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Viral Causes of Gastroenteritis in the Era of Widespread Rotavirus Vaccination

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By
Dame Idossa
2016

VIRAL CAUSES OF GASTROENTERITIS IN THE ERA OF WIDESPREAD ROTAVIRUS VACCINATIONS. Dame Idossa, Novagrami George, and Virginia Pierce (Sponsored by Marietta Vázquez). Department of Pediatrics, Yale University, School of Medicine, New Haven, CT.

Background: It is unknown if widespread use of Rotavirus (RV) vaccine will impact the primary causative agent of viral gastroenteritis (VGE).

Objective: To identify changes in epidemiology of VGE affected by vaccination, determine factors associated with higher severity of illness, and assess agreeability between two clinical severity-grading scales; Clark and Vesikari.

Methods: We analyzed fecal samples of children, 6 months-5 years of age, evaluated at YNHH for VGE. Fecal samples were tested using a real-time PCR assay. Primer and probe sequences targeted conserved regions of the genome for RV, Norovirus GI/GII, Adenovirus, Astrovirus, and Sapovirus. Data were analyzed using SPSS.

Results: Of the 268 fecal samples analyzed, 215 (80%) were positive for at least one viral pathogen. Of those, 133 (62%) had a single viral pathogen identified and 82 (38%) had multiple pathogens. The frequencies of pathogens were: RV in 132 (61%), Norovirus GI/GII in 93 (43%), Astrovirus in 32 (15%), Adenovirus in 24 (11%), and Sapovirus in 21 (10%). For subjects <12 months of age the frequency of viral pathogens were: RV 48 (41%), Norovirus GI/GII 41(35%), Astrovirus 12 (10%), Adenovirus 10 (9%), and Sapovirus 7 (6%). State of being infected by any pathogen, having educated caretakers, infection with RV, and not being vaccinated for RV were associated with greater severity of diarrheal illness. In contrast, difference in severity of illness seen with Hispanic ethnicity, Black race, and coinfection with multiple pathogens was not statistically significant. Lastly, Clark and Vesikari clinical severity grading scales were shown to have poor agreeability (k=0.309), which was not improved by modification.

Conclusions: We conclude that in the era of widespread use of RV vaccine, the epidemiology of VGE may be changing. We've identified several factors that may be associated with higher severity of illness, which may help guide clinicians in improving care and directing resources. Lastly, we confirm the poor agreeability between the Clark and Vesikari scales, which may guide future researchers to standardize use of clinical severity scales.

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INTRODUCTION

Diarrheal illnesses are one of the most common causes of morbidity and mortality in children worldwide. Acute diarrhea accounts for 2 to 3 million deaths per year with most occurring in young children in developing countries [1]. Each year in the United States, gastroenteritis is responsible for approximately 600,000 outpatient medical visits, 55 to 70,000 hospitalizations, and 20 to 60 deaths [2, 3]. The costs associated with diarrheal illnesses are also very high with total annual direct and indirect costs of approximately $1 billion [4-6]. Below we review the literature on the epidemiology, risk factors, and clinical severity grading scales for gastroenteritis, with particular attention to viral gastroenteritis.

Type of Pathogens Causing Gastroenteritis: Parasitic, Bacterial and Viral

The causes of acute diarrhea in children vary depending on multiple factors such as location, season, and population studied. The infections responsible for causing gastroenteritis in children can be divided into parasitic, bacterial, and viral infections.

Parasitic causes of diarrhea are not as common in developed countries as they are in the developing world, however, they still account for 1% to 8% of cases of diarrhea in pediatric patients. In the United States, *Giardia* and *Cryptosporidium* infections are the most common parasitic causes of disease [7]. They are usually acquired via fecal to oral transmission. Most community-wide epidemics have resulted from a contaminated water supply. Person to person transmission can also occur, most commonly in daycare centers. Prognosis tends to be very good with proper management of disease.

Bacterial pathogens are the second most common cause of gastroenteritis in pediatric patients in developed countries, accounting for 2% to 10% of cases [7]. The most common species include *Salmonella, Shigella,* and *Campylobacter*. Other notable bacteria also includes enterohemorrhagic *Escherichia coli* (EHEC) and with the increasing use of antibiotics, *Clostridium difficile*, although this tends to be uncommon in the pediatric population [7]. With proper management, the prognosis for bacterial gastroenteritis tends to be very good, especially in developed countries. Mortality is usually due to dehydration and malnutrition from a protracted course.

Viral pathogens are by far the most common cause of diarrheal illnesses in the United States, accounting for 75-90% of pediatric gastroenteritis [8]. Before initiation of the RV vaccination program in 2006, nearly every child in the United States was infected with RV by age 5 years; the majority had gastroenteritis, resulting in significant morbidity and mortality [2]. Other viruses also known to cause gastroenteritis include Norovirus, Adenovirus, Astrovirus, and Sapovirus. Transmission for all of these viruses is presumed to be fecal to oral. The morbidity and mortality associated with viral gastroenteritis has significantly decreased since the initiation of RV vaccination program. The effectiveness of the RV vaccine is consistent with clinical trials estimates [9]. Studies from the United States, Europe, and Australia have demonstrated effectiveness of up to 100% (95% CI 85%-100%) associated with decreased hospitalizations for RV gastroenteritis. Healthcare utilization (hospitalizations and emergency-department visits) was also reduced by up to 90% [10, 11]. Although the efficacy of RV vaccine is reduced in developing countries, there are still significant reductions in burden of VGE infections. In studies conducted in

Latin America, RV vaccine has resulted in a 17–51% reduction in gastroenteritis-associated hospitalizations and 59–81% reduction in RV hospitalization among children younger than five years of age [12].

Characteristics of Viral Agents

Below we review characteristics of the following viruses that were examined in this study: Rotavirus (RV), Norovirus, Sapovirus, Adenoirus, and Astrovirus.

RV is a double-stranded RNA virus, in the family of Reoviridae, which is classified into various groups, subgroups, and serotypes. There are at least 15 different serotypes of RV; presently, 5 serotypes of RV (G1, G2, G3, G4, and G9) account for the majority of the strains circulating worldwide. Surface antigens VP7 (G protein) and VP4 (P protein) are 2 important structural viral proteins involved in eliciting the immune response through neutralizing antibodies. Rotaviruses have substantial diversity, with a possible 132 separate G-P combinations [3]. This great diversity of serotypes and surface antigens creates an opportunity for multiple assortment and combination of RV serotypes, allowing for the potential emergence of new serotypes of the virus.

Caliciviruses are a family of single-stranded, nonenveloped RNA viruses. The two recognized genera that cause diarrheal disease in humans are noroviruses (Norwalk-like viruses) and sapoviruses (Sapporo-like viruses). Sapoviruses have the typical calicivirus morphology that on electron microscopy reveals the “Star of David” appearance, similar to many animal caliciviruses. The surface structure of Noroviruses is smooth and

normally does not reveal the “Star of David” appearance. Noroviruses are known as “small round structured viruses” [13]. Both Noroviruses and Sapoviruses are genetically diverse, and multiple strains with distinct genetic identities circulate within a community at the same time. In the Norovirus genus, the GII strains have been found to be more common than the GI strains worldwide [14, 15].

Human adenoviruses (HAdVs) are classified in the family Adenoviridae, genus Mastadenovirus, which contains seven known species, from A to G [16]. They are double-stranded, linear, nonenveloped DNA viruses. To date there are over 60 distinct serotypes known to cause human infections. Adenoviruses are known to cause many types of illnesses including respiratory, ocular, and urinary tract infection [17]. The most common serotypes that are associated with gastroenteritis are 40, 41, and, to a lesser extent, 31 [7].

Astrovirus is a nonenveloped, single-stranded RNA viruses with a characteristic starlike appearance by electron microscopy. Eight human antigenic types are currently known. Astroviruses have a worldwide distribution and multiple antigenic types are known to co-circulate in the same region [18].

Introduction of Rotavirus Vaccine Program

In 1998, RotaShield^{R}, a rhesus RV tetravalent vaccine became the first RV vaccine to be licensed in the United States. Unfortunately, it was recalled shortly after its licensure due to a rare side effect of intussusception among those vaccinated, resulting in a major set

back for RV vaccination program [3]. After several years, two different vaccines were developed, licensed, and approved for use by the US Food and Drug Administration. Rotateq[R] (RV5) is a pentavalent human-bovine reassortant live-attenuated oral vaccine licensed in 2006; and Rotarix[R] (RV1) is a monovalent live-attenuated human strain vaccine, licensed in 2008, which shares neutralizing epitopes against the most common RV serotypes. Universal RV vaccination was recommended for U.S. infants by the Advisory Committee on Immunization Practices (ACIP) in February of 2006. 3 doses of the pentavalent RV vaccine [RV5], RotaTeq[R] (Merck and Company) were to be given at ages 2, 4, and 6 months [2, 3]. Since the initiation of this widespread RV vaccination, the number RV cases and its complications in the United States have been significantly reduced[19, 20]. Studies from middle and low income countries have also seen many improvements, including reduction of VGE associated mortality of 22-41% [12].

Clinical Severity Grading Systems

The Vesikari and Clark clinical severity scales were developed and routinely used to assess severity of cases and assist in investigations of diarrhea and dehydration in RV vaccine clinical trials.

The Vesikari clinical severity grading system (VSS) is a 20-point scale that is classified into two categories (non-severe and severe) [21]. Scores greater than or equal to 11 are considered severe [22]. The Clark clinical severity grading system (CSS) is a 24-point scale that is classified into three categories (mild, moderate and severe) [23]. Scores less than 9 are considered mild, scores between 9 and 16 moderate, and scores above 16

points severe [22]. Both severity scales assess clinical information, such as the magnitude and duration (in days) of diarrhea and vomiting, and the maximum temperature during illness. In addition to these parameters, the Clark scale assesses the magnitude and duration of behavioral symptoms such as irritability and lethargy, and the duration of a temperature greater than 38.0°C, whereas the Vesikari scale assesses dehydration and treatment (rehydration or hospitalization) [22].

Several studies have concluded that the Clark scale is less likely to identify a disease as severe, compared with the Vesikari Clinical Severity Scoring System [24]. Thus it tends to have a less sensitive measure of severity of illness. This is because among the five common items included in both scoring systems (# of stools/day, duration of diarrhea, # of emesis/day, duration of emesis, rectal temperature), the Vesikari scale provided a higher score for each item compared to the Clark scale, with the exception of temperature [25]. Thus higher value is reached with a lower frequency of episodes or number of days of duration with the Vesikari scale, resulting in greater proportion of cases being classified as “severe”.

STATEMENT OF PURPOSE

Since the initiation of the RV vaccine program in 2006, cases of severe gastroenteritis have significantly decreased [2]. Nevertheless, diarrheal illness and complication associated with them still account for thousands of outpatient/ED visits and hospitalization [8]. The current distribution pattern of viral pathogens causing pediatric gastroenteritis in era of widespread RV vaccination program still remains unknown. Is the RV still the most common cause or have other viral pathogens, such as astrovirus or norovirus taken its place?

Understanding the current pathogenic distribution of viral pathogens is of great importance to improving care for infants and children who suffer from diarrheal illness. There have been many studies that explore the viral causes of gastroenteritis, both in developed and developing countries [1, 13-16, 26-28]. There have also been several studies of active surveillance of certain viruses such as RV, post vaccination program [3, 11, 29]. However, there haven't been many studies that explore the epidemiology of viral pathogens known to cause gastroenteritis in the era of wide spread RV vaccination. This study will seek to identify any changes, if any, in the distributive pattern of viral gastroenteritis, since the initiation of widespread use of the RV vaccine.

Specific Aim 1: To identify any changes in the epidemiology of viral gastroenteritis.

Hypothesis #1: We predict that RV will still be responsible for causing viral gastroenteritis in notable portion of children <5 years of age. However, we predict it will no longer be the most common cause of viral gastroenteritis in children <5 years of age.

Other viral pathogens, notably Norovirus, will likely take its place.

Specific Aim 2: To determine factors associated with higher severity of illness as graded by Clark and Vesikari Clinical Severity Scales.

Hypothesis #2: We predict that having infected status, Hispanic ethnicity, Black race, lower education status of carers of child, infection with RV, no vaccination for RV, and infection with multiple pathogens will be associated with greater severity of illness.

Specific Aim 3: To determine the agreeability between the clinical severity grading systems in children seen at Yale New Haven Hospital (Emergency department and inpatient units).

Hypothesis #3: We predict that there will be poor agreeability between the Clark and Vesikari clinical severity grading systems.

MATERIALS AND METHODS

Participants Selection Process

Eligible children were prospectively enrolled into match-controlled study of RV vaccine effectiveness in children (6 months – 5 years of age) at Yale-New Haven Children's Hospital in New Haven, Connecticut January 2010 through June 2011. Eligible children were those who met all the following criteria:

1) Presented to the hospital with acute gastroenteritis (≥3 looser-than-normal stools in a 24 –hour period during the illness, and onset of diarrhea ≤ 10 days at presentation).
2) Diarrhea as the main or one of the main reasons for the visit and managed as an emergency department (ED) patient or inpatient.
3) Eligible to have received at least one RV1 or RV5 dose ≥14 days before presentation.
4) Lived in the usual catchment area of the hospital.

Children with immunocompromising condition such as a malignancy or HIV infection were not eligible, because this made them ineligible from receiving the RV vaccines. After informed consent was obtained, a standardized questionnaire was administered to the parent/guardian (which queried demographics, symptoms, name and location of all immunization providers, and general household information) and a stool sample was collected. Children were classified as either a RV case or a "RV-negative" gastroenteritis control based on the RV antigen enzyme immunoassay result. The study was in collaboration with researchers at the CDC.

Subjects were interviewed to collect clinical and demographic information. Medical records were reviewed to assess previous vaccination with RV vaccine and to assess clinical severity of disease using both the Clark and Vesikari clinical severity scales. Enrollment was performed approximately 40 hours per week and included evening and weekend periods. The project was approved by the institutional review boards the hospital and reviewed for human subjects protection at CDC.

Study Design

We conducted a retrospective cohort study using leftover frozen stool samples from a previous study that were sent to Children's Hospital of Philadelphia (CHOP) to be tested for the presence of viral stool pathogen known to cause VGE such as RV, Adenovirus, Astrovirus, Sapovirus, and Norovirus using a new real-time PCR assay, developed by the collaborators at CHOP.

Procedure

A total of 293 frozen specimens [stool samples or diaper lining soaked in viral transport medium (VTM)] from pediatric patients were tested. All samples had been previously characterized as either positive (n = 93) or negative (n = 200) for RV by RV-specific enzyme immunoassay, conducted as part of the case-control investigation by the CDC. Each stool specimen (both RV positive and negative specimens) was rapidly thawed in a 37°C water bath and then prepared as a 5% suspension in 1.0 ml of nuclease free water, vigorously vortexed for 30 sec, and clarified by centrifugation at maximum speed for 5 min in a microcentrifuge.

Real-time PCR Assays

Nucleic acids were extracted from 200 µl of each clinical specimen (prepared 5% stool suspension supernatant or VTM-soaked diaper lining) by standard procedures using the MagNA Pure LC automated instrument (Roche Diagnostics, Indianapolis, IN) and corresponding Roche total nucleic acid isolation kit. Extracted product was heated to 95°C for 5 min and then immediately placed in ice for 1 min. Individual real-time PCR assays were performed in 50-µl volumes on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using 5 µl of eluted nucleic acid; universal master mixes for either RNA (Ambion AgPath-ID One-Step RT-PCR master mix; Applied Biosystems) or DNA (TaqMan universal master mix; Applied Biosystems); universal amplification conditions consisting of 1 cycle for 10 min at 45°C and 1 cycle for 10 min at 95°C, followed by 45 two-step cycles of 15 s at 95°C and 45 s at 45°C; and TaqMan fluorogenic chemistry for detection. Positive and negative controls were processed with each batch of clinical specimens from extraction of nucleic acids through the detection of amplified products. Negative controls consisted of 1.0 x 10^6 cells/ml of an uninfected human lung carcinoma cell line (A549 cells; ATCC CCL-185), and positive controls were prepared as a mixture of clinical material from previously positive patients. No-template controls were included in each reaction plate for all sets of primers and probes. Primer and probe sequences targeted conserved regions of the genome for each virus (Table 1) and were based on the published literature for Adenovirus types 40 and 41[30], Astrovirus [31], Norovirus genogroups I and II [32], RV [30], and Sapovirus [33]. Specimens and controls were considered positive when the generated fluorescence signal at the threshold cycle (C_T) exceeded a defined threshold limit. Specimens that reached

the threshold before 38 cycles were considered positive without further testing, and those that reached the threshold at or after 38 cycles but before the last of 45 cycles were considered positive only if, upon duplicate repeat testing of separate aliquots of stored original specimen, at least one of the two repeat tests also reached the threshold before 45 cycles.

Table 1. Nucleotide sequences of real-time PCR primers and probes[a]

Primers and probes	Nucleotide sequences (5'-3')	Gene target
Adenovirus 40 and 41		
Forward	TTC CAG CAT AAT AAC TC**W** GGC TTT G	Hexon
Reverse	AAT TTT TTC TG**W** GTC AGG CTT GG	
Probe[b]	(FAM)-CC**W** TAC CCC CTT ATT GG-(MGBNFQ)	
Astrovirus		
Forward	CC**D** GCC AG**R** CTC ACA GAA GAG	Capsid protein precursor
Reverse	GAC TTG CTA GCC ATC ACA CT**Y** C	
Probe[c]	(FAM)-ACT CCA TCG-(ZEN)-CAT TTG GAG GGG AGG ACC-(IABkFQ)	
Norovirus genogroups I and II		
Genogroup I forward	CG**Y** TCC ATG CG**N** TT**Y** CAT GA	Polymerase/capsid junction
Genogroup I reverse	CTT AGA CGC CAT CAT CAT T**Y**A C	
Genogroup I probe A	(FAM)-AGA T**Y**G CGA TC**Y** CCT GTC CA-(TAMRA)	
Genogroup I probe B	(FAM)-AGA TCG CGG TCT CCT GTC CA-(TAMRA)	
Genogroup II forward	CA**R** GA**R** **B**C**N** ATG TT**Y** AG**R** TGG ATC AG	
Genogroup II reverse	TCG ACG CCA TCT TCA TTC ACA	
Genogroup II probe	(VIC)-TGG GAG GGC GAT CGC AAT CT-(TAMRA)	
Rotavirus		
Forward 1	GGA TGT CCT GTA CTC CTT GTC AAA A	Inner capsid protein VP6
Forward 2	GGA GGT TCT GTA CTC ATT GTC AAA AA	
Reverse 1	TCC AGT TTG GAA CTC ATT TCC A	
Reverse 2	TCC AGT TTG AAA GTC ATT TCC ATT	
Probe 1	(FAM)-ATA ATG TGC CTT CGA CAA T-(MGBNFQ)	
Probe 2	(FAM)-AAT ATA ATG TAC CTT CAA CAA T-(MGBNFQ)	
Sapovirus		
Forward 1	GAC CAG GCT CTC GC**Y** ACC TAC	Polymerase/capsid junction
Forward 2	TTG GCC CTC GCC ACC TAC	
Reverse	CCC TCC AT**Y** TCA AAC ACT A**W**T TTG	
Probe	(FAM)-TGG TT**Y** ATA GG**Y** GGT AC-(MGBNFQ)	

[a]Abbreviations: FAM, 6-carboxyfluorsescein; MGBNFQ, minor groove binder/non-fluorescent quencher; VIC, proprietary formulation, Applied Biosystems; IABkFQ, Iowa Black FQ quencher, proprietary formulation, Applied Biosystems; TAMRA, 6-carboxytetramethylrhodamine. International Union of Biochemistry base codes: W = A or T, D = A or G or T, R = A or G, Y = C or T, B = C or G or T, N = A or C or G or T.
[b]Logan et al. used two probes; we used one probe with mixed bases. [c]We incorporated an internal ZEN quencher from Integrated DNA Technologies, Coralville,

Resolution of Discordant Results

When there was discordance between real-time PCR and enzyme immunoassay result, the real-time PCR assay was repeated again using the original sample. If the initial real-time PCR result was positive and one or both of the duplicate retests were positive, the final PCR result was reported as positive. Conversely, if the initial real-time PCR result was negative and one or both of the duplicate retests were negative, the final PCR result was reported as negative.

Omissions

Out of the 293 samples, 18 were duplicates (2 samples collected from same subject). In 1 of the 9 subjects with duplicate samples, one sample was collected from a diaper lining and the other was collected from a whole stool. In another 3 of the 9 subjects, both samples were collected from a diaper lining, but were collected on different days. There was 1 subject in which both samples were from a whole stool, but were collected on 2 different days. In these 5 subjects with duplicate samples, the PCR result for the two samples gave different results. Thus these 10 samples (from 5 subjects with discordant results) were omitted from the final analysis. The remaining 4 subjects had a PCR results that were the same for both of their samples. Because they had a total of 8 samples, 4 of those samples were omitted from the final analysis to insure that each subject's sample was only counted once. The 11 Rota only samples were also omitted in order not to skew the results towards RV. These were samples tested by EIA and known to have only RV. This left 268 samples (293-10 discordant-4duplicate-11rota only) to be included in the analysis of the study.

Analysis

Data were analyzed with SPSS 14.0 software for Windows. Categorical data were analyzed using the χ^2 test. Continuous variables were analyzed using the *t*-test procedure. Cohen kappa statistics used to measure the agreement between the two severity scoring scales by adjusting both scales to have the same number of categories. A *P*-value of <0.05 was considered significant.

Statement of Responsibility

This researcher, Dame Idossa, was responsible for creating a database and compiling all of all the available data into that database. Novagrami George, BS was responsible for participant screening and recruitment from January through June 2010 and 2011. Virginia Pierce MD, our collaborator from CHOP conducted the real-time PCR for all of the samples and detailed out the methods that were used to do this. This researcher performed the statistical analysis for all of the data. Dame Idossa, Novagrami George, BS and Marietta Vázquez, MD interpreted the results.

RESULTS

Participants

Table 2 presents an overview of the social, demographic, insurance status, parental education, prematurity, and vaccination status of the 268 analyzed subjects. Overall, participants were approximately 18 months of age at intake (M=18.12, SD 12.2) and predominately comprised of males (61.2%). Almost half of the participants (47%)

TABLE 2. Characteristics for N=268 Subjects

Characteristics	Total Cases N= 268 (%)	Infected N=215 (%)	Not Infected N=53 (%)
Age at Intake (months)			
Mean (SD)	18.12 (12.2)	18.03 (11.9)	15.72 (12.6)
Median	16	16	13
Gender			
Male	164 (61.2%	137 (63.7%)	27 (50.1%)
Female	104 (38.8%)	78 (36.3%)	26 (49.1%)
Ethnicity			
Hispanic/Latino	127 (47.4%)	99 (46.0%)	28 (52.8%)
Non Hispanic/Latino	141 (52.6%)	116 (54.0%)	25 (47.2%)
Race			
White	114 (42.5%)	94 (43.7%)	20 (37.7%)
Black	57 (21.3%)	44 (20.5%)	13 (24.5%)
American Indian	3 (1.1%)	3 (1.40%)	0 (0%)
Asian	8 (3.0%)	4 1.86%)	4 (7.5%)
Other	84 (31.3%)	67 (31.2%)	17 (32.1%)
Insurance			
Private	78 (29.1%)	70 (32.6%)	8 (15.1%)
Public	182 (67.9%)	138 (64.2%)	44 (84.9%)
None	8 (3.0%)	7 (3.26%)	1 (1.9%)
Parental education level			
<High school	67 (25%)	51 (23.7%)	16 (30.2%)
High school/GED	123 (45.9%)	96 (44.7%)	27 (50.1%)
College	50 (18.7%)	44 (20.5%)	6 (11.3%)
Graduate	28 (10.4%)	24 (11.2%)	4 (7.5%)
Prematurity			
Full term	233 (86.9%)	187 (87.0%)	46 (86.8%)
Premature	35 (13.1%)	28 (13.0%)	7 (13.2%)
Vaccinations			
Rota	195 (72.8%)	148 (68.8%)	47 (88.7%)
Tdap + other vax	266 (99.2%)	213 (99.1%)	53 (100%)
Unknown	2 (.75%)	2 (.93%)	0
Clinical Severity score			
Mean Clark Clinical Severity scale	10.90 ± 3.4	11.13 ± 3.4	10.09 ± 3.4
Mean Vesikari Clinical Severity scale	10.38 ± 3.6	10.65 ± 3.6	9.24 ± 3.7

identified as Hispanic/Latino ethnicity. 42.5% of the participants identified as Caucasian, with the rest identifying as Black (21.3%), American Indian (1.1%), Asian (3.0%), and other (31.3%). The majority of the participants had health insurance coverage through public (67.9%) or private (29.1%) institutions. A quarter of the parents of participants had less than a high school level of education with the rest having high school diploma/GED (45.9%), College degree (18.7%) or Graduate degree (10.4%). Most of the children were born at full term (86.9%) and had received RV vaccine (72.8%) and other childhood vaccines (99.2%). The mean clinical severity score as determined by the Clark scale was 10.9 ± 3.4 and by Vesikari scale was 10.38 ± 3.6. The stratified characteristics for the participants infected with any pathogen and for those not infected with any tested pathogen were also similar and as listed in Table 2.

Pathogens and Severity

Tables 3, 4, and 5 describe the pathogens detected in subjects who were infected. Of the 215 infected subjects, 133 (49.5%) were single infections and 82 (30.6%) were multiple infections. Overall, Adenovirus was detected in 24 (11.2%), Astrovirus in 32 (14.9%), Norovirus GI in 4 (1.9%), Norovirus GII in 89 (41.4%), RV in 132 (61.4%), and Sapovirus in 21 (9.8%) of the participants.

Of the 215 infected participants, RV caused the greatest severity of illness as measured by the Clark Clinical Severity scale 11.7 ± 3.4, followed by Adenovirus, Norovirus GII, Astrovirus, Sapovirus, and Norovirus GI (Table 3). However, Adenovirus caused the greatest severity of illness as measured by the Vesikari Clinical Severity scale 12 ± 3.4,

followed by RV, Norovirus GII, Astrovirus, sapovirus, and lastly Norovirus GI, as shown in Table 3.

TABLE 3. Pathogens Detected in Infected subjects

Pathogens Detected	Total Cases N= 215 (%)	Mean Clark Scale (SD)	Mean Vesikari Scale (SD)
Adenovirus	24 (11.2)	11.5 (3.1)	12 (3.4)
Astrovirus	32 (14.9)	10.2 (3.2)	9.44 (3.9)
Norovirus GI	4 (1.9)	8.8 (4.5)	7.25 (3.6)
Norovirus GII	89 (41.4)	10.9 (3.3)	10.66 (3.4)
Rotavirus	132 (61.4)	11.7 (3.4)	11 (3.6)
Sapovirus	21 (9.8)	9.5 (2.7)	9.19 (3.4)

** Total adds up to > 215 because 82 subjects were coinfected with multiple pathogens and hence counted >1 times*
Clark: <9 "mild", 9-16 "moderate", >16 "severe" Vesikari: Scores <11 "non severe", ≥11 "severe"

Single Viral Infections

Of the 133 subjects infected with a single virus, 8 (6%) were infected by Adenovirus, 8 (6%) by Astrovirus, 42 (32%) by Norovirus GII, 66 (50%) by RV, and 9 (7%) by Sapovirus. In these subjects, Adenovirus caused the greatest severity of illness as measured by the Clark Clinical Severity scale 12.1 ± 3.7, followed by RV, Norovirus GII, Astrovirus, and Sapovirus (Table 4). However, RV caused the greatest severity of illness as measured by the Vesikari Clinical Severity scale 12.5 ± 3.3, followed by Adenovirus, Norovirus GII, Astrovirus, Sapovirus, as shown in Table 4.

TABLE 4. Pathogens Detected in Single Infections

Pathogens Detected	Total Cases N= 133 (%)	Mean Clark Scale (SD)	Mean Vesikari Scale (SD)
Adenovirus	8(6)	12.1 (3.7)	10.4 (2.1)
Astrovirus	8(6)	8.6 (2.8)	9.6 (2.4)
Norovirus GI	-	-	-
Norovirus GII	42(32)	10.1 (3.1)	10.1 (3.3)
Rotavirus	66(50)	11.6 (3.6)	12.5 (3.3)
Sapovirus	9(7)	8.2 (2.3)	8.6 (3.2)

Clark: <9 "mild", 9-16 "moderate", >16 "severe" Vesikari: Scores <11 "non severe", ≥11 "severe"

Multiple Viral Infections

Of the 82 subjects who were co-infected by multiple viruses, 15 (18.3%) were infected by Adenovirus, 26 (31.7%) by Astrovirus, 4 (4.9%) by Norovirus GI, 49 (59.8%) by Norovirus GII, 71 (86.6%) by RV, and 12 (14.6%) by Sapovirus. In these subjects, Adenovirus caused the greatest severity of illness as measured by the Clark Clinical Severity scale 12.3 ± 3.5, followed by Norovirus GII, RV, Astrovirus, Sapovirus and Norovirus GI (Table 5). Similarly Adenovirus caused the greatest severity of illness when measured by the Vesicari Clinical severity score 12.1 ± 3.5, followed by Norovirus GII, RV, Sapovirus, Astrovirus, and Norovirus GI (Table 5).

TABLE 5. Pathogens Detected in Multiple Infections

Pathogens Detected	**Total Cases N= 133 (%)**	**Mean Clark Scale (SD)**	**Mean Vesikari Scale (SD)**
Adenovirus	15(18.3)	12.33 (3.5)	12.13 (3.5)
Astrovirus	26(31.7)	10.42 (3.4)	9.54 (4.2)
Norovirus GI	4(4.9)	8.75 (5.2)	7.25 (4.1)
Norovirus GII	49(59.8)	11.61 (3.3)	11.24 (3.5)
Rotavirus	71(86.6)	11.14 (3.4)	10.51 (3.5)
Sapovirus	12(14.6)	10.17 (2.4)	9.92 (4.1)

** Total adds up to > 82 because all of these subjects were coinfected with multiple pathogens*
Clark: <9 "mild", 9-16 "moderate", >16 "severe" Vesikari: Scores <11 "non severe", ≥11 "severe"

Pathogens by Age

When we stratified participants by age, the children between 0-12 months of age were most commonly infected by RV 48 (40.7%), followed by Norovirus GII 40 (33.9%), Astrovirus 12 (10.2%), Adenovirus 10 (8.5%), Sapovirus 7 (5.9%), and Norovirus GI 1 (0.85%). This trend was similar for children between 12-24 months and 24-36 months. For children who were ≥ 36 months of age, RV 8 (72.7%) caused the majority of the infections found. Astrovirus, Norovirus GII, and Sapovirus all caused 1 (9.1%) of the

infections detected in this age group. Adenovirus and Norovirus GI did not cause any infections (Table 6).

TABLE 6. Pathogens by Age

Pathogens Detected	0-12 mo N= 118 (%)	12-24 mo N= 122 (%)	24-36 mo N= 57 (%)	36+ mo N= 11 (%)
Adenovirus	10 (8.5)	9(7.4)	4 (7)	0
Astrovirus	12 (10.2)	15 (12.3)	6 (10.5)	1 (9.1)
Norovirus GI	1 (.85)	0	3 (5.3)	0
Norovirus GII	40 (33.9)	38 (31.1)	11 (19.3)	1 (9.1)
Rotavirus	48 (40.7)	53 (43.4)	47.4)	8 (72.7)
Sapovirus	7 (5.9)	7 (5.7)	6 (10.5)	1 (9.1)

** Total adds up to > 264 because of coinfections*

<u>RV Vaccination Status' Effects on Infection With Pathogens and Severity of Illness</u>

Of the 268 participants, 195 (72.8%) were vaccinated against RV with at least one dose of Rotateq[R] or Rotarix[R] vaccine. In these participants, RV was the most common viral pathogen detected 83 (38.4%), followed by Norovirus GII 73 (33.8%), Astrovirus 27 (12.5%), Sapovirus 16 (7.4%), Adenovirus 13 (6.02%), and Norovirus GI 4 (1.85). Adenovirus had the highest severity of illness when measured using the Clark Clinical Severity scale 11.5 ± 2.8, followed by RV, Norovirus GII, Astrovirus, Sapovirus, and Norovirus GI (Table 7). Adenovirus also caused the greatest severity of illness as measured by the Vesikari Clinical Severity scale 11.9 ± 3.6, followed by Norovirus GII, RV, Astrovirus, Sapovirus, and Norovirus GI (Table 7).

TABLE 7. Pathogens Detected in Rotavirus Vaccinated Participants

Pathogens	N=216 (%)	Clark Scale (SD)	Vesikari Scale (SD)
Adenovirus	13 (6.0)	11.54 (2.8)	11.92 (3.6)
Astrovirus	27 (12.5)	9.82 (3.1)	9.18 (4.2)
Norovirus GI	4 (1.9)	8.87 (4.5)	7.25 (3.6)
Norovirus GII	73 (33.8)	10.52 (3.2)	10.52 (3.3)
Rotavirus	83 (38.4)	10.73 (3.1)	9.87 (3.2)
Sapovirus	16 (7.4)	9.56 (2.8)	9 (3.6)

** Total adds up to > 195 because of coinfections*
Clark: <9 "mild", 9-16 "moderate", >16 "severe" Vesikari: Scores <11 "non severe", ≥11 "severe"

Of the 73 (27.2 %) participants who were not vaccinated with at least one dose of Rotateq^{R} or Rotarix^{R} vaccine, RV was the most common viral pathogen detected 48 (58.5%), followed by Norovirus GII 15 (18.3%), Adenovirus 11 (13.4%), Astrovirus and Sapovirus both detected in 4 (4.9%) of the stool samples. Norovirus GI did not cause any infections in these participants. RV caused the greatest severity of illness as measured by the Clark Clinical Severity scale 13.4 ± 3.0, followed by Astrovirus, Norovirus GII, Adenovirus, and Sapovirus (Table 8). Conversely, Astrovirus caused the greatest severity of illness as measured by the Vesikari Clinical Severity scale 13.3 ± 2.1, followed by RV, Adenovirus, Norovirus GII, and Sapovirus (Table 8).

TABLE 8. Pathogens Detected Rotavirus Non Vaccinated Participants

Pathogens	N=82 (%)	Clark Scale (SD)	Vesikari Scale (SD)
Adenovirus	11 (13.4)	11.45 (3.5)	12.09 (3.1)
Astrovirus	4 (4.9)	13.25 (2.5)	13.25 (2.1)
Norovirus GI	0	Na	Na
Norovirus GII	15 (18.3)	12.67 (3.6)	11.33 (3.8)
Rotavirus	48 (58.5)	13.44 (3.0)	12.98 (3.3)
Sapovirus	4 (4.9)	9.75 (2.4)	10.75 (2.3)

** Total adds up to > 73 because of coinfections*
Clark: <9 "mild", 9-16 "moderate", >16 "severe" Vesikari: Scores <11 "non severe", ≥11 "severe"

Factors Associated with Higher Severity of Illness

We explored various factors that may be associated with higher severity of illness using T-test. Both the Clark and Vesikari severity scales were used to measure severity. Table 9 depicts the p values of the various characteristics tested.

State of being infected by any pathogen, having educated caretakers (some college or greater level of education), infection with RV, and not being vaccinated for RV were associated with greater severity of illness. In contrast, having multiple infections, being of Hispanic ethnicity and being of Black race were not associated with greater severity of illness (Table 9).

Table 9. Factors association with Severity of Illness

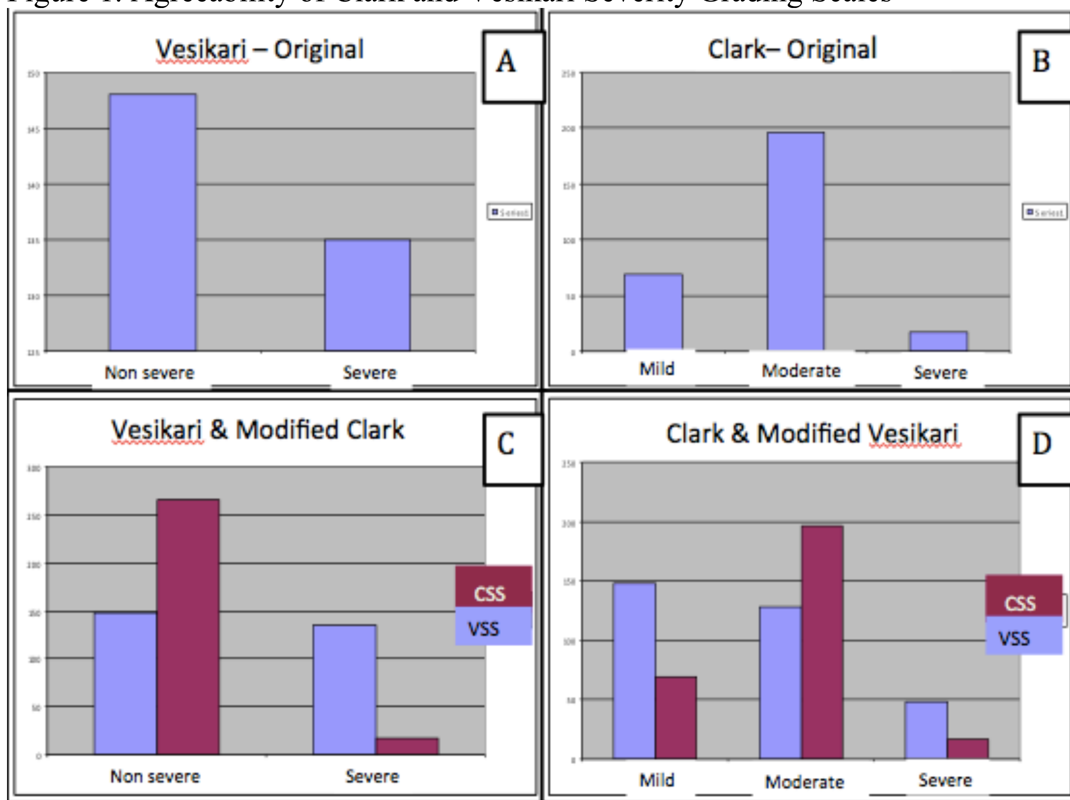
Characteristics	**Mean CSS**	**P-value CSS**	**Mean VSS**	**P-value VSS**
Non Vaccinated vs Vaccinated	12.59 vs 10.31	<0.001	12.29 vs 9.70	<0.001
Rotavirus vs non Rotavirus	12.53 vs 9.88	<0.001	11.64 vs 9.88	0.002
Rotavirus vs Norovirus GII	12.53 vs 10.00	<0.001	11.64 vs 10.00	0.013
Rotavirus vs Sapovirus	12.53 vs 8.55	0.001	11.64 vs 8.22	0.006
Rotavirus vs Astrovirus	12.53 vs 9.63	0.018	11.64 vs 8.63	0.023
≤HS diploma vs >HS diploma	10.75 vs 11.70	0.035	9.83 vs 12.05	<0.001
Infected vs non Infected	11.13 vs 10.09	0.047	10.65 vs 9.25	0.011
Rotavirus vs Adenovirus	12.53 vs 10.38	0.076	11.64 vs 12.13	0.718
Black vs non Black race	10.67 vs 11.59	0.100	10.25 vs 11.34	0.062
Public vs Private Insurance	10.85 vs 11.54	0.130	9.88 vs 11.76	<0.001
Non Hispanic vs Hispanic	10.71 vs 11.00	0.668	10.65 vs 9.25	0.011
Single vs Multiple Infections	11.14 vs 10.74	0.944	11.11 vs 10.55	0.741

Agreeability Between Vesikari and Clark Severity Scales

Figure 1 shows all cases (N=268) graded by both the Vesikari and Clark severity grading scales individually (Panels A and B). The Clark Severity Scale was modified into a 2 category scale by grouping the mild and moderate cases into “non severe.” This modified

Clark scale was then compared to the Vesikari Scale as in shown in panel C. Lastly the Vesikari scale was modified into a 3-category system by regrouping into mild, moderate, and severe groups. This modified Vesikari scale was compared the original scale as shown in panel D.

Figure 1. Agreeability of Clark and Vesikari Severity Grading Scales



A and B show cases as graded by Vesikari and Clark scale respectively. C shows cases as graded by Vesikari and modified 2-category Clark scale. D shows cases as graded by Clark and modified 3-category Vesikari scale. Blue=Clark, Red=Vesikari.

The comparison between the two scoring scales cannot be analyzed statistically because the distribution categories are not even. The Clark scale is divided into 3 ranges (<9, 9–16 and >16) while the Vesikari scale is divided into 2 ranges only (<11 and ≥11). Thus we modified Clark scale into a 2-category scale and the Vesikari into a 3-category scale to make the comparisons. The Cohen's Kappa coefficient [34] was used to determine the

agreeability between the Clark and Vesikari severity grading scales. Kappa values can range from -1 to 1. Values <1 suggest poor agreement, 0-0.20 slight agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.8 substantial agreement, and 0.81-1 almost perfect agreement. As depicted in Table 10, the Kappa scores for the original and modified Vesikari and Clark scales show there is slight or fair agreement at best between the two grading systems.

Table 10. Agreeability between Vesikari and Clark grading systems

Type of scale	Kappa value	95%CI	P-value
Original	.306	(0.218,0.394)	<0.001
Modified Clark	.111	(0.050-0.172	<0.001
Modified Vesikari	.326	(0.244-0.408)	<0.001

DISCUSSION

RV vaccines have significantly reduced the burden of RV disease both in the United States and globally. Studies in the United States have shown a decline of approximately 85% to 95% in RV cases during the 2008 season compared with previous seasons [2]. In addition, data from the Natural Respiratory and Enteric Virus Surveillance System (NREVSS), network of U.S. laboratories that provide the CDC with weekly reports of the number of tests performed and positive results obtained for a variety of pathogens, have shown a >50% decrease of RV positive samples in 2007–2008 season of RV when compared to the season of 1991–2006 [29]. Studies from developing countries have shown lower efficacy of the vaccine; nonetheless, great reductions in severe diarrheal illness in children who received the RV vaccine. For example, studies from Bangladesh and Vietnam have shown the vaccine to be 48% efficacious against severe disease in young infants [35, 36]. RV vaccine has undoubtedly reduced the mortality and morbidity associated with viral gastroenteritis in young children.

There have also been studies that have demonstrated a shift in the seasonality of RV since initiation of RV vaccine [37]. In addition, the usual spread of RV associated gastroenteritis from southwest to northeast has not been shown post RV vaccine initiation. The RV season has also been noted to be later in the year, shorter, and less pronounced when compared to pre-vaccination era [29]. All of this information suggests that the morbidity and mortality associated with RV gastroenteritis is increasingly becoming better managed.

Interesting results in regards to distribution of pathogens post RV vaccination program have emerged from our study. Overall, our data shows RV remains the most common cause of both single and co-infections causing viral gastroenteritis in children in CT. Given the successes of the RV vaccination program [38], this is unexpected, although not surprising. There are many different types of the RV serotypes and surface antigens [39]. This great diversity of serotypes and surface antigens creates an opportunity for multiple assortment and combination of RV serotypes, allowing for the potential emergence of new serotypes of the virus. The current vaccines available cannot protect against all of the possible RV serotypes or any new serotypes that emerge.

In subjects who were previously vaccinated for RV, the frequency of RV infection was significantly decreased, as expected (38%), and we have noted the frequency of Norovirus GII has increased (34%). The data from those who were not vaccinated for RV seems to be similar to data from pre RV vaccination era, with RV accounting for nearly 60% of the viral gastroenteritis. In this group Norovirus GII is found to cause only 18% of disease. An interesting result from this study is the change in the frequency of Norovirus infections when the data were stratified by ages. Norovirus GII frequency is increased mostly in the children <12months, who are the target population for RV vaccination programs. This may suggests this trend may be a direct result of RV being better controlled in this age group. A recent active surveillance study, since the introduction of RV vaccines, found that Norovirus has become the leading cause of medically attended acute gastroenteritis in U.S. children and is associated with nearly 1 million health care visits annually. They estimated treatment cost associated with

Norovirus is >$270 million and expected to continue rising [40]. Given the recent advances in the development of candidate Norovirus vaccines [27, 41], the need to determine the burden of gastroenteritis associated with Norovirus and other viruses is increasingly more important.

Several factors associated with increased severity of illness emerged from our study. Primarily, the detection of any viral pathogen tested was associated with greater severity of illness as expected. Subjects who exhibited symptoms of viral gastroenteritis but did not shed any of the viruses tested could have been infected with parasitic, bacterial, or viral agents that were not tested for. Regardless, these subjects had less severe disease manifestations than their viral infected counterparts. As predicted subjects who were not vaccinated against RV had a greater severity of illness (P=<0.001) (regardless of what pathogen they were infected with) when compared to those that were vaccinated against RV. We also found, higher education of status of caretakers was found to be associated with higher severity of illness as measured by both Clark (11.70 p=0.035) and Vesikari ((12.05 p=<0.001) Scale. Given the usual association of higher level of education with higher annual income and, typically, better access to care/better health outcomes, this result was unexpected. A possible explanation for this could be that caretakers with higher level of education could potentially have more demanding jobs and presumably present their child to the ED/hospital at later or more severe stages of disease. This could result in detection or ascertainment bias. Lastly, subjects who were infected with RV, as either a single infection of a co-infection, were found to have greater severity of illness as

expected. Other studies have also shown that RV associated gastroenteritis cause the greatest severity of illness in children <5 years old [42, 43].

Health disparities in the United States are pervasive and present in almost all realms of medicine. In primary care, minority children are more likely to receive poorer quality of care in terms of provider interactions, preventive services and management of common conditions [44]. However, contrary to our hypothesis, the results of this study did not support his fact. Hispanic ethnicity did not result in higher severity of illness, in fact non-Hispanics were found to have greater severity of illness only by the Vesikari scale (p=<0.001). The difference in severity measured by the Clark Scale was found to be not statistically significant (p=0.095). Black race was also not associated with greater severity of illness, when measured by both the Clark (p=0.100) and Vesikari (p=0.062) scale. Lastly, subjects with public insurance were also not found to have higher severity of illness. In fact, subjects with private insurance were found to have a greater severity of illness as measure only by the Vesikari scale (p=<0.001). The data from this study did not demonstrate disparities in severity of illness among minority or poor participants.

Interestingly, being infected with more than one pathogen also did not result in greater severity of illness as predicted. In fact our data demonstrated the difference between severity of illness of those with single and multiple infections was not statistically significant when measured by both the Clark (p=0.944) and Vesikari (p=0.741) scale.

As predicted the Clark and Vesikari severity grading systems had poor agreeability even with modification of the scales. The results obtained using the two severity scales differ significantly and can disrupt comparisons [25]. The original scales were found to have only fair agreement as measured by the kappa score. When the Clark three-category scale was transformed into a two-category scale by combining mild and moderate categories as non-severe, the agreeability actually worsened. Modifying the Vesikari two-category scale into a three-category scale by further subdividing the severe category into two parts, provided a better correlation between the two severity scales, but still did not achieve a good level of agreement. This suggests that there either needs to be a development of a new severity grading system or standardized use of only one of the existing scales in subsequent research/clinical trials.

Limitations

There are several limitations of this study. The greatest limitation is our choice of modality for detecting pathogens in the stool samples. The real time PCR assay was designed to detect sequences of conserved regions of the genome for RV, Norovirus GI/GII, Adenovirus, Astrovirus, and Sapovirus. Thus other viral pathogens, if present, would not be detected via this method. However, previous studies support the notion that the selected viral agents are known to cause the majority of viral gastroenteritis in the United States and worldwide [30-32, 45, 46] Additionally, the real time PCR does not detect other causative agents of gastroenteritis such as bacteria or parasitic agents. The subjects who were exhibiting symptoms of gastroenteritis, but did not show infection by the tested pathogens likely had infection with bacterial, parasitic, or other viral pathogens. Secondly, the study analyzed data collected from January-June. There is a

possibility that some viruses that are seen in the other months were not fully accounted for in our study. Lastly, there were some samples that were omitted from the study due to differing PCR results. There are a number of reasons why the PCR results for two different samples from the same subject might give different results. Primarily, the timing of sample collection is important. Samples obtained on different days are more likely to give different results. Secondly, the sample type is also important. Stool vs. diaper lining squeezed out into viral transport medium (which dilutes the specimen) is more likely to give different results. In addition, the samples could have had low viral load. One test might be positive and the other negative in different samples if the PCR target was present in only very small amounts. Lastly, there is always the possibility of contamination of the sample and problems with the sample quality/specimen handling. These are some possible reasons for why we had these samples from the same subject produce different PCR results.

Clinical implications

Currently, there are no studies that identify the epidemiological changes in the causative pathogens of VGE in post the widespread RV vaccination programs. Based on results from our study, we conclude that in the era of widespread use of RV vaccine, the most common agent causing VGE at YNH children's hospital still remains RV. Norovirus frequency seems to be increasing, mostly in the younger children who are the target population for RV vaccination program. This suggests the epidemiology of VGE may indeed be changing as more children are protected from RV. We've identified several factors that may be associated with higher severity of illness, which may help guide clinicians in improving care and directing resources. Lastly, we've confirmed the poor

agreeability between the Clark and Vesikari scales, which may guide future researchers to standardize use of clinical severity scales.

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