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A Comparison of Viral Fitness and Virulence between Emergent Adenovirus 14p1 and Prototype Adenovirus 14p Strains

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Abstract

Background—Epidemiological studies from the last decade have suggested that the morbidity and mortality associated with a newly emergent strain of human adenovirus (HAdV-14p1) is greater than other, more prevalent, adenovirus strains. Recent molecular analysis identified very minor genetic differences in HAdV-14p1 compared to prototype HAdV-14p. No studies have evaluated how these differences may affect virulence.

Objective—To compare HAdV-14p1 and HAdV-14p strains for competitive fitness and virulence.

Study Design—We performed *in vitro* and molecular assays to evaluate growth kinetics, cellular infectivity, cytotoxicity, and plaque morphology of the two strains.

Results—Growth kinetic data showed no viral replication at 30°C and minimal differences at 37°C for both strains. Cellular infectivity data showed propagation capabilities for both strains in a diverse array of cell lines, with human lung and kidney cells having the highest propagation potential. Cytotoxicity data indicated cellular distress differences induced by both strains of virus in the first 12 hours, but similar distress levels between 12 and 48 hours. Plaque morphology assays showed some differences in average plaque diameter.

Conclusions—These data suggest that the increase in morbidity and mortality observed in recent HAdV-14p1 infections is not due to viral growth or cellular infectivity differences from the prototypic HAdV-14 strain. While there were some statistically important differences detected between strains in cytotoxicity and plaque morphology assays, it seems more likely that other factors, such as environmental stressors, co-infections, or individual host response are likely contributing to the increase in morbidity.

Keywords

adenoviruses; virology; viral diseases; virulence

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Ethical approval: Not required

BACKGROUND

Human adenovirus (HAdV) is a highly prevalent pathogen classified into 7 different species (A-G) with at least 52 unique serotypes.¹ Adenovirus infections are particularly common among military recruits, adolescents, the elderly, and immunocompromised populations.²

In 2006-2007, a relatively rare adenovirus subspecies B2 serotype 14 was found to be associated with 10 deaths and 140 respiratory illnesses in New York, Oregon, Washington, and Texas.³ This strain of adenovirus 14 was soon recognized in other geographical locations, identified as a causative agent in several community outbreaks where infected individuals presented with seemingly more severe lower respiratory disease than that usually seen with other human adenoviruses.⁴⁻¹⁰

Isolates of the strain have since been named HAdV-14p1,¹¹ sequenced, and genetically compared to the first described subspecies B2 HAdV-14 strain from the 1950's, differing only with respect to a 6 base pair deletion on the knob region of the fiber gene and a 3 base pair insertion on the E1A gene region.^{11, 12} No studies have evaluated whether these minor genetic differences affect virulence.

OBJECTIVE

Using *in vitro* and molecular assays, we sought to compare the recently isolated strain, HAdV-14p1, with the prototypic HAdV-14 strain, looking for viral characteristics that might explain an increase in virulence.

STUDY DESIGN

Viruses

Human adenovirus HAdV-14p, a prototype strain isolated from Dutch military recruits in the 1950's, was kindly provided by Dr. Dean Erdman of the Centers for Disease Control and Prevention (CDC).¹³ Human adenovirus HAdV-14p1 was collected March 2005 from the nasal swab of a 3-year-old male in Missouri, typed by previously described molecular techniques.¹⁴

Cells

Human lung cells, A549 (ATCC catalog # CCL-185), human cervix cells, HeLa (ATCC catalog # CCL-2), human liver cells, HepG2 (ATCC catalog # HB-8065), human vascular cells, EY.hy926 cells (ATCC catalog # CRL-2922), human intestinal cells, HCT-8 (ATCC catalog # CCL-224), and human kidney cells, HEK-293 (ATCC catalog # CRL-1573) were propagated in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1× non-essential amino acids (NEAA), 0.292 mg/ml glutamine, 1mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin. All cell lines were housed in a 37°C incubator with 5% CO₂.

Plaque assay

A plaque assay was used to quantify virus growth and evaluate plaque morphology for both viruses. The assay was performed using standard 6-well or 12-well cell culture plates seeded with A549 cells to reach 100% confluency upon infection. Cells were inoculated and then rocked at room temperature for one hour after which the inoculum was removed and replaced with 1 ml of 1% methyl cellulose (Catalog # M0512) mixed 1:1 with 2× MEM (20% FBS, 2× NEAA, 0.584 mg/ml glutamine, 2mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin), and placed in a 37°C incubator with 5% CO₂ for 7 days. Seven days post-infection, plates were removed, fixed, permeabilized, and stained using a

1:1 acetone/methanol solution containing 10 mg/ml crystal violet. Plates were incubated for 15 minutes at 4°C after which the fixative was decanted and the plates rinsed three times in cold tap water. Plates were dried overnight at room temperature.

Growth Kinetics/Yield

As HAdV-14p1 has been known to cause acute lower respiratory tract infections, growth kinetic assays were run at two different temperatures.^{15, 16} We used a temperature of 30°C to simulate the upper respiratory environment and a temperature of 37°C to simulate the lower respiratory and internal tissue environments. Cells were infected at a multiplicity of infection (MOI) of 0.1 with either HAdV-14p or HAdV-14p1 in serum free MEM and rocked at room temperature for one hour, after which the inoculum was removed and cells were rinsed with sterile PBS to remove excess virus. Cells were then overlaid with 1 ml of MEM with 10% FBS and incubated at either 37°C or 30°C with 5% CO₂ in air atmosphere.

Culture supernatant and cells were sampled at 0, 8, 24, 48, 72, 96, 120, and 144 hours post-infection and titrated using the plaque assay described above. Results were presented from one of two independent trials with four replicates each.

Cellular Infectivity

Since adenovirus is known to cause clinical symptoms in several different organ systems,¹⁷ the infectivity of HAdV-14p and HAdV-14p1 was evaluated in several cell lines to determine if there were differences in tissue types each virus could infect. Six cell lines (A549, HeLa, HepG2, EA.hy926, HCT-8 and HEK-293) were infected at an MOI of 1.0 with either HAdV-14p or HAdV-14p1 in serum-free MEM and rocked at room temperature for one hour, after which the inoculum was removed and the monolayers were rinsed with sterile PBS to remove excess virus. Cells were then overlaid with 1 ml of MEM with 10% FBS and incubated at 37°C with a 5% CO₂ in air atmosphere.

At 48 hours post-infection, cells were homogenized, filtered and then viral nucleic acid was extracted from filtrate. Real-time polymerase chain reaction (rPCR) was conducted utilizing the iQ5 multicolor real-time PCR detection system (Bio-Rad: Hercules, CA). The IScript One-Step RT-PCR Kit for probes was used (Bio-Rad: Hercules, CA) with Ad Hex forward (5'-GGGTTGAAACTACTGAAGAACG-3'), reverse (5'-CATCTGTATCAGTCCACG ATT-3') primers, and probes that target hypervariable regions 1–6 of the hexon gene previously described.¹⁸ The following cycling conditions were followed: initial denaturing cycle at 95.0°C for 15 minutes followed by 45 cycles of denaturation at 95.0°C for 1 s, annealing at 60.0°C for 15 s, and extension at 72.0°C for 10 s. Three different independent assays were run in triplicate.

Cytotoxicity assay

The cytotoxicities of the virus strains were measured by monitoring mitochondrial reductase activity using a MTT cell proliferation assay (Millipore Inc.: Billerica, MA) according to the manufacturer's instructions. Absorbance was measured using a PowerWave HT microplate spectrophotometer (Biotek: Winooski, VT).

Standard 96-well flat bottom cell culture plates were seeded with A549 cells in MEM with 10% FBS at a cell density of 2.0×10^4 cells/well and incubated at 37°C with 5% CO₂ in air atmosphere overnight. Once cells reached confluency, media was removed, replaced with either HAdV-14p or HAdV-14p1 diluted to an MOI of 0.1 or 10 in MEM with 2% FBS, and returned to a 37°C incubator with 5% CO₂. Cell viability was assayed at 12, 20, and 50 hours post-infection.

Results were presented as cell viability, defined as the ratio of optical density (OD) readings at a wave-length of 570 nm of infected cells and controls adjusted for background OD readings at a reference wave-length of 630 nm. Cell viability reflected the inverse of cytotoxicity, and was expressed as the mean (95% CI) of 5 replicates. Twenty-four, 2-sample, non-paired t-tests were run to compare differences in cell viability between virus strains, virus concentrations (MOI), and time points.

Plaque morphology

The plaques of HAdV-14p and HAdV-14p1 were evaluated for differences in size, which has previously been presented as an effective virulence indicator for other viruses.^{19, 20} A549 cells were infected at an MOI of 0.1 in a standard 6-well cell culture plate. Cells were inoculated and rocked for one hour at room temperature after which the inoculum was removed and the cell monolayers were rinsed with PBS. Cells were then overlaid with 4ml of 1% methyl cellulose mixed 1:1 with 2× MEM (20% FBS, 2× NEAA, 0.584 mg/ml glutamine, 2mM sodium pyruvate, 100 IU/ml penicillin, and 100 µg/ml streptomycin), and incubated at 37°C with 5% CO₂ in air atmosphere. Eight days post-infection, cells were fixed, permeabilized, and stained by the addition of 1:1 methanol and acetone containing 10 mg/ml crystal violet for 15 minutes at 4°C. The stain was decanted and the plates were rinsed three times in cold tap water. Plates were dried overnight at room temperature.

Plaque diameters were expressed as the mean plaque diameter (mm) from a randomly selected sample size of n=60 for each virus. Continuous variables were compared using a t-test, with the Satterthwaite unequal variance method used where appropriate. For comparisons of ordinal variables and where normality assumption was violated, the non-parametric Wilcoxon two-sample test, exact method, was used.

RESULTS

Growth Kinetics

HAdV-14p and HAdV-14p1 exhibited similar growth kinetics in both intracellular and extracellular samples at 37°C (Figure 1 panels a & b). Overall, there was no substantial difference in growth between HAdV-14p and HAdV-14p1 at 37°C. Neither virus propagated at 30°C.

Cellular Infectivity

Real-time PCR analysis showed positive threshold cycle (Ct) values in viral samples for both viruses in all six cell lines (A549, HeLa, HepG2, EA.hy926, HCT-8 and HEK-293) (Table 1). A comparison utilizing a Kruskal-Wallis rank test indicated that at 48 hours HAdV-14p1 had more genome copies present than HAdV-14p in A549 cells (p=0.0001), and HAdV-14p had more genome copies present than HAdV-14p1 in HeLa cells (p=0.03).

Cytotoxicity Assay

Mitochondrial reductase activity, as an indicator of cell death, showed that at an MOI of 0.1, cell viability of HAdV-14p1 was 52% higher than that of HAdV-14p (p=0.001), 12 hours post-infection. Between 20 and 50 hours, both HAdV-14p (p<.0001) and HAdV-14p1 (p<.0001) showed a decrease in cell viability, with reductions of 61% and 58%, respectively (Figure 2 panel a).

At an MOI of 10, cell viability of HAdV-14p1 was 36% higher than that of HAdV-14p (p<.0001), 20 hours post-infection. Between 20 and 50 hours, both HAdV-14p (p<.0001) and HAdV-14p1 (p<.0001) showed a decrease in cell viability, with reductions of 66% and 81%, respectively (Figure 2 panel b).

Plaque morphology

Upon comparison, the mean plaque diameters between viruses after 8 days incubation were found to be significantly different ($p = 0.04$), with the mean diameter for HAdV-14p (0.36mm) being 14% larger than the mean diameter for HAdV-14p1 (0.31mm).

DISCUSSION

We hypothesized that observed differences in the collection of assays between the two viruses could infer virulence differences. Growth kinetics data indicated that temperatures greater than 30°C are necessary for virus replication and suggested that HAdV-14p and HAdV-14p1 complete one replication cycle in 48-72 hours. At 37°C there were no substantial differences in the overall replication of each virus.

Cellular infectivity data was consistent with previously described literature, indicating that HEK-293's and A549's are optimal cell lines for *in vitro* adenovirus propagation.^{21, 22} There were no major differences in viral propagation by cell lines.

Cytotoxicity data provided some evidence of differences between strains. In the first 12 hours, HAdV-14p showed larger reductions in cell viability than HAdV-14p1, suggesting that time to apoptosis is faster for HAdV-14p. This seemed counter-intuitive, with the expectation that if cytotoxicity was the virulence mechanism, then HAdV-14p1 would have larger reductions in cell viability. It is possible that HAdV-14p1 could gain a competitive advantage in a slower time to apoptosis, allowing the virus more time to propagate, or evade a host response. Since yield results, however, showed similar growth curves, this hypothesis seems unlikely. Furthermore, beyond 12 hours, both viruses showed comparable cell viability levels, suggesting no apparent advantages.

Plaque morphology was similar to cytotoxicity results, showing some significant differences, but not in favor of HAdV-14p1. HAdV-14p was shown to have a greater average plaque diameter. It is unclear how this may affect virulence.

Our study is limited in that viral characteristics were only assayed *in vitro*, not accounting for the often complex interactions that can occur in real populations. These interactions, such as serotype-specific host immunity, could have influenced clinical outcomes. Furthermore, type specific surveillance of adenovirus is relatively poor, limiting the availability of epidemiological data prior to the recent outbreaks. This makes it difficult to say that HAdV-14 has not been circulating since the 1950's.

Case-reports and research of HAdV-14p1 outbreaks have shown viral transmission and clinical symptoms to be non-uniform and spread out across geographical locations and populations.^{3, 4, 7, 9, 12, 23} Our results seem to agree with these reports in that neither strain had a consistent advantage by our assays. Hence, it seems likely that factors other than the viral characteristics assessed in this study, such as environment, host response, or co-infections may be responsible for the observed increase in virulence in HAdV-14p1 outbreaks. Our finding could also support the position that the two viruses we have studied here are essentially the same virus.

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REFERENCES

1. Jones MS 2nd, Harrach B, Ganac RD, Gozum MM, Dela Cruz WP, Riedel B, et al. New adenovirus species found in a patient presenting with gastroenteritis. *J Virol.* Jun; 2007 81(11):5978–84. [PubMed: 17360747]
2. Lynch JP 3rd, Fishbein M, Echavarria M. Adenovirus. *Semin Respir Crit Care Med.* Aug; 2011 32(4):494–511. [PubMed: 21858752]
3. Acute respiratory disease associated with adenovirus serotype 14--four states, 2006-2007. *MMWR Morb Mortal Wkly Rep.* Nov 16; 2007 56(45):1181–4. [PubMed: 18004235]
4. Metzgar D, Osuna M, Kajon AE, Hawksworth AW, Irvine M, Russell KL. Abrupt emergence of diverse species B adenoviruses at US military recruit training centers. *J Infect Dis.* Nov 15; 2007 196(10):1465–73. [PubMed: 18008225]
5. Gray GC, Chorazy ML. Human adenovirus 14a: a new epidemic threat. *J Infect Dis.* May 15; 2009 199(10):1413–5. [PubMed: 19351263]
6. Lewis PF, Schmidt MA, Lu X, Erdman DD, Campbell M, Thomas A, et al. A community-based outbreak of severe respiratory illness caused by human adenovirus serotype 14. *J Infect Dis.* May 15; 2009 199(10):1427–34. [PubMed: 19351259]
7. Tate JE, Bunning ML, Lott L, Lu X, Su J, Metzgar D, 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.* May 15; 2009 199(10):1419–26. [PubMed: 19351260]
8. Lessa FC, Gould PL, Pascoe N, Erdman DD, Lu X, Bunning ML, et al. Health care transmission of a newly emergent adenovirus serotype in health care personnel at a military hospital in Texas, 2007. *J Infect Dis.* Dec 1; 2009 200(11):1759–65. [PubMed: 19842979]
9. Outbreak of adenovirus 14 respiratory illness--Prince of Wales Island, Alaska, 2008. *MMWR Morb Mortal Wkly Rep.* Jan 15; 2010 59(1):6–10. [PubMed: 20075838]
10. Esposito DH, Gardner TJ, Schneider E, Stockman LJ, Tate JE, Panozzo CA, et al. Outbreak of pneumonia associated with emergent human adenovirus serotype 14--Southeast Alaska, 2008. *J Infect Dis.* Jul 15; 2010 202(2):214–22. [PubMed: 20533881]
11. Kajon AE, Lu X, Erdman DD, Louie J, Schnurr D, George KS, et al. Molecular epidemiology and brief history of emerging adenovirus 14-associated respiratory disease in the United States. *J Infect Dis.* Jul 1; 2010 202(1):93–103. [PubMed: 20500088]
12. Hough HS, Gong H, Kajon AE, Jones MS, Kuschner RA, Lyons A, et al. Genome sequences of human adenovirus 14 isolates from mild respiratory cases and a fatal pneumonia, isolated during 2006-2007 epidemics in North America. *Respir Res.* 2010; 11:116. [PubMed: 20738863]
13. Van Der Veen J, Kok G. Isolation and typing of adenoviruses recovered from military recruits with acute respiratory disease in The Netherlands. *Am J Hyg.* Mar; 1957 65(2):119–29. [PubMed: 13410904]
14. Lu X, Erdman DD. Molecular typing of human adenoviruses by PCR and sequencing of a partial region of the hexon gene. *Arch Virol.* Aug; 2006 151(8):1587–602. [PubMed: 16502282]
15. Lang V, Marjuki H, Krauss SL, Webby RJ, Webster RG. Different incubation temperatures affect viral polymerase activity and yields of low-pathogenic avian influenza viruses in embryonated chicken eggs. *Arch Virol.* Jun; 2011 156(6):987–94. [PubMed: 21302122]
16. Chen P, Shakhnovich EI. Thermal adaptation of viruses and bacteria. *Biophys J.* Apr 7; 2010 98(7):1109–18. [PubMed: 20371310]
17. Mena KD, Gerba CP. Waterborne adenovirus. *Rev Environ Contam Toxicol.* 2009; 198:133–67. [PubMed: 19253037]
18. 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(9):e7081. [PubMed: 19759905]

19. Schloer GM, Hanson RP. Relationship of plaque size and virulence for chickens of 14 representative Newcastle disease virus strains. *J Virol.* Jan; 1968 2(1):40–7. [PubMed: 4911841]
20. Madansky CH, Bratt MA. Relationships among virus spread, cytopathogenicity, and virulence as revealed by the noncytopathic mutants of Newcastle disease virus. *J Virol.* Dec; 1981 40(3):691–702. [PubMed: 7321100]
21. Brown M, Petric M. Evaluation of cell line 293 for virus isolation in routine viral diagnosis. *J Clin Microbiol.* Apr; 1986 23(4):704–8. [PubMed: 3009540]
22. Smith CD, Craft DW, Shiromoto RS, Yan PO. Alternative cell line for virus isolation. *J Clin Microbiol.* Aug; 1986 24(2):265–8. [PubMed: 3018038]
23. Louie JK, Kajon AE, Holodniy M, Guardia-LaBar L, Lee B, Petru AM, et al. Severe pneumonia due to adenovirus serotype 14: a new respiratory threat? *Clin Infect Dis.* Feb 1; 2008 46(3):421–5. [PubMed: 18173356]

Figure 1.

Panel a (left) -Relative virus titers (4 replicates each, mean $\pm$ SEM) of 37°C intracellular samples 0, 8, 24, 48, 72, 96, 120, and 144 hours post-infection, HAdV-14p vs. HAdV-14p1. Panel b (right) Relative virus titers (4 replicates each, mean $\pm$ SEM) of 37°C extracellular samples 0, 8, 24, 48, 72, 96, 120, and 144 hours post-infection, HAdV-14p vs. HAdV-14p1. Variability among replicates was often too small for error bars to be visible.

Figure 2.

Panel a (left) -Changes in cell viability (5 replicates each, mean $\pm$ SEM) for low multiplicity of infection (MOI) conditions at 12, 20, and 50 hrs, HAdV-14p vs. HAdV-14p1. Panel b (right) -changes in cell viability (5 replicates each, mean $\pm$ SEM) for high multiplicity of infection (MOI) conditions at 12, 20, and 50 hrs, HAdV-14p vs. HAdV-14p1. *Statistically significant difference

Table 1

Cellular infectivity results calculated using mean Ct values with 95% confidence intervals, p-values calculated from the Ct value differences of HAdV-14p and HAdV-14p1

Cell Type	HAdV-14p	HAdV-14p1	p-value
A549	24.3 (23.4,25.2)	21.9 (21.2,22.5)	0.0001
HeLa	32.3 (31.9,32.8)	33.3 (32.5,34.1)	0.03
HepG2	28.8 (26.1,31.4)	30.5 (28.3,32.8)	0.3
Endo	30.8 (28.3,33.4)	30.2 (28.8,31.7)	0.6
HCT	13.2 (12.4,14.1)	14.3 (13.4,15.2)	0.07
Hek293	17.8 (16.2,19.5)	19.5 (15.8,23.3)	0.4

Ct= cycle threshold