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Original Article

Acute viral gastroenteritis following rotavirus vaccine implementation in Venezuela: Is rotavirus still a cause for concern?

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Abstract
Introduction: Acute gastroenteritis (AGE) remains a major public health concern for the pediatric population. Diarrheal surveillance in Venezuela following the implementation of the rotavirus vaccines has been discontinuous, resulting in a lack of knowledge of the true epidemiological burden. This study investigated retrospectively the occurrence of enteropathogenic virus infections and potential changes in the etiological pattern of diarrheal disease in Venezuelan children during the post-vaccination period.
Methodology: Stool samples from 150 children with AGE and 148 controls under five years old collected in 2012-2013, were analyzed using molecular assays by rotavirus, norovirus, human adenovirus, human astrovirus, and Aichi virus. Clinical and sociodemographic associations were assessed.
Results and Conclusions: At least one virus was found in 66 (44%) of the children with AGE, and in 12 (8.1%) of the control group ($p <$ 0.0001), mostly under 24 months old. Norovirus and rotavirus prevailed significantly in the AGE group (19.3% and 18%, respectively) compared to the control group (4.7% and 0%, respectively) ($p < 0.001$). Astrovirus, adenovirus, and Aichi virus were found in 5.3% or less of the children. Malnutrition, lack of breastfeeding, absence of rotavirus vaccination, and lower socioeconomic status were more frequent among AGE children than in controls ($p = 0.014$). This study suggests that rotavirus continued circulating widely even after vaccine introduction. It emphasizes the importance of norovirus and other viruses (adenovirus, astrovirus, and Aichi virus) as potentially emerging causes of pediatric diarrhea. Future strategies for precise health management and prevention of viral diarrhea should include surveillance using molecular methods alongside sanitation efforts and measures to reduce poverty and malnutrition.

Key words: Acute gastroenteritis; Venezuela; molecular surveillance; rotavirus; norovirus; vaccine.

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Introduction

Diarrhea or acute gastroenteritis (AGE) is a main cause of pediatric morbidity and mortality worldwide, especially in developing countries. Despite the recent decline in mortality rates, pediatric AGE remains a significant global health concern. It accounts for around 8.5% of pediatric deaths [1]. It has been estimated that in 2019, diarrheal disease caused around 450,000 deaths worldwide among children under five years old [1]. Repetitive episodes of AGE not only increase the risk of secondary bacterial infections but also contribute to various long-term consequences, including delayed growth and impaired brain development, which are further exacerbated by malnutrition, especially in children residing in low- and middle-income countries [2].

Most AGE in children is caused by viruses belonging to four distinct families, with the human group A rotavirus (RV) and norovirus (NoV) being the most common agents. Some viruses, such as human adenovirus (AdV), human astrovirus (HAstV), and enteroviruses (EV), have also been described as causes of AGE among infants and young children, with prevalence varying considerably depending on the geographical area [3,4].

Rotaviruses are the most frequent cause of AGE in infants younger than 24 months old, leading to around 35.2% of under-five diarrhea deaths around the world [5]. In 2021, rotavirus resulted in approximately 120,000 (83,100–169,000) deaths among children under 5 years worldwide [5]. Interventions to reduce diarrhea-associated morbidity and mortality, such as the RV vaccination, are essential to achieve a global reduction in the incidence of RV infections. Two RV vaccines were first licensed in 2006, Rotarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq® (Merck & Co., West Point, PA, USA), which were successfully introduced in many countries, including Venezuela, as a highly effective measure to prevent severe infections in children [6–8]. Rotarix® is administered in two doses (at 2 and 4 months of age) as part of the national immunization program, making it the most widely used in Venezuela. Studies conducted in 2010 suggested that the RV vaccine had limited effectiveness, possibly due to the low vaccination coverage (49%) at that time [9].

Following the introduction of universal immunization against RV, concerns have been raised regarding the pathogen replacement of RV with other gastroenteritis viruses. An active surveillance system is essential to monitor the epidemiological burden of RV and other diarrhoea-associated viruses. However, the limited data may pose a challenge for some countries, including Venezuela, when interpreting postvaccination trends in RV disease [10,11].

Since the global introduction of RV vaccines, NoV has been reported to rapidly replace RV, becoming the most important cause of AGE among children younger than 5 years of age in some countries [12–15]. The marked variability of NoV almost certainly favors fitness and transmissibility in humans and provides new challenges to epidemiological studies. Human NoV of genogroup GII.4 has been able to cause outbreaks worldwide over the past two decades. To date, no norovirus vaccine or antiviral is available.

Human adenovirus gastroenteritis, usually mild and self-limiting, is most frequently associated with types 40 and 41 of species F, also known as enteric adenoviruses. Studies in low- and middle-income countries have demonstrated the under-appreciated burden of disease caused by adenovirus F40/41 [16]. Earlier studies indicated that AdV was the third most common cause of AGE in Venezuelan children before RV vaccine implementation [17,18]. However, in other studies conducted in 2007-2013 was shown that the incidence of AdV infection rose significantly to 24%, compared to 11.5% in previous years [19].

Human Aichi virus (AiV), initially described as a cause of oyster-associated nonbacterial gastroenteritis [20], has also been associated with AGE in infants and children, with detection rates ranging between 0.5 and 4% in several continents [21]. The emergence of infections caused by this virus, as well as others occasionally described in AGE as cosavirus and klassevirus, is not well understood and generates concerns [20,22,23]. The finding of AiV in a major river in Caracas polluted with sewage discharges in 2007-2008 [24] highlights the need to investigate the epidemiologic burden in Venezuela.

The scope of this study was to examine the prevalence, clinical impact, and potential epidemiological pattern shifts of the most common diarrhea-associated viruses in a pediatric population of the city of Barcelona (Venezuela) after the introduction of universal RV immunization.

Methodology

Patients and clinical information

From May 2012 to May 2013, a total of 298 stool samples were obtained: 150 from children presenting AGE symptoms and 148 from control subjects. This was accomplished through a stratified random sampling, based on the enrollment of children aged 1 month to 5 years old, admitted via the emergency department or outpatient clinic at the *Complejo Hospitalario Universitario "Dr. Luis Razetti*" in the city of Barcelona, Anzoátegui State (Venezuela). Children from 6 different municipalities (*Sotillo, Bolivar, Bruzual, Freites, Guanta,* and *Libertad*) participated. The control group consisted of children visiting the outpatient clinic for non-diarrheal diseases, matched by the day of attendance. The AGE group was composed of children with ≥ 3 watery or looser-than-normal stools, or ≥ 1 episode of vomiting within 24 hours, with symptom duration of ≤ 7 days at presentation. Children with allergic or eosinophilic enterocolitis, neoplasia, chronic inflammatory bowel disease, or known underlying neurological, genetic, immunological, or immunosuppressive conditions were excluded from the study.

Inpatient treatment was defined as the admission of AGE children to either the emergency room to receive oral rehydration therapy or to the regular pediatric wards of the hospital for a longer time [25]. Information from the clinical history and physical examination was collected: age, gender, nutritional status, type of treatment (outpatient/community cases or inpatient/hospital-based), and rotavirus vaccination status were recorded, together with the estimation of dehydration level, assessed according to WHO (World Health Organization) criteria [26]. The disease severity was evaluated based on the duration of diarrhea, vomiting, fever, and dehydration level using the Ruuska–Vesikari scaling system. This provides a cumulative score (with a maximum value of 20) for the severity of gastrointestinal infections [27] and classifies severity into three categories: mild (score < 7), moderate (7-10), and severe (> 11-20). Children who received both doses of the Rotarix® vaccine were considered fully vaccinated, while those with only one dose were partially vaccinated.

Socio-demographic data were obtained through parental interviews. The socioeconomic status was determined for everyone via a modified Graffar methodology, where the highest number corresponds to the lowest socioeconomic status [28].

The study was cleared by the Ethics Committee of the *"Dr. Luis Razetti*" Hospital and the *Instituto Venezolano de Investigaciones Científicas* (IVIC), the latter being the site of sample handling. At enrollment, written informed consent was obtained from each child's parents or legal guardians.

Clinical specimen collection and preparation

Stool samples were collected from the children within 48 hours of ambulatory care or hospital admission to exclude nosocomial cases and stored at −20 ˚C until processing. All of them were systematically examined for the presence of RV, NoV, AdV, HAstV, and Aichi virus. For this purpose, viral DNA and RNA were simultaneously extracted from 300 µL of diluted fecal suspensions (10% w/v in phosphate buffer saline) prepared from each stool sample, using Ribospin™ vRD (Geneall Biotechnology Co., LTD., Seoul, South Korea), based on a spin-column procedure according to the manufacturer's guidelines. Briefly, samples were lysed in the presence of VL lysis buffer (provided), transferred onto a column (provided), washed, and then the viral nucleic acids were eluted in 50 µL of DNase/RNase-free water for direct use in amplification reactions or for storage at −70 °C.

Reverse transcription

A preliminary reverse transcription (RT) reaction was performed using *RevertAid® First Strand cDNA Synthesis Kit* (Thermo Fisher Scientific Inc., USA), following the manufacturer's instructions. The reverse transcription reaction mix was prepared on ice by combining 8 µL total RNA, 1 µL of 0.2 µg/µL random hexamers, and 3 µL nuclease-free water in a reaction tube. The mixture was incubated at 80 °C for 3 min, followed by placing the tubes on ice for 2 min. Next, 4 µL of 5X reaction buffer, 2 µL of 10 mM dNTP mix, 1 µL of 20 U/µL RNase inhibitor (provided), and 1 µL of 200 U/µL RevertAid M-MuLV reverse transcriptase (provided) were added. The reaction tubes were incubated at 37 °C for 90 min and subsequently at 94 °C for 2 min using an Applied Biosystems 2720 Thermal Cycler (PerkinElmer, USA). They were then placed on ice for 2 min and briefly centrifuged. After thermocycling, the cDNA was stored at 4 °C overnight or at -20 °C for prolonged storage.

Commercial kit for the detection of astrovirus, adenovirus, rotavirus, and norovirus

All samples were tested using a commercial polymerase chain reaction (PCR) multiplex system, the *Seeplex® diarrhea-V ACE detection assay* (Seegene Inc., South Korea), which allows the simultaneous amplification of DNA/cDNA for HAstV [whose PCR product is 650 base pairs (bp)], species F of AdV (411 bp), RV group A (541 bp), genogroup I and genogroup II NoV (304 bp and 214 bp, respectively), following the manufacturer's instructions. Briefly, a working reaction mix was prepared by combining primer mixture, 8-8-methoxypsoralen solution, multiplex master mix (provided), and 3 µL of DNA/cDNA. Negative and positive internal controls were included. Amplification was performed following cycling parameters: 94 °C for 15 min, followed by 40 cycles at 94 °C for 0.5 min, 60 °C for 1.5 min, 72 °C for 1.5 min, with a final cycle at 72 °C for 10 min. After thermocycling, PCR products were analyzed by 2% agarose gel electrophoresis and SYBR safe® staining (Invitrogen, USA).

Molecular detection of Aichi virus

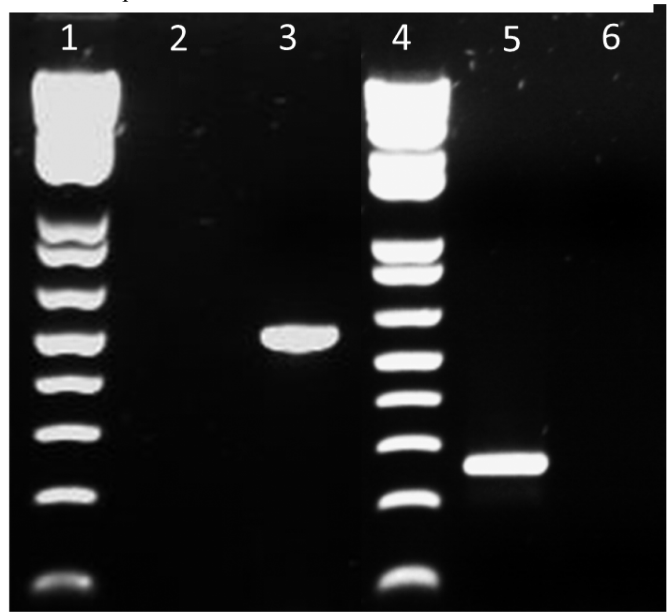
The presence of AiV was investigated using a modified PCR method, starting from 5 µL of cDNA in

Table 1. Oligonucleotide primers and amplification conditions used in this study for the molecular detection of Aichi virus.

Primer name	Region	PCR	Sequence 5'-3'	Nucleotide position	Amplicon size (bp)	Reference
6261	3C-3D	1R	ACACTCCCACCTCCCGCCAGTA	6261-6283	519 pb	[22]
6779			GGAAGAGCTGGGTGTCAAGA	6760-6779		
C94b-(mod)2F		2R	GACTTCCCCGGAGTYGTCGTCT	6398-6419	266 pb	In this study
246k-(mod)2R			GACATYCGGTTGACGTTGAC	6644-6663		

two rounds (nested PCR). Primer set 6261 and 6779 was used in the first round to amplify the 3C-3D junction region of the AiV genome and generate a 519-bp product, as described previously [22] (Table 1). The second PCR round was performed from 1 µL of the first-round amplification product using primers described previously [29] and additionally modified in this study, named C94b(mod)2F and 246K(mod)2R (Table 1), based on the genome sequence of AiVs earlier found in Venezuela [24], generating a product of 266 bp (Figure 1). Both primer pairs were used at a final concentration of 0.2 µM each in a mix containing 1.2 mM MgCl2, 0.2 mM each deoxynucleoside triphosphate, and 1 U of KAPA Taq DNA Polymerase (KAPA BIOSYSTEM, Boston, Massachusetts, USA) in PCR buffer to reach a final volume of 50 µL. Cycling conditions used were adapted as follows: for the first round, 95 °C for 1 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, with a final elongation at 72 °C for 10 min. The second round was conducted under the same cycling conditions, but with a different annealing temperature, which was 55 °C, and primer set. All the PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Figure 1. Electrophoresis in 1.5% agarose gel showing the PCR products obtained from Aichi virus-positive stool samples using a nested PCR protocol with two rounds.



Lanes 1 and 4: molecular weight [1 Kb plus DNA ladder (Invitrogen, USA)]; lane 3: first round PCR product (519 bp) from one sample positive for Aichi virus; lane 5: Second round PCR product (266 bp) in a sample positive for Aichi virus; lanes 2 and 6: negative control for the PCR reaction.

Table 2. Sociodemographic and clinical features of the pediatric patients studied in Barcelona, Venezuela. The study included children with acute gastroenteritis symptoms (AGE group) and children who attended the hospital for reasons other than diarrhoeal disease (control group).

Group	**AGE**		**Control**		***p***
N.	150		148		
Median age (m)	11.5		12		0.057
Age range (m)					
< 24	121	(80.7)	103	(69.6)	0.027
24-59	29	(19.3)	45	(30.4)	
Gender					
Male	91	(60.7)	81	(54.7)	0.3
Female	59	(39.3)	67	(45.3)	
Graffar					
1	0	(0)	0	(0)	-
2	1	(0.7)	0	(0)	1.0
3	13	(8.7)	7	(4.7)	0.174
4	110	(73.3)	131	(88.5)	0.0008
5	26	(17.3)	10	(6.8)	0.005
Nutritional status					
Well-nourished	135	(90)	147	(99.3)	0.0004 *
Malnourished	15	(10)	1	(0.7)	
Maternal breastfeeding					
Exclusive	47	(31.3)	73	(49.3)	0.0023
Formula or Mixed	103	(68.7)	75	(50.7)	
RV vaccination status **					
Partially vaccinated	62	(41.3)	85	(57.4)	
Fully vaccinated	22	(14.7)	20	(13.5)	
Non-vaccinated	66	(44.0)	43	(29.1)	0.014 *

m: months. Data are n (%) of children studied and were analyzed using $\chi 2$ (or * χ^2 with Yates correction) (two-tailed, 95% confidence intervals) when the sample size was less than 5 (Statgraphics, Centurion XV.I (Statgraphics Technologies, Virginia). The significance of the age differences was calculated by Mann-Whitney U (Wilcoxon). $p < 0.05$ was considered statistically significant. The scale used for the Graffar socioeconomic level was based on a modified methodology described by Méndez Castellano *et al.* [28]. ** Fully vaccinated children received both doses of the Rotarix® vaccine, while partially did not complete the schedule.

Statistical analysis

Data were analyzed using 2 × 2 tables with χ^2 test, or Fisher's exact test (2-tailed, 95% confidence intervals) by Statgraphics, Centurion XV.I. (Statgraphics Technologies, Virginia). The Mann-Whitney U (Wilcoxon) and Kruskal-Wallis tests were applied to nonparametrically distributed values and group comparisons. All tests were two-tailed, and a $p < 0.05$ was considered significant. For the comparison of the viral etiological pattern, only municipalities with a statistically acceptable sample size were considered.

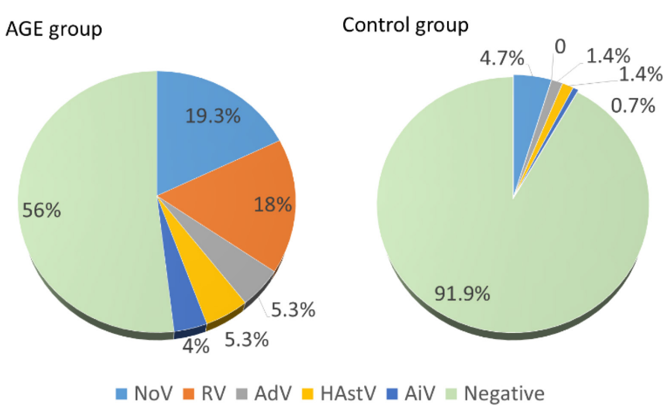
Results

Population characteristics

Of the 298 pediatric patients tested for diarrhea-related viruses, 172 (57.7%) were male and 126 (42.3%) were female. The age distribution of children under investigation ranged from 1 to 57 months (median age 12 months), and the majority (75.2%) were younger than 24 months. The median age of the 150 AGE cases and 148 controls was similar ($p > 0.05$, 11.5 vs.12 months, respectively) (Table 2). Children less than 24 months old were significantly more numerous in the AGE group than among control subjects (80.7% vs. 69.6%, $p = 0.027$). The gender difference in both groups was not statistically significant ($p > 0.30$).

The proportion of malnourished children and those belonging to the extreme poverty stratum was significantly higher among the cases of AGE compared to the controls (17.3% vs. 6.8%, $p = 0.005$, and 10% vs 0.7%, $p = 0.0004$, respectively). A significantly higher proportion of controls than cases had been exclusively breastfed and vaccinated against RV ($p = 0.002$ and $p = 0.014$, respectively) (Table 2).

Figure 2. Comparison of virus detection rates between the AGE and control group from pediatric patients studied in Barcelona, Venezuela, during 2012-2013.



A total of 150 AGE cases and 148 control children were tested for gastroenteritis viruses. Diagrams show the percentage of children infected with each single virus. NoV: norovirus, RV: rotavirus, HAstV: human astrovirus, AdV: human adenovirus, AiV: Aichi virus. Statistical comparison was performed using the χ^2 or Fisher's exact test (2-tailed, 95% confidence intervals), where $p < 0.05$ was considered statistically significant. Viral infections caused by NoV and RV were significantly more frequent in AGE children than in controls ($p = 0.001$).

Prevalence of enteric viruses

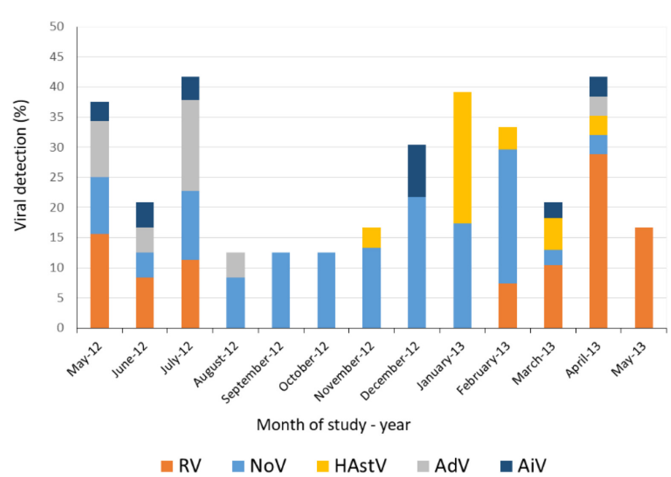
Overall, 78 (26.2%) of the 298 children tested positive for one or more viruses, as at least one virus was demonstrated in 66 (44%) of the 150 children with AGE, while only in 12 (8.1%) of the 148 controls, revealing a significant difference ($p < 0.001$). The median age in the AGE group did not differ statistically between virus-positive children (15 months) and virus-negative ones (10 months) ($p = 0.5$).

NoV and RV were found in 29 (19.3%) and 27 (18%) children, respectively, of the 150 AGE cases, and in 7 (4.7 %) and none of the controls (Figure 2). Both viruses were notably more prevalent than others in the AGE group compared to controls ($p < 0.0001$ for each). The detection rates of HAstV and AdV (each 5.3%) and of AiV (4%) in AGE children were similar to those in the control group (1.4% for AdV and HAstV, and 0.7% for AiV), with $p \geq 0.05$ for each comparison (Figure 2).

Seasonality of gastroenteritis viruses

Detection of gastrointestinal viruses showed temporal variability across the study duration (2012-

Figure 3. Temporal distribution of infections caused by gastrointestinal viruses in 298 children from Barcelona (Anzoátegui State, Venezuela) studied between May 2012 and May 2013.



The figure shows the percentage (%) of viral infections in the total examined per month. RV: rotavirus, NoV: norovirus, HAstV: human astrovirus, AdV: human adenovirus, AiV: Aichi virus.

Table 3. Gastrointestinal viruses detected in 12 children with coinfection from 150 exhibiting diarrhea in Barcelona, Venezuela (2012-2013).

Viral coinfections detected	n (%)
NoV + HAstV	3 (25)
RV + HAstV	2 (16.7)
RV + AiV	2 (16.7)
RV + AdV	2 (16.7)
NoV + RV	1 (8.3)
NoV + AdV	1 (8.3)
NoV + AiV	1 (8.3)

NoV: norovirus; HastV: human astrovirus; RV: rotavirus; AiV: Aichi virus; AdV: human adenovirus

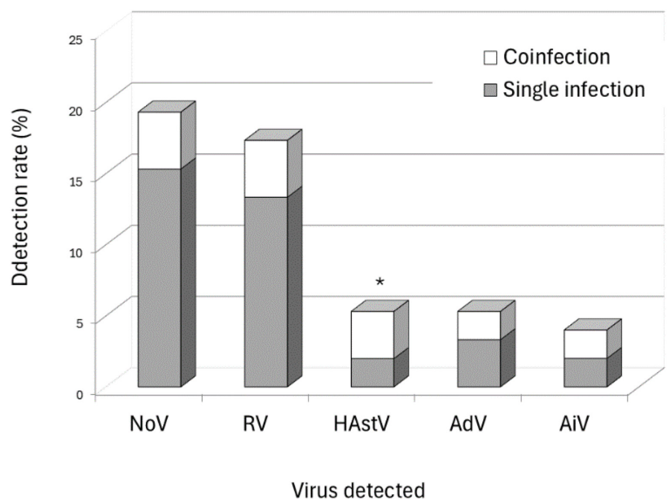
2013) in both groups, children with AGE and controls, with a detection rate exceeding 35% during peak periods (Figure 3).

RV and NoV were the predominant viruses throughout the study, although their prevalence showed seasonal variations. RV was present from May to July 2012, absent between August 2012 and January 2013, and then reemerged during the 2013 dry season, achieving a peak positivity rate of 28.8% in April. On the other hand, NoV was present for almost the entire study period with a frequency that varied from 2.6% to 22.2%. It predominantly circulated from August 2012 to February 2013, coinciding with periods of increased rainfall. HAstV detection increased in January, the coolest and driest month. AiV was detected intermittently.

Single infection and coinfections caused by gastroenteritis viruses

Of the 66 infected children with AGE, 54 (81.8%) had a single viral infection, while 12 (18.2%) showed mixed infections (or coinfections, involving more than one virus). There were no coinfections in the control group.

Figure 4. Proportion of gastrointestinal viruses involved in single infection or coinfection among children with AGE from Barcelona (Venezuela), 2012-2013.



NoV: norovirus, RV: rotavirus, HAstV: human astrovirus, AdV: human adenovirus, AiV: Aichi virus. Statistical analysis was performed using the Fisher-Freeman-Halton test (95% confidence interval). HAstV was significantly more involved in coinfections than in single infections ($p < 0.0001$), which was indicated with an asterisk (*)

The most common coinfection was caused by NoV and HAstV (25%) (Table 3). Figure 4 illustrates the proportion of single infections and coinfections caused by each virus during the AGE episodes. When analyzed individually, HAstV was significantly more involved in coinfections than in single ones (62.5 vs 37.5%, $p = 0.0001$) compared with the other viruses. In contrast, NoV, RV, AdV, and AiV most frequently caused a single infection (Figure 4).

Mixed infections occurred throughout the entire

Table 4. Demographical and clinical characteristics of single- and co-infected AGE patients.

	Single infection		Co-infection		*p*
N	54	(81.8)	12	(18.2)	
Median age (m)	14		20.5		0.228
Age range (m)					
< 24	44	(81.5)	8	(66.7)	0.264
24-59	10	(18.5)	4	(33.3)	
Gender					
Male	33	(84.6)	6	(77.8)	0.528
Female	21	(15.4)	6	(22.2)	
Nutritional status					
Well-nourished	52	(96.3)	12	(100)	1.0
Malnourished	2	(3.7)	0	(0)	
Maternal breastfeeding					
Exclusive	45	(80.3)	11	(91.7)	0.675
Formula or Mixed	9	(16.7)	1	(8.3)	
Severity score (median)	11		14		0.085
Type of treatment					
Inpatient	15	(27.8)	4	(44.4)	0.086
Outpatient	39	(72.2)	5	(55.6)	

m: months. Data are n (%) of children studied and were analyzed using Mann Witney (Wilcoxon) text for median age and severity, and Fisher's exact test (95% confidence intervals) for qualitative variables. $p < 0.05$ was considered statistically significant. The disease severity was assessed using the Ruuska–Vesikari scaling system (maximal value score = 20) [27], and the socioeconomic status by a modified Graffar methodology [28].

Table 5. Sociodemographic and clinical characteristics in AGE children according to the virus detected.

	NoV		**RV**		**AdV**		**HAstV**		**AiV**		***p* (*)**
N.	23		20		5		3		3		
Median age (m)	12		13.5		15		23		24		0.202
Age range (m)											
< 24	21	(91.3)	17	(85)	3	(60)	2	(66.6)	1	(33.3)	0.073
24-59	2	(8.7)	3	(15)	2	(40)	1	(33.3)	2	(66.6)	
Gender											
Male	14	(60.9)	12	(65)	2	(40)	2	(66.6)	0	(0)	0.715
Female	9	(39.1)	8	(35)	3	(60)	1	(33.3)	3	(100)	
HRV vaccination status											
Vaccinated	13	(56.5)	13	(67.3)	2	(40)	1	(33.3)	2	(66.6)	0.836
Non-vaccinated	10	(43.5)	7	(33.7)	4	(60)	2	(66.6)	1	(33.3)	
Nutritional status											
Well-nourished	23	(100)	19	(95)	5	(100)	3	(100)	2	(66.6)	0.143
Malnourished	0	(0)	1	(5)	0	(0)	0	(0)	1	(33.3)	
Vesikari score (median)	11		12		7		12		9		0.173
Type of treatment											
Inpatient	6	(26.1)	8	(40)	0	(0)	1	(33.3)	0	(0)	0.148
Outpatient	17	(73.9)	12	(60)	5	(100)	2	(66.6)	3	(100)	

m: months; NoV: norovirus; RV: rotavirus; AdV: adenovirus; HAstV: human astrovirus; AiV: Aichi virus. Data are n (%) of children with single viral infection studied and were analyzed using the Kruskal Wallis test for median age and severity, and Fisher-Freeman-Halton's exact text (95% confidence intervals) for qualitative variables. (*) Because of the small numbers, statistical calculations did not include AdV, HAstV, and AiV. $p < 0.05$ was considered statistically significant. The disease severity was assessed using the Ruuska–Vesikari scaling system (maximal value score = 20) [27].

period studied. Overall, both single infection and coinfections were more frequent in children under 24 months of age. The median age of the single-infected children was lower than that of the coinfected children, but the difference was not statistically significant (p = 0.228) (Table 4). The median Vesikari severity score and the proportion of inpatient children were higher in the coinfected group, although the differences were not statistically significant ($p > 0.05$). There were also no significant differences in gender, nutritional status, feeding habits, or type of treatment received between the two groups.

Viral pathogen and clinical severity in children with AGE

The sociodemographic and clinical characteristics of children did not differ significantly based on the virus detected in cases of single infection (Table 5). The median age was higher in HAstV- and AiV-infected children (23 and 24 months, respectively) than in others (Table 5). Rotavirus and NoV infected a higher proportion of children under 24 months of age than the other viruses, with RV determining more frequent hospitalization than NoV (40% vs 26.1%) (Table 5). However, the differences in severity score or any other sociodemographic parameters (age, gender, HRV vaccination, or nutritional status) were not statistically significant ($p > 0.05$).

Demographic pattern of gastroenteritis viral infections

Gastrointestinal viruses were detected in children from four of the six municipalities included in the study, specifically from Bolivar, Sotillo, Guanta, and Libertad, which were also the most densely populated [30] (Figure 5A). The following analysis focused on Bolívar and Sotillo due to their numerical representation. The total viral infection rate (including AGE and control children) was comparable (p = 0.07) between Bolívar (30.4%) and Sotillo (21.3%). The RV vaccination rate was slightly, although not significantly, higher in Sotillo compared to Bolívar (68.8% vs. 59.5%, p = 0.09). The percentage of children with AGE was considerably higher in Bolívar (66.9%, 99/148 subjects) compared to Sotillo (31.9%, 45/141) ($p < 0.0001$). The analysis of the viral etiology among the AGE cases from these municipalities revealed that the proportions of both NoV (42% vs. 35.3%) and RV (34% vs. 23.5%) were higher in Bolivar than in Sotillo. Conversely, AdV was detected with a significantly higher frequency in Sotillo (20.6%) ($p < 0.05$) (Figure 5B).

Discussion

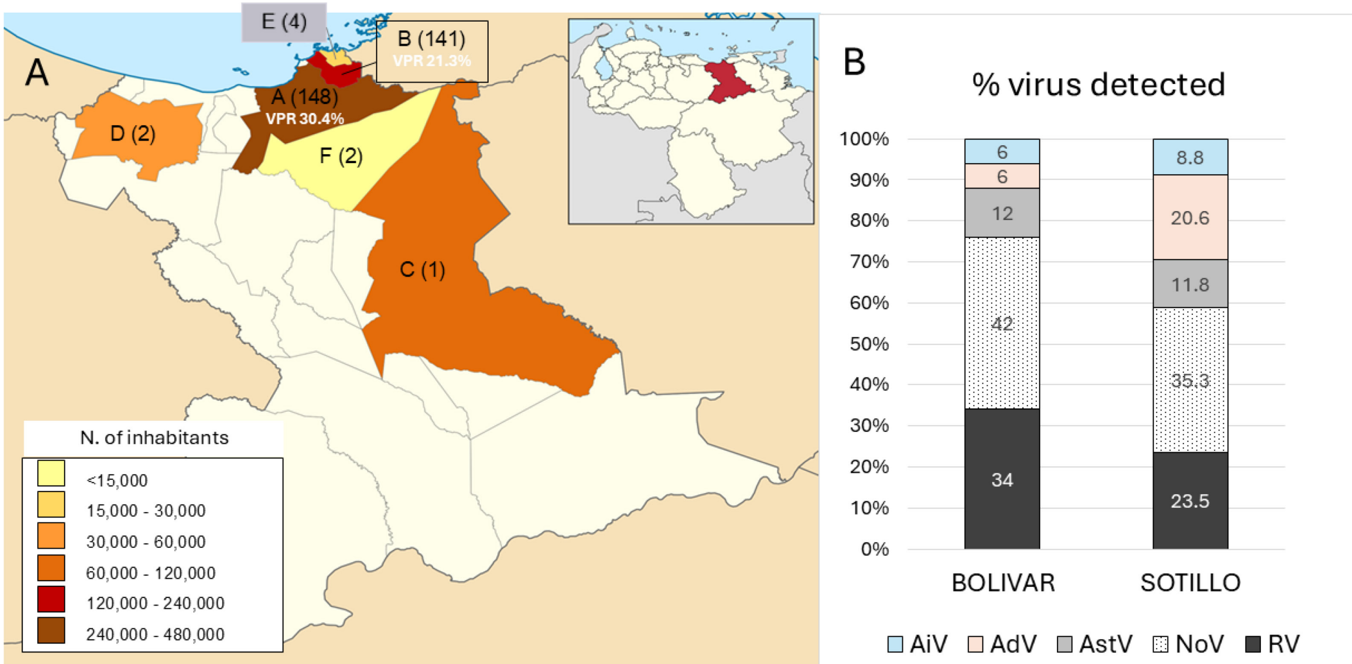
Acute gastroenteritis remains one of the main causes of death in children under 5 years, despite the reduction in diarrhea mortality by over 60% since the year 2000 due to improved social, economic, and environmental conditions of the population [31,32]. Regardless of the heavy burden of AGE in Latin American countries, only a few published studies have described the role of gastrointestinal viruses in sporadic diarrhea events by molecular epidemiology based on highly sensitive molecular methods [17,33–35]. In Venezuela, more than 1,200 children of all ages died of diarrheal diseases in 2013 [36], but data about the causes of diarrhea are limited, even after the introduction of the rotavirus vaccine [19].

The current study was conducted in a hospital in Barcelona, the largest and most important city in the Eastern Region of Venezuela, near the Caribbean Sea. Patients from all over Anzoategui state visited this hospital. Based on the XIV national population census (2011), this state had a population of about 1,469,747 inhabitants (http://www.redatam.ine.gob.ve/Censo2011/index.html) [30] (Figure 5). While the results may not be representative of the entire population, as they only encompassed individuals who sought medical attention during the study period, they reveal a high viral infection rate in children with AGE. This is consistent with findings from neighboring countries [35,37] and a previous study conducted in Valencia (Venezuela) before the RV vaccine implementation [17]. This study demonstrates the significant involvement of norovirus and rotavirus in diarrheal infections among Venezuelan children, with other gastrointestinal viruses such as astrovirus, adenovirus, and Aichi virus, contributing to a lesser extent. This confirms the active circulation of these pathogens within the population [18]. The results agree with those reported elsewhere, indicating that NoV and RV remain the most common infectious agents associated with moderate to severe AGE in children under 5, even in the post-rotavirus vaccine era [37,38].

The significant absence of RV activity and increased circulation of NoV during the rainy season confirms the seasonal pattern previously observed for these viruses in other cities in Venezuela [10,17]. Although there are variations from year to year, in Venezuela the rainy season is usually from May to October, and the dry season runs from November to April, encompassing the coldest and driest months. The observed pattern shows that RV frequency peaks in the dry season, and highlights the role of water in influencing the transmission efficiency of other agents, such as noroviruses. No clear seasonal pattern was identified for HAstV, Aichi virus, and AdV. Notably, a recent report from wastewater from Caracas, Venezuela, indicated widespread circulation of norovirus and the continuous presence of AdV consistently around the year [39].

Although enteric viruses can be found in children with and without diarrhea [40], the higher detection rate in AGE cases compared with controls highlights their significant pathogenic role in children under 5 years

Figure 5. Map of Venezuela showing study location. A) The top right panel is a map of Venezuela showing the location of the Anzoátegui State, which has an area of 43,300 km² and represents 4.7% of the national territory. It is composed of 21 municipalities. Patients screened for gastroenteritis viruses included in this study were from five of them (colored, indicated with a letter (A: Bolivar, B: Sotillo, C: Freites, D: Bruzual, E: Guanta, and F: Libertad).



The population size of the municipalities studied is shown in the box on the left, while the number of samples collected from each area is in parentheses ( ) on the map. VPR (viral positivity rate) for the two municipalities whose sample size was valid for statistical calculation, is indicated on the map. B) The proportion (%) of viruses detected among the positive samples of AGE children of two of the municipalities (Bolivar and Sotillo) is shown. RV: rotavirus, NoV: norovirus, HAstV: human astrovirus, AdV: human adenovirus, AiV: Aichi virus old, especially those under 24 months.

This study found that malnutrition and high poverty status were significantly associated with diarrhea. Malnourished children from lower economic strata are more likely to experience diarrhea in the first two years of life, due to a combination of factors that include a weaker immune system, limited access to clean water and sanitation, and higher exposure to pathogens. In developing countries, most viral gastrointestinal infections occur at younger ages, further increasing their vulnerability [5]. On the other hand, breastfeeding and RV vaccination had a protective effect. These measures, alongside improved global access to safe drinking water, adequate sanitation, and oral rehydration therapy, can contribute to significantly reducing the burden of this preventable illness [32].

Following the introduction of universal rotavirus immunization programs, norovirus has become the most common cause of pediatric AGE in some countries, such as the USA [14], and it has gained importance in some, but not all, developing countries [12,34,40-42]. Variable NoV prevalences have been described, probably due to climatic or socioeconomic discrepancies, depending on the country. In this study, the NoV infection rate found in the AGE group was similar to that reported in Valencia (Venezuela) in children with diarrhea before the RV vaccine implementation [18]. It is plausible that this rate is underestimated, as our case definition was based on having three or more watery stools within 24 hours, whereas norovirus patients presenting only with vomiting could have been overlooked. The results confirm the relevant epidemiological role of noroviruses, which should be actively monitored to facilitate the implementation of appropriate control measures.

Several reports indicate a global decline in RV-related diarrhea hospitalizations after the vaccine implementation [7,8,44], but current data for Venezuela are limited. The moderately high RV prevalence in this study (18%) generates concern about the efficacy of the vaccine implementation program. This prevalence, derived from inpatients and outpatients with diarrhea, was slightly higher than that reported in one sentinel RV surveillance study (< 16.9%), carried out during the period 2004-2012, which only included hospitalized children [11]. Diverse evidence supports that rotavirus vaccine efficacy can differ across locations [45]. The vaccine coverage rate in Venezuela was estimated to be approximately 75% in 2013, rising to 84% in 2015 [46]. Since then, the rate has decreased, and its current status is uncertain. The data here collected suggest that RV remains a significant cause of diarrhea in this country and that the vaccine has not been as effective as initially expected. These findings can serve as an important reference for subsequent research efforts aimed at further investigating this issue.

The occurrence of astroviruses, adenoviruses, and Aichi viruses in both AGE and controls children draws interest. They were involved in mixed infections exclusively in children with diarrhea, especially astrovirus, whose significant role has also been described in a previous study in Venezuela [18], and elsewhere [47,48]. Mixed infections are common in AGE children, particularly where hygiene is poor [33]. In settings with a strong circulation of enteric viruses, viral coinfections may probably impair vaccine efficacy for other viruses. The frequency of coinfections among children with AGE was consistent with rates described in studies from developing countries [33,37,47]. Although the sample size was insufficient for robust statistical analysis, a slightly increased severity of diarrhea was noted in children experiencing coinfections compared to those with single infections. The enrolled children could not be tested for non-viral pathogens causing diarrhea, thus the presence of other concurrent infections responsible for a worsening clinical manifestation could not be considered. The results show that infections with multiple pathogens lead to dehydration and enhanced comorbidity in AGE. Other authors have described coinfections in older children [33]. Behavioral factors, along with other unidentified socioeconomic elements, may contribute to an increased risk of infection. Additionally, the possibility that passive or acquired immunity may decline after the first year of life cannot be discounted.

More populated municipalities had higher rates of gastrointestinal viral infection, possibly due to crowded living conditions, increased human contact, and sanitation challenges. The lower proportion of children with AGE in the municipality of Sotillo can be attributed to improved economic conditions of its inhabitants, linked to the development of the tourism industry, which implies better access to medical care and a higher overall quality of life. On the other hand, the significantly greater proportion of AdV infection in the Sotillo municipality is not surprising, as it coincides with the slightly higher RV vaccination rate compared to Bolívar. This could reflect a changing epidemiological landscape of AGE, in which a population vaccinated against rotavirus is infected with other pathogens, like AdV, in agreement with previous observations [19]. Research on the factors affecting viral transmission and seasonality may enhance our understanding of the relationship between geographic distribution and infection risk for these pathogens.

Conclusions

The global consensus emphasizes the need to scale up RV vaccination programs through a structured approach that includes data collection, promotion, and expansion of the initiative, with appropriate incentive strategies and continued evaluation, particularly in countries eligible for support. Current results from eastern Venezuela show that pediatric gastroenteritis is caused by a wide range of viruses, with co-infections correlating with a more severe illness. Comprehensive diagnostic tests capable of detecting multiple enteric viruses should be implemented, in addition to RV and norovirus, for a more accurate clinical diagnosis and epidemiological surveillance. Moreover, understanding community transmission dynamics, disease severity, and vaccine efficacy for these pathogens remains crucial. On the other hand, pathogen-specific interventions require localized evaluation of their effectiveness, as they may not significantly affect the overall incidence of diarrhea across different populations. The development of more effective and targeted prevention and control strategies must focus on improving vaccination coverage, optimizing water sanitation, and expanding access to healthcare. It also demands a centralized data collection system and digital platforms, incorporating trained personnel and encompassing sentinel sites in outlying and remote territories, as well as wastewater surveillance. These findings can serve as a valuable reference for future studies and highlight the need for ongoing surveillance of acute gastroenteritis, guiding evidence-based public health interventions.

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Ethics statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the *"Dr. Luis Razetti"* Hospital (Aval 11/30/2012) and *Instituto Venezolano de Investigaciones Científicas* (IVIC) (DIR-0893/1568/2016 on July 20th, 2016). At enrollment, written informed consent was obtained from each child's parents or legal guardians.

Availability of data

The data that support the results are available from the corresponding author upon reasonable request.

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Authors contributions

JZ: Conceptualization, funding acquisition, investigation, methodology, formal analysis, acquisition of data, writing – original draft. AJM: Conceptualization, funding acquisition, supervision, investigation, methodology, formal analysis, writing – review & editing. MZS and AJ: Investigation, methodology. RF: investigation, methodology, acquisition of data. RER: Investigation, methodology. MG: Formal analysis, visualization, writing – review & editing. EV: Formal analysis, investigation, supervision, methodology, resources, funding acquisition, writing – original draft, and writing – review & editing. All authors have read and approved the manuscript.

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Conflict of interest

No conflict of interest is declared.

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