Human Adenovirus in Children with Acute Gastroenteritis in the New Vaccine Surveillance Network (NVSN)

by

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Abstract

Acute gastroenteritis (AGE) is a leading global cause of pediatric morbidity and mortality. Human adenoviruses (HAdV) are a cause, but the burden is not fully defined. We sought to determine the prevalence and clinical features of HAdV F40/41 in pediatric patients at seven U.S. children's hospitals. We enrolled children with AGE or age-matched healthy controls between 12/5/2016-11/30/2017 in the Centers for Disease Control and Prevention (CDC) New Vaccine Surveillance Network (NVSN). Seven NVSN sites enrolled a total of 4,831 subjects between inpatient, ED, and healthy controls. Demographic and clinical data and stool samples were prospectively collected. Stool samples were tested for HAdV using multiplex PCR. HAdV-positive samples from Pittsburgh and Kansas City sites were sequenced for genotyping. A total of 1,980 subjects were included in the study. In the ED and inpatient settings, 108 (16.7%) and 77 (10%) patients tested positive for HAdV, respectively, while only 19 (3.4%) healthy control patients tested positive. Among all subjects with AGE, the overall average age was 2.1 years old (SD ± 3.1y), but inpatients were older (3 ± 4.1 years) compared to healthy controls (1.6 ± 2.3y) and ED patients (1.2 ± 1.7y). The most common symptoms of HAdV in both inpatient and ED settings were fever, diarrhea, and vomiting. HAdV+ children experienced significantly more diarrhea than HAdV- children in both inpatient and ED. Six genotypes were detected: B3 (2/120), C1 (5/120), C2 (2/120), C5 (1/120), F40 (4/120) with majority F41 (106/120). There was no

difference in genotypes by site or clinical setting. HAdVs are a leading cause of AGE in young children, often resulting in hospitalization. The majority of cases were type F41, which has implications for vaccine development.

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1.0 Introduction

1.1 Acute Gastroenteritis

Acute gastroenteritis (AGE) is a leading cause of morbidity and mortality in children worldwide. In 2016, it was estimated that AGE accounted for 1.1 billion episodes, 450,000 deaths and 40 million disability-adjusted life-years in children less than 5 years old (1). Children are at a higher risk of AGE due to close proximity at childcare centers and schools, the lack of acceptable hygiene and sanitation practices as well as the absence of a fully developed immune system. While majority of global deaths occur in developing countries, AGE is a common reason for Emergency Department admissions and hospitalizations in young children globally.

Gastroenteritis is an inflammation of the stomach, small intestine, or large intestine. Symptoms include vomiting, nausea, fever, abdominal pain, and diarrhea, which can lead to severe dehydration. Dehydration can be associated with electrolyte disturbance and metabolic acidosis, a dangerous and life-threatening complication (2). Diarrhea is defined by a change in consistency of stools to loose or liquid as well as increased frequency of more than 3 per day (3). Symptoms of AGE can range from mild to severe and are typically self-limiting in an immunocompetent host. In an immunocompromised host, particularly bone marrow transplant patients, gastroenteritis can be life threatening (4) (5). The routine use of antibiotics, antidiarrheal agents and antiemetics is not recommended as a treatment of AGE in kids and may cause harm (2). With limited treatments avaliable, prevention of AGE is key such as good hand hygiene, sanitation, and vaccination.

Improvements in prevention measures have greatly reduced the global burden of AGE in recent years, but progress is still needed. Unfortunately, reductions in AGE deaths have not been evenly

distributed, with a substantial number still occurring in developing nations. AGE is currently the second leading cause of childhood mortality in developing nations (6). Gastroenteritis and diarrheal diseases account for one in eight deaths among children less than 5 years old annually in Africa, Asia and South America (7, 8). The burden of AGE in developing countries is difficult to calculate and often underestimated. Diarrhea due to AGE can negatively affect early childhood development through enteric dysfunction and impaired uptake of macronutrients and micronutrients (9). In a study of Nepali children, those exposed to diarrhea at an early age were 2.5 times more likely to have a disability compared to children without early childhood diarrhea exposure (10). These developmental issues can result in many detrimental effects on not only the individual, but their family and future as well which can contribute to the cycle of poverty.

In developed countries, gastroenteritis is looked at more as a nuisance than a life-threatening occurrence. In these countries, mortality due to AGE is low, instead resulting in numerous episodes, general practitioner consultations and hospitalizations (11). There are both direct and indirect cost of AGE in kids, resulting in a burden not only on the individual, but the family, society, healthcare, and economic system. There are direct costs such as hospital bills, transportation costs, and missed work of the child's parent. Indirect costs include the child missing school, loss of productivity of the parent and more. While the mortality burden of AGE in developed nations has significantly decreased, it is difficult to calculate the overall burden of the direct and indirect costs of AGE.

AGE is caused by a variety of viral, bacterial, and parasitic pathogens, resulting in difficultly of establishing cause and treatment. Bacterial causes of AGE include *Campylobacter*, *Clostridium difficile* toxin A/B, *Escherichia coli* 0157, Enterotoxigenic *E. coli* (ETEC), Shiga-like Toxin producing *E. coli* (STEC), *Salmonella*, *Shigella*, *Vibrio cholerae*, *Yersinia enterocolitica.*

Common parasites associated with AGE are *Cryptosporidium*, *Entamoeba histolytica* and *Giardia.* Viral agents are the most common cause of AGE and include Adenovirus, Rotavirus, Norovirus, Astrovirus and Sapovirus. Prior to 2006 and the approval of the first Rotavirus vaccine, it was the leading cause of severe childhood gastroenteritis (12). After the implementation of the Rotavirus vaccine, it is estimated that there was a reduction of 382,000 hospitalizations and $1.228 billion in medical costs (13). With the reduction of Rotavirus associated AGE, Norovirus and Adenovirus have emerged as leading causes of AGE in children, resulting in substantial healthcare associated costs.

1.2 Human Adenovirus

Human adenovirus (HAdV) is a known cause of AGE in pediatric populations; however, the burden is not fully defined. HAdV is easily spread from person to person via inhalation, direct contact with small aerosol droplets, or fecal-oral transmission (14). HAdVs are readily transmissible and can be highly contagious. In low-resource countries, poor sanitation can result in outbreaks, but outbreaks have also occurred in close-proximity populations such as military bases and hospital wards (15, 16). HAdVs are recognized as a significant viral pathogen associated with high morbidity and mortality among immunocompromised patients (17).

HAdV is a non-enveloped, double-stranded DNA virus in the genus *Mastadenovirus* in the family *Adenoviridae*. The icosahedral capsid of the virus consists of 252 capsomeres, with 240 hexons forming the faces and 12 pentons bearing a slender fiber at the vertices (18). HAdVs are hardy viruses that can last on surfaces for days or even weeks. HAdV is classified into 7 species (A-G) with >70 different types (19). Each species is associated with a different clinical

manifestation. HAdV can rarely result in hemorrhagic cystitis, hepatitis, hemorrhagic colitis, pancreatitis, nephritis or encephalitis (20) but are most commonly associated with infections of the upper or lower respiratory tract, conjunctivitis or AGE. HAdV types B, C, and E are commonly associated with respiratory infections (21). Certain types can also be associated with disease severity, for instance HAdV type C is often associated with mild respiratory illness, while type B is associated with more severe and disseminated infection of the respiratory tract (21). Adenovirus type D is commonly associated with severe conjunctivitis (22, 23). HAdV types F40/41 are most associated with AGE.

HAdV types A, B, C, D and G have all been detected in cases of gastroenteritis, but the etiological roles and potential of viral shedding from other infections make their role in AGE unclear (24). While HAdV types F40/41 have long been established as causative roles of AGE, the burden remains to be difficult to fully define. Certain studies indicate that the burden of HAdV may be as low in hospitalized kids as 5% (25), while others suggest the burden being as high as almost 30% (24). Recent studies suggest that while types F40 and F41 are both associated with AGE, there has been a shift towards most cases being caused by HAdV F41 (26).

Currently, the only HAdV vaccine targets common respiratory illness causing types 4 and 7, and is only given to military recruits (27). While antiviral therapies are in development, there is currently no specific antiviral drug for the treatment of enteric HAdV infection (28). The only means of current protection against enteric adenovirus infection is means of prevention including avoiding contaminated food and water, good hand-hygiene and improved sanitation. A vaccine targeted at enteric adenovirus would vastly reduce the disease burden and hospitalizations in pediatric populations within the United States.

1.3 New Vaccine Surveillance Network (NVSN)

The New Vaccine Surveillance Network (NVSN) focuses on the two leading causes of morbidity and mortality in kids <5 years old, Acute Respiratory Infection (ARI) and AGE. The NVSN is an active, prospective, population-based surveillance system with the purpose of data collection and analysis on the impact of vaccines and vaccine policies in relation to ARI and AGE. This case-control surveillance system utilizes the collection of pediatric respiratory samples and gastroenteritis samples from Inpatient, Emergency Department (ED) and healthy controls to further understand and define hospitalizations associated with these diseases. The NVSN conducts surveillance at seven pediatric hospitals across the United States including Vanderbilt University Medical Center, University of Rochester School of Medicine and Dentistry, Cincinnati Children's Hospital Medical Center, Texas Children's Hospital, Seattle Children's Hospital, Children's Mercy Hospital, and UPMC Children's Hospital of Pittsburgh. We sought to explore the contribution of HAdV and species type to AGE among a prospective cohort of children in this national multi-center study.

2.0 Materials and Methods

2.1 Patients and Specimen Collection/Preparation

Subjects were prospectively enrolled year-round between 12/5/2016 and 11/30/2017 as a part of the New Vaccine Surveillance Network (NVSN) AGE study conducted by the Centers for Disease Control and Prevention (CDC). The NVSN study methods have been reported in detail previously (29, 30). Participating NVSN sites included Texas Children's Hospital (Houston, TX), University of Rochester Medical Center (Rochester, NY), Cincinnati Children's Hospital and Medical Center (Cincinnati, OH), Vanderbilt University Medical Center (Nashville, TN), Children's Mercy Hospital (Kansas City, MO), Seattle Children's Hospital (Seattle, WA), and UPMC Children's Hospital of Pittsburgh (Pittsburgh, PA).

Patients presenting with AGE symptoms including non-bloody diarrhea ($\geq$3 loose stools in a 24-hour period) and/or vomiting ($\geq$1 episode in a 24-hour period) were enrolled at all sites as inpatients or in the Emergency Department (ED). Healthy controls were enrolled during well-child visits with no history of AGE symptoms during the 14 days prior to enrollment; healthy controls were matched by age within 6 months and season within 2 weeks. Demographic and medical data were collected from parents/guardians prospectively using a standardized questionnaire. Informed consent was obtained and the study was approved by the University of Pittsburgh Institutional Review Board (IRB). Stool or diaper specimens were collected from children older than 14 days through 18 years of age for inpatients, through 5 years old for ED patients, and up to 11 years old for healthy controls. Patients were eligible for the study if they experienced AGE symptoms for $\leq$ 10 days and were residents of the participating hospital's county. Samples were collected and

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sent to the laboratory within 10 days of symptom onset, where the samples were stored at -20˚C until processing. Samples that were received as liquid specimens in diapers were eluted from the diaper liner using Earle's Balanced Salt Solution (EBSS).

2.2 Extraction and Adenovirus Detection

Total Nucleic Acid (TNA) extractions were performed on a MagMAX-96 express automated instrument (ABI) using the MagMAX Total Nucleic Acid Isolation Kit (Thermo Fisher). Multi-pathogen detection of stool samples was performed using the Luminex xTAG Gastrointestinal Pathogen Panel (GPP) according to the manufacturers' instructions with analysis software TDAS version 2.40. The GPP tests for pathogens commonly associated with AGE including: *Campylobacter*, *Clostridium difficile* toxin A/B, *Escherichia coli* 0157, Enterotoxigenic *E. coli* (ETEC), Shiga-like Toxin producing *E. coli* (STEC), *Salmonella*, *Shigella*, *Vibrio cholerae*, *Yersenia enterocolitica*, Adenovirus 40/41, Norovirus GI/GII, Rotavirus A, *Cryptosporidium*, *Entamoeba histolytica* and *Giardia*.

2.3 PCR and Sequencing

Specimens that tested positive for HAdV F40/41 on the Luminex GPP were analyzed by conventional PCR. PCR was performed at 95˚C for 15 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 1 min, and 72˚C for 2 min with a final extension at 72˚C for 7 min and then held at 4˚C. The partial hexon gene was amplified using the forward primer F1 (5'

TICTTTGACATICGIGGIGTICTIGA 3') and reverse primer R1 (5' CTGTCIACIGCCTGRTTCCACA 3'). Samples were visualized on a 1.5% agarose gel by gel electrophoresis. Samples that did not produce a band were amplified further through nested PCR utilizing the forward primer F2 (5' GGYCCYAGYTTYAARCCCTAYTC 3') and reverse primer R2 (5' GGTTCTGTCICCCAGAGARTCIAGCA 3'). Nested RT-PCR was performed at 95˚C for 15 min, followed by 35 cycles of 94˚C for 30 sec, 45˚C for 1 min, and 72˚C for 2 min with a final extension at 72˚C for 7 min and then held at 4˚C. The samples were visualized on a 1.5% agarose gel. Samples were sent to Macrogen Corp. for PCR purification and sequencing. Results were analyzed in MacVector version 16.0.10. Phylogenetic analysis performed using MegaX.

3.0 Results

3.1 HAdV in the NVSN
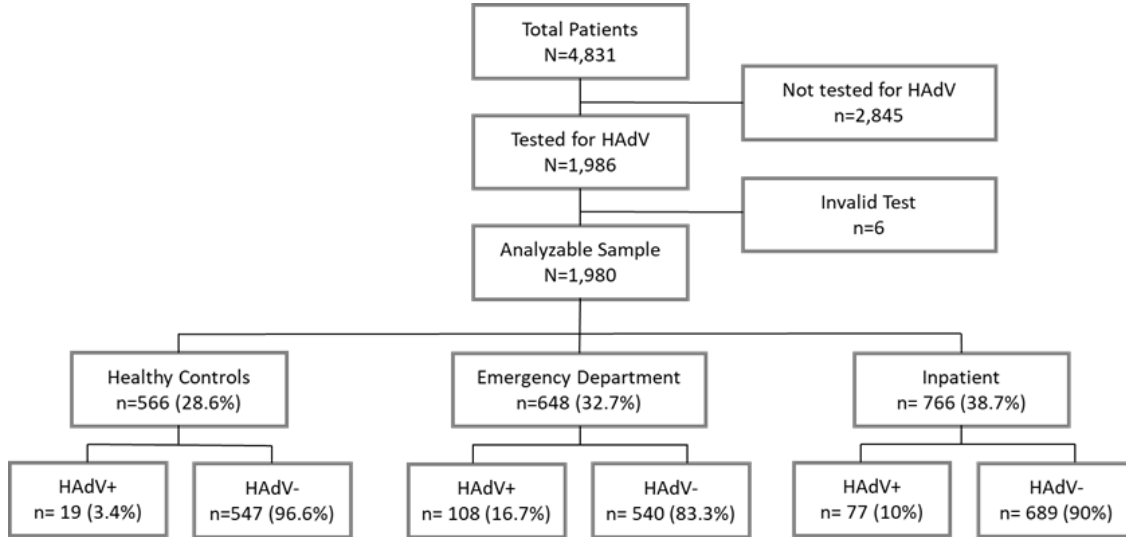
Figure 1. Flow chart of patients enrolled and HAdV testing results for all NVSN sites between 12/2016 - 12/2017

There was a total of 4,831 subjects with AGE from all NVSN sites; of those, 1,980 had stools collected and tested for HAdV (Figure 1). Of the 1,980 specimens, 566 (28.6%) were healthy controls, 648 (32.7%) ED, and 766 (38.7%) inpatients. Among the healthy controls, 3.4% of patients tested positive for HAdV while in the ED and inpatient clinical settings, 16.7% and 10% of patients tested positive for HAdV, respectively.

3.2 HAdV Seasonality

We analyzed seasonality data at the Pittsburgh site during the included study year (2017) but also had the seasonality data for 2018 (Figure 2). We noted a difference in the number of HAdV positive (HAdV+) and HAdV negative (HAdV-) samples in both inpatient and the ED between 2017 and 2018. From December 2016 to January 2018, we detected 47 HAdV+ patients in the ED and 31 HAdV+ inpatients. While there were steady case numbers in 2017, after samples peaked in January 2018, HAdV+ samples dropped to only 6 inpatient and 3 in the ED.
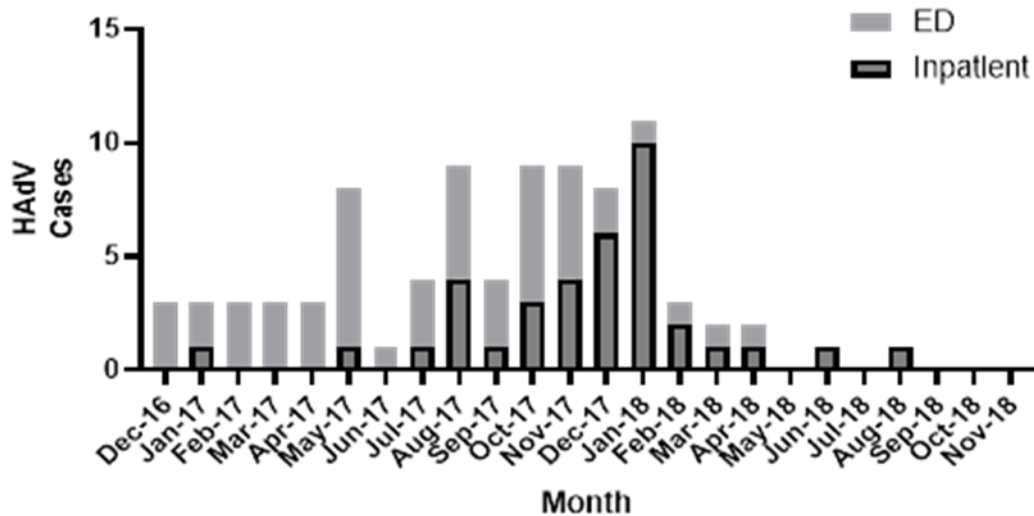

Figure 2. Seasonality of the Pittsburgh HAdV positive cases between December 2016 and November 2018 in inpatient and ED samples

3.3 HAdV Coinfection

Out of the 87 samples that tested positive for HAdV F40/41 at the Pittsburgh site, 21 samples (24%) had another pathogen co-detected (Table 1). Six subjects had co-detection of >1 of the tested pathogens. The most common co-detections with HAdV were norovirus (NoV), rotavirus (RV), and *C. difficile*.

Table 1. Coinfections of Positive HAdV specimens

Coinfections:	
Norovirus	1
Norovirus/Rotavirus	1
Norovirus/*E. coli* 0157	1
Rotavirus	5
Rotavirus/*Giardia*	1
Rotavirus/*C. difficile*	2
C. difficile	6
C. difficile/Shigella	1
E. coli 0157	2
Salmonella	1

3.4 Baseline Characteristics of Subjects with AGE

We analyzed the demographic characteristics of all subjects included in the study (Table 2). There was an even distribution of males and females in the healthy controls, ED, and inpatients. Overall, the race of most patients was White (49.6%), followed by Black (33.3%) and Others (17.1%) with a majority being non-Hispanic (76.1%). A similar distribution was seen in the inpatient and ED settings, though more healthy controls were Black (51.6%). Most patients were on public insurance (69.5%), and this was true across all clinical settings, though a higher

proportion of inpatients had private insurance than ED or healthy controls. The majority of samples were collected from the Rochester (18.7%), Cincinnati (19.3%), Houston (22.1%) and Pittsburgh (21.9%) NVSN sites. While the overall average age for AGE patients was 2.1 years old (SD ± 3.1), inpatients were older (3 ± 4.1 years) compared to healthy controls (1.6 ± 2.3 years) and ED subjects (1.2 ± 1.7 years).

Table 2. Baseline characteristics of AGE subjects by clinical setting

Characteristics	Total	Clinical setting			P value
	N=1,980	Healthy	ED	Inpatient	
	N (%)	n=566 (28.6%)	n=648 (32.7%)	n=766 (38.7%)	
Sex					0.2661
Female	941 (47.5)	276 (48.8)	291 (44.9)	374 (48.8)	
Male	1,039 (52.5)	290 (51.2)	357 (55.1)	392 (51.2)	
Race					<.0001
White	981 (49.6)	185 (32.7)	282 (43.5)	514 (67.0)	
Black	660 (33.3)	292 (51.6)	242 (37.4)	126 (16.5)	
Others	339 (17.1)	89 (15.7)	124 (19.1)	126 (16.5)	
Hispanic					<.0001
No	1,454 (76.1)	467 (84.3)	450 (71.5)	537 (73.7)	
Yes	458 (23.9)	87 (15.7)	179 (28.5)	192 (26.3)	
Insurance Status					<.0001
Public	1,352 (69.5)	420 (74.2)	481 (76.5)	451 (60.1)	
Private	514 (26.4)	135 (23.8)	125 (19.9)	254 (33.9)	
Both	37 (1.9)	6 (1.1)	8 (1.3)	23 (3.1)	

None	42 (2.2)	5 (0.9)	15 (2.3)	22 (2.9)	
Clinical Site					<.0001
Vanderbilt	118 (6.0)	19 (3.4)	50 (7.7)	49 (6.4)	
Rochester	371 (18.7)	63 (11.1)	185 (28.5)	123 (16.1)	
Cincinnati	383 (19.3)	227 (40.1)	93 (14.4)	63 (8.2)	
Seattle	44 (2.2)	0 (0.0)	1 (0.2)	43 (5.6)	
Houston	438 (22.1)	138 (24.4)	105 (16.2)	195 (25.5)	
Kansas City	193 (9.8)	0 (0.0)	72 (11.1)	121 (15.8)	
Pittsburgh	433 (21.9)	119 (21.0)	142 (21.9)	172 (22.4)	
Age, mean (SD), Yrs	2.1 (3.1)	1.6 (2.3)	1.2 (1.7)	3 (4.1)	<.0001

3.5 HAdV in the ED Clinical Setting

Next, we compared HAdV- andHAdV+ subjects with AGE in the ED and inpatient clinical settings (Table 3). In the ED setting, there were 540 HAdV- subjects (83.3%) and 108 HAdV+ (16.7%). There was no significant difference between HAdV- and HAdV+ ED subjects as far as sex and race, except fewer HAdV+ subjects were Hispanic (p=0.0016). ED subjects were more likely to have public rather than private insurance (p=0.0449). There were significantly more HAdV+ samples from the Rochester and Pittsburgh ED sites. HAdV is also associated with acute respiratory infection (ARI), and subjects with both AGE and ARI symptoms could be enrolled in the NVSN; therefore, we examined whether any HAdV+ subjects had both illnesses. Most subjects were not experiencing ARI symptoms at the time of AGE presentation, and this did not differ

between HAdV- and HAdV+. Both HAdV+ and HAdV- subjects with AGE predominantly experienced fever, diarrhea, and vomiting. There was no significant difference between positive and negative subjects for rotavirus co-infection. The average age of subjects with AGE in the ED did not vary between HAdV- (1.3 $\pm$ 1.8 years) and HAdV+ (1.1 $\pm$ 1.1 years). The number of days since onset of illness before ED arrival was longer in HAdV- children (7.6 $\pm$ 16.7 days) than HAdV+ children (4.4 $\pm$ 8.7 days).

3.6 HAdV in the Inpatient Clinical Setting

In the Inpatient setting, there were 689 HAdV- (89.9%) and 77 HAdV+ (10.1%). While there was no difference in sex between HAdV- and HAdV+ inpatients, most subjects were White, non-Hispanic, and had public insurance. There was a higher occurrence of HAdV+ subjects in the inpatient setting compared to ED for the Rochester and Pittsburgh sites. The majority of subjects with AGE did not have a concurrent ARI. Both HAdV+ and HAdV- subjects experienced fever, diarrhea, vomiting, and dehydration. More HAdV- subjects experienced abdominal pain (14.9%) compared to HAdV+ subjects (5.2%) in the inpatient setting. Subjects with rotavirus coinfection were not common in either group but were slightly more common among inpatients. HAdV- subjects tended to be older (3.2 $\pm$ 4.2 years) compared to HAdV+ subjects (1.3 $\pm$ 1.6 years). The number of days since the onset of illness was longer in the HAdV+ group (7.4 $\pm$ 15.9 days) compared to the HAdV- group (4.9 $\pm$ 9.4 days).

3.7 HAdV Positives in Inpatient vs. ED

Next, we compared the HAdV+ subjects between the inpatient and ED settings. In both settings, we saw similar demographic data between HAdV+ children as far as sex, race, and insurance status. In both settings, there tended to be more HAdV+ subjects from the Rochester and Pittsburgh sites, potentially indicating geographic differences in HAdV infection. Most subjects in both inpatient and ED did not have coexisting ARI symptoms. While the most common symptoms in both settings were fever, diarrhea, and vomiting, more children admitted to inpatient experienced dehydration (18.2%) compared to the ED (0.9%). HAdV+ subjects tended to be younger in both settings with the average age of 1.1 years in the ED and 1.3 years for inpatient. While the days of symptom onset in the ED was longer in HAdV- subjects compared to HAdV+, this was reversed in the inpatient setting.

Table 3. Baseline characteristics of AGE subjects by clinical setting and HAdV+ vs. HAdV-. The HAdV-category includes samples that may have tested positive for other AGE causing pathogens on the GPP Panel.

Characteristics	ED Setting		P value	Inpatient Setting		P value
	HAdV- n=540(83.3%)	HAdV+ n=108 (16.7%)		HAdV- n=689 (89.9%)	HAdV+ n=77 (10.1%)	
Sex			0.5962			0.3874
Female	240 (44.4)	51 (47.2)		340 (49.3)	34 (44.2)	
Male	300 (55.6)	57 (52.8)		349 (50.7)	43 (55.8)	
Race			0.9352			0.1430
White	236 (43.7)	46 (42.6)		470 (68.2)	44 (57.1)	
Black	200 (37.0)	42 (38.9)		109 (15.8)	17 (22.1)	
Others	104 (19.3)	20 (18.5)		110 (16.0)	16 (20.8)	
Hispanic			0.0016			0.3312
No	360 (69.0)	90 (84.1)		479 (73.1)	58 (78.4)	
Yes	162 (31.0)	17 (15.9)		176 (26.9)	16 (21.6)	
Insurance Status			0.0449			0.7917
Public	408 (77.7)	73 (70.2)		406 (60.2)	45 (59.2)	
Private	96 (18.3)	29 (27.9)		226 (33.5)	28 (36.8)	
Both	6 (1.1)	2 (1.9)		21 (3.1)	2 (2.6)	

None	15 (2.9)	0 (0.0)		21 (3.1)	1 91.3)	
Clinical Site			<.0001			0.0014
Vanderbilt	50 (9.3)	0 (0.0)		46 (6.7)	3 (3.9)	
Rochester	131 (24.3)	54 (50.0)		101 (14.7)	22 (28.6)	
Cincinnati	83 (15.4)	10 (9.3)		58 (8.4)	5 (6.5)	
Seattle	0 (0.0)	1 (0.9)		37 (5.4)	6 (7.8)	
Houston	98 (18.1)	7 (6.5)		187 (27.1)	8 (10.4)	
Kansas City	72 (13.3)	0 (0.0)		112 (16.3)	9 (11.7)	
Pittsburgh	106 (19.6)	36 (33.3)		148 (21.4)	24 (31.2)	
ARI			0.1933			0.5646
No	465 (86.1)	98 (90.7)		592 (85.9)	68 (88.3)	
yes	75 (13.9)	10 (9.3)		97 (14.1)	9 (11.7)	
Symptoms						
Fever	306 (56.7)	56 (51.8)	0.3576	432 (62.7)	43 (55.8)	0.2398
Diarrhea	367 (68.0)	86 (79.6)	0.0158	515 (74.8)	67 (87.0)	0.0169
Vomiting	460 (85.2)	95 (88.0)	0.4523	599 (86.9)	69 (89.6)	0.5055
Abdominal pain	34 (6.3)	5 (4.6)	0.5062	103 (14.9)	4 (5.2)	0.0192
Dehydration	20 (3.7)	1 (0.9)	0.1367	132 (19.2)	14 (18.2)	0.8361
Enteritis/Rotavirus	1 (0.2)	0 (0.0)	---	15 (2.2)	5 (6.5)	0.0243
Age, in years, mean (SD)	1.3 (1.8)	1.1 (1.1)	0.2846	3.2 (4.2)	1.3 (1.6)	<.0001
Number of days since onset of illness, Mean (SD)	7.6 (16.7)	4.4 (8.7)	0.0042	4.9 (9.4)	7.4 (15.9)	0.1850

3.8 Adenovirus Subtyping

We sequenced the partial hexon gene 120 HAdV positives from Pittsburgh (81/120) and Kansas City (39/120) (Figure 3). Six different types were identified: B3 (2/120), C1 (5/120), C2 (2/120), C5 (1/120), F40 (4/120) with the majority F41 (106/120). There was no significant difference in subtypes between the two sites as well as between inpatient and ED samples.
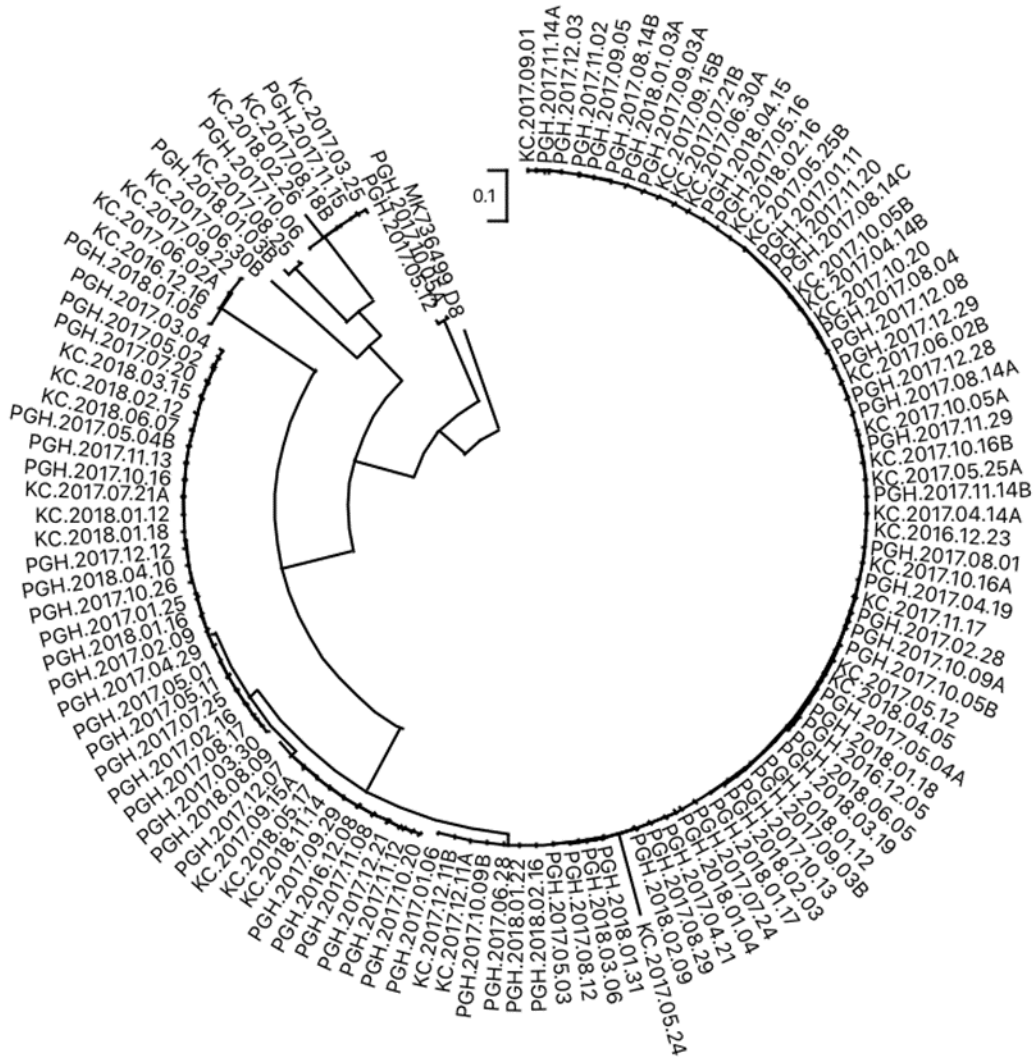
Figure 3 Phylogenetic analysis of HAdV samples from Kansas City and Pittsburgh

4.0 Discussion

We sought to define the burden of HAdV among U.S. children with AGE. We found that HAdV was associated with a substantial number of AGE cases in children seeking medical attention. We observed that the most common symptoms in both inpatient and ED settings were fever, diarrhea, and vomiting, while more children admitted to inpatient experienced dehydration than in the ED. Previous studies found a longer duration of vomiting associated with HAdV infection compared to astrovirus and rotavirus infections (31). Consistent with our findings and these reports of more prolonged emesis, HAdV has been associated with repeat ED visits and severe disease (32). As shown by the low numbers of HAdV in healthy controls, there is an association with HAdV as a causative factor of severe clinical disease resulting in hospitalization. This low prevalence of HAdVs in healthy children has been noted elsewhere (32).

Some studies reported a higher HAdV positivity rate in patients <5 years of age (33, 34). Our findings showed this as well, with HAdV+ subjects tending to be younger in both the inpatient and ED settings with the average age of 1.1 years in the ED and 1.3 years for inpatient. This emphasizes the importance of HAdV infection in early childhood. We did not find a difference in HAdV infection between males and females, consistent with other reports (35).

Previous studies have investigated the seasonality of HAdV infection to see if there is a correlation between seasons, weather conditions and HAdV prevalence, and some found no correlation (33, 36). While some studies showed no set seasonality, others found that HAdV infections can occur in oscillatory patterns, showing minor peaks of 1 year and then more significant peaks for the following two-three years (34). Our data indicates that during certain

years, there was a pattern for higher HAdV prevalence. Longer time-series analysis of HAdV infection is needed to further verify HAdV seasonality.

At the Pittsburgh site, 21 (24%) out of the 87 HAdV+ samples were coinfected with another pathogen from the testing panel. Previous studies have shown a coinfection rate of other pathogens with HAdV as high as 44.8% in diarrheal cases (33, 37). At the Pittsburgh site, coinfections of other known AGE causing pathogens such as rotavirus and norovirus was common, which has been observed in other studies (38). *C. difficile* was also a common pathogen coinfecting HAdV+ patients. High rates of asymptomatic colonization with *C. difficile* in pediatric populations has been reported, so it is a challenge to determine its role in coinfections in children (39, 40).

We found HAdV species B, C, and F were associated with AGE, with type F41 being the most prevalent, consistent with other reports (33, 34, 41). This predominance of one type could provide a potential target for vaccine development. While there is a vaccine approved for the U.S military for HAdV that cause respiratory infections, there is still no vaccine to treat AGE caused by HAdV (42). While types F40/41 were the most common, other species have also been found to be associated with AGE. For example, species D was found in AGE in Kenya, and there are other reports of outbreaks of types typically not associated with AGE (43, 44). While some have been directly associated with outbreaks, the relevance of viral shedding of non-enteric HAdV types from non-AGE infections is unclear.

There are some limitations to this work. First, the molecular assays used targeted HAdV 40/41, though these assays are capable of detecting other species. Thus, we may have failed to detect non-40/41 types in children with AGE. In addition, we cannot clinically determine if the other subtypes detected caused the AGE or were simply being shed in the stool while the AGE was caused by another undetected pathogen (45). However, the rate of HADV detection was much

lower in healthy controls subjects, suggesting a causative role. There is also the potential that not all causative factors of AGE, whether viral, bacterial, or parasitic, were detected in the multiplex panel used on the stool sample. There have also been concerns in other studies of the sensitivity of the assay in detecting these pathogens (46). Strengths of the study include the multi-center prospective nature, use of molecular diagnostics, and inclusion of matched healthy controls.

4.1 Implications to Public Health

We found that HAdV caused a substantial proportion of medically attended AGE in children, including hospitalizations. This places a strain not only on the hospital system, but society overall. From hospital costs, missed work, school and more, the financial and social burden due to AGE is substantial, and one that is difficult to calculate. We sought to further define the demographic and clinical characteristics of AGE caused by HAdV, to get a more accurate picture of the burden using a prospective, multi-site surveillance study. We found that most children with HAdV associated AGE were younger, and that although other types were detected, the vast majority were HAdV41, suggesting potential benefit from a vaccine.

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