiple diverse HAdV infections after allogenic stem cell transplantation, although relatively few of these were coinfections, but rather sequential HAdV infections of different HAdV types. Metzgar et al. (2007)²⁷ found 2% HAdV coinfection among recruits that presented with febrile acute respiratory disease. We found HAdV coinfection and intermediate strains prevalences of 3.5% and 0.6% respectively (pilot study and intermediate strain surveillance sub-study combined). Immunocompromised and Immunocompetant patients suffered both types of HAdV infections. Unlike the previously reported HAdV 35+HAdV11 recombinant in 2004²⁸, we found one intermediate strain that had the hexon gene from a HAdV11 and the fiber gene from a HAdV35.

Our study had a number of limitations. If clinical specimens were contaminated with one or more secondary HAdV strains at the providing laboratories, we would have incorrectly classified the specimen as clinically coinfecting. However, all the laboratories involved in our national surveillance were very accomplished clinical laboratories with active clinical proficiency programs. When a coinfecting clinical specimen is cultured through multiple passages, one virus may grow more rapidly than other viruses and thus appear as a pure sample. Thus, when we cultured virus, we sought to reduce this potential problem by performing analyses using virus from the lowest possible passage. Our study is further limited in that our algorithm often depends upon viral culture for definitive HAdV characterization. If a novel clinical HAdV does not grow well in the study cells lines, it would be missed or very difficult to characterize. Some enteric HAdVs have such poor growth characteristics and could be missed. Our algorithm will additionally miss the detection of novel intermediate strains in which the recombination involves genetic areas independent of the hexon and fiber regions. Finally, as many laboratories have supplanted cell culture diagnostic methods with molecular techniques our algorithm will only be useful to those laboratories perform culture and plaque purification.

As the sequence genotyping results for HVR1-6 agreed with that of HVR7 in our pilot study, we concluded that recombination was not occurring between HVR1-6 and HVR7. Based on this, amplification and sequencing of HVR7 was not performed with the sequentially collected specimens. All the HAdV recombination we observed occurred between the hexon and fiber regions, and not within the hexon region.

Using the study algorithm to analyze 337 HAdV positive clinical samples, we identified 12 specimens that contained multiple virus types and 2 with intermediate strains. This clinically important finding reinforces the notion that the “one disease one pathogen” mantra taught to clinicians for many years is less accurate than was assumed, and that novel strains may be more prevalent than one would expect.

In conclusion, herein we have presented an algorithm that may be used to screen clinical specimens for evidence of HAdV coinfection and intermediate HAdV strains. Until high throughput whole genome sequencing techniques become less expensive and more available, this algorithm may help epidemiologists investigate HAdV epidemics and cases of unusually severe disease.

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Fig. 1.

An algorithm for detecting the presence of multiple HAdV types or novel intermediate HAdV strains in clinical samples. 1st time here? = the first time the unknown virus has been at this point in the logic flow diagram.

Table 1

Distribution of HAdV types in pilot (n=86) and sequential surveillance (n=256) specimen samples (total n=342) using HVR1-6, HVR7, and fiber gene sequence typing methods.

Sequence typing method			Interpretation
HVR1-6 ^a (n)	Fiber gene (n)	HVR7 ^{a,b} (n)	
HAdV1 (28)	HAdV1 (28)	HAdV1 (5)	
HAdV2 (50)	HAdV2 (50)	HAdV2 (7)	
HAdV2 (1)	HAdV5 (1)	-	coinfection
HAdV3 (1)	HAdV1 (1)	-	coinfection
HAdV3 (3)	HAdV19 (3)	-	coinfection
HAdV3 (87)	HAdV3 (87)	HAdV3 (9)	
HAdV3 (1)	HAdV31 (1)	-	coinfection
HAdV3 (1)	HAdV4 (1)	-	coinfection
HAdV4 (1)	HAdV3 (1)	-	coinfection
HAdV4 (66)	HAdV4 (66)	HAdV4 (6)	
HAdV5 (1)	HAdV19 (1)	-	coinfection
HAdV5 (7)	HAdV5 (7)	HAdV5 (5)	
HAdV6 (3)	HAdV6 (3)	HAdV6 (3)	
HAdV6 (2)	HAdV9 (2)	HAdV6 (2)	coinfection
HAdV7 (1)	HAdV4 (1)	-	Coinfection
HAdV7 (23)	HAdV7 (23)	HAdV7 (4)	
HAdV11 (4)	HAdV11 (4)	HAdV11 (4)	
HAdV11 (1)	HAdV35 (1)	HAdV11 (1)	intermediate strain
HAdV12 (5)	HAdV12 (5)	HAdV12 (4)	
HAdV14 (10)	HAdV14 (10)	HAdV14 (6)	
HAdV19 (11)	HAdV19 (11)	HAdV19 (6)	
HAdV21 (10)	HAdV21 (10)	HAdV21 (6)	
HAdV31 (5)	HAdV31 (5)	HAdV31 (4)	
HAdV34 (1)	HAdV11 (1)	HAdV34 (1)	intermediate strain
HAdV34 (7)	HAdV34 (7)	HAdV34 (5)	
HAdV35 (5)	HAdV35 (5)	HAdV35 (4)	
HAdV41 (7)	HAdV41 (7)	HAdV41 (4)	

^aHVR1-6, HVR7 = hypervariable regions 1–6 and 7 of the HAdV hexon gene, respectively.

^bOnly the specimens in the pilot study were typed using sequences of HVR7.

Table 2

Comparison of clinical characteristics of patients infected with adenovirus (HAdV)

Variables	n (%)			
	Total (N =218)	Single HAdV strain (n=207)	Multiple HAdV strains (coinfection) (n=9)	Intermediate HAdV strain (n=2)
Gender				
Male	125	120 (58)	5 (55.6)	0 (0)
Female	93		4 (44.4)	2 (100)
Age group				
< 7 years old	194	185 (89.4)	8 (88.9)	1 (50)
7 years or older	23	21 (10.1)	1 (11.1)	1 (50)
Unknown	1	1 (0.5)	0 (0)	0 (0)
Severity ^a				
Death	4	2 (1)	2 (22.2)	0 (0)
ICU	16	15 (7.3)	1 (11.1)	0 (0)
Hospitalized	78	73 (35.3)	3 (33.3)	2 (100)
Others	119	116 (56)	3 (33.3)	0 (0)
Unknown	1	1 (0.5)	0 (0)	0 (0)
Transplant type ^a				
Bone marrow	21	17 (8.2)	2 (22.2)	2 (100)
Heart	1	1 (0.5)	0 (0)	0 (0)
Kidney	2	2 (1)	0 (0)	0 (0)
Liver	4	3 (1.5)	1 (11.1)	0 (0)
Lung	3	3 (1.5)	0 (0)	0 (0)
None	176	170 (82.1)	6 (66.7)	0 (0)
Others	2	2 (1)	0 (0)	0 (0)
Unknown	9	9 (4.4)	0 (0)	0 (0)
Culture Sites ^a				
respiratory tract	149	145 (70.1)	4 (44.4)	0 (0)
respiratory tract	5	4 (1.9)	1 (11.1)	0 (0)
Gastrointestinal	33	30 (14.5)	3 (33.3)	0 (0)
Urine	13	11 (5.3)	0 (0)	2 (100)
Blood	2	2 (1)	0 (0)	0 (0)
Ocular	4	3 (1.5)	1 (11.1)	0 (0)
Others	12	12 (5.8)	0 (0)	0 (0)

^a Significant difference between groups at 95% confidence level with fisher exact test (unknown not considered for testing)