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Epidemiology of enteric virus infections in children living in the Amazon region

Alberto Ignacio Olivares Olivares[a,b], Gabriel Azevedo Alves Leitão[a,c], Yan Cardoso Pimenta[a,c], Carina Pacheco Cantelli[a], Tulio Machado Fumian[a], Alexandre Madi Fialho[a], Sergio da Silva e Mouta Junior[a], Isabella Fernandes Delgado[c], Johan Nordgren[d], Lennart Svensson[d], Marize Pereira Miagostovich[a], José Paulo Gagliardi Leite[a,1], Marcia Terezinha Baroni de Moraes[a,1,*]

[a] Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Fiocruz, Avenida Brasil, Rio de Janeiro, RJ, Brazil
[b] Post-Graduate Program in Parasite Biology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Fiocruz, Avenida Brasil, Rio de Janeiro, RJ, Brazil
[c] Post-Graduate Program in Sanitary Surveillance, National Institute for Quality Control in Health, Oswaldo Cruz Foundation, Fiocruz, Avenida Brasil, Rio de Janeiro, RJ, Brazil
[d] Division of Molecular Virology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

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ABSTRACT

Objectives: To verify the frequency of viruses causing acute gastroenteritis (AGE) in association with the histo-blood group antigen (HBGA) and Rotarix™ vaccination coverage in children from the Amazon region.

Design: Fecal and saliva samples were collected from children with AGE (n = 485) and acute respiratory infection (ARI) (n = 249) clinical symptoms. Rotavirus A (RVA), norovirus, human adenovirus (HAdV), and sapovirus (SaV) were verified in feces by molecular detection. Saliva samples were used for HBGA phenotyping/FUT3 genotyping. Blood group types, clinical aspects and Rotarix™ RVA vaccination data were recorded.

Results: Norovirus remained the most prevalently detected cause of AGE (38%, 184/485 and ARI 21.3%, 53/249). High HAdV frequencies were observed in AGE children (28.6%, 139/485) and ARI children (37.3%, 93/249). RVA was the third most prevalent virus causing AGE (22.7%, 110/485 and ARI 19.3%, 48/249) and a low RV1 coverage (61%, 448/734) was verified. The SaV frequencies were lower (7.2%, 35/485 for AGE and 6.8%, 17/249 for ARI). Secretor children were HBGA susceptible to HAdV infection (OR 1.5, 95% CI 1.0–2.3; $P = 0.04$) but not to RVA, norovirus or SaV infection.

Conclusions: Norovirus could be considered the main etiological agent of AGE. No association was verified for HBGA susceptibility to RVA, norovirus and SaV. Secretor children showed a slight susceptibility to HAdV infection and the Le (a-b-) heterogeneous SNPs on the *FUT3* gene.

Introduction

Infectious diseases are responsible for more than half of childhood deaths and an even greater level of morbidity worldwide. Almost all children who are infected (99%) live in low-income and middle-income countries. Controlling infectious diseases in the Amazon region has been a challenge and this low-income region has frequently suffered, with deforestation being a practice that negatively impacts the health of the younger children living there; acute gastroenteritis (AGE) corresponds to 12% of these deaths (GBD, 2015). Rotavirus A (RVA) and norovirus are the most prevalent viral agents causing AGE, and sapovirus (SaV) has recently been recognized as an important AGE causative agent (Becker-Dreps et al., 2020). Human adenovirus (HAdV) frequently causes AGE outbreaks (GBD, 2015) and acute respiratory infection (ARI) (Frenkel, 2018). In 2006, both monovalent (Rotarix™ = RV1)

* Corresponding author at: Laboratory of Comparative and Environmental Virology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Fiocruz, Avenida Brasil, 4365-Manguinhos, Rio de Janeiro, RJ, Brazil.
E-mail addresses: baroni@ioc.fiocruz.br, marciaterezinha4@gmail.com (M.T.B. de Moraes).
[1] Jose Paulo Gagliardi Leite and Marcia Terezinha Baroni de Moraes contributed equally to this study.

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and pentavalent (RotaTeq™ = RV5) vaccines were licensed and recommended by the World Health Organization (WHO) and Pan American Health Organization (PAHO). Six Latin American countries, including Brazil, introduced one of these vaccines into their National Immunization Program (NIP) (PATH, 2015). The ABO (H), secretor (*FUT2*) and Lewis (*FUT3*) system genes control the expression of part of the histo-blood group antigens (HBGA) acting as genetic host susceptibility factors. HBGAs mediate norovirus and RVA infections and might impact RV1 vaccine efficacy (Cantelli et al., 2020; Sharma et al., 2020; Nordgren and Svensson, 2019; Heggelund et al., 2017; Desselberger, 2017).

This study investigated the detection frequency of RVA, norovirus, HAdV, and SaV in the feces collected from 734 children – 485 with AGE and 249 with ARI –, the latter being the most common infection affecting Amazonian children (Moraes et al., 2019). The HBGA phenotype and RV1 vaccination coverage were defined for the 734 children. This study aimed to verify the frequency of viruses causing AGE in Amazonian children.

Methods

Ethics statement and study population

This study was approved by the Federal University of Roraima Ethical Research Committee (CEP No: 1.333.480 from 23 November 2015). A total of 734 children aged ≤5 years were enrolled in this study, corresponding to a total of 1.468 fecal and saliva samples collected in parallel from each child from October 2016 to October 2017. The children with severe AGE (n = 485) or ARI (n = 249) exclusively attended the emergency care unit of "Hospital da Criança de Santo Antonio" (HCSA). The inclusion criteria, according to WHO (2005, 1990), were: AGE: ≥three liquid/semi-liquid evacuations in a 24-h period and dehydration; and ARI: high fever ≥39 °C, cough, dyspnea, and dehydration. The HCSA is the only hospital placed in Boa Vista, state of Roraima (RR), that attends children living in the extreme north of Brazil and the limit border with Venezuela and Guyana, including those living in the Amazon rainforest in demarcated indigenous areas. The pediatrician responsible for collecting the samples immediately after the children's admission was in attendance every day at the HCSA. Each child was examined, and the child's parents or guardians were interviewed to collect data and fill out a form containing clinical and epidemiological information for each child. All saliva samples containing epithelial cell samples were collected at least 1 h before or after breastfeeding.

RV1 vaccination data, method of determination of RV1 coverage, and blood type data

The vaccination card was verified for each child. The vaccination coverage was determined through the method recommended by the Ministry of Health in Brazil for the most accurate record of the reality of the local vaccine situation. The method is named "Monitoring of Vaccination Coverage" (MVC), which is calculated considering the total number of children vaccinated divided by the total number of children in the study (either vaccinated or unvaccinated) (Brasil et al., 2012). Blood type data collection was performed at "Hospital Maternidade de Boa Vista" or "Maternidade Nossa Senhora de Nazaré" located in Boa Vista City, RR state.

Samples processing

All samples were sent together with clinical-epidemiological records to the Regional Rotavirus Reference Laboratory–Laboratory of Comparative and Environmental Virology (RRRL–LVCA). This laboratory is part of the ongoing national network for AGE surveillance and is coordinated by the General Coordination of Public Health Laboratories, Brazilian Ministry of Health. Samples were strictly kept under –20 °C until the moment of processing. Then, 10% fecal suspensions in pH 7.4 phosphate-buffered saline (PBS) solutions were prepared (WHO, 2009). The saliva samples were processed using 1.2 mL of PBS to a final dilution of 1:5, as previously described (Moraes et al., 2019).

Virus detection

Total viral nucleic acid was obtained from 140 μL of each supernatant of 10% fecal suspension sample that was subjected to an automatic nucleic acid extraction procedure using a QIAamp® Viral RNA Mini kit (QIAGEN, CA, USA) and a QIAcube® automated system (QIAGEN), according to the manufacturer's instructions, with a final elute sample volume of 60 μL. The total viral nucleic acids isolated were immediately stored at −80 °C until molecular analysis. Monoplex reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed for detection of RVA (Zeng et al. 2008) and SaV (Fioretti et al., 2016; Oka et al., 2006), as previously described. For norovirus detection, a duplex (GI/GII) RT-qPCR was performed (Kageyama et al., 2003). For HAdV, a quantitative polymerase chain reaction (qPCR) assay was carried out according to that recently presented (Leitão et al., 2020). All the reactions were conducted on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, California USA) using SuperScript™ III Platinum™ One-Step qRT-PCR (Invitrogen, Carlsbad, California, USA), according to the manufacturer's recommendations, in a total volume of 20 μL and with optimized thermal cycling conditions as described. All samples that showed signals crossing the threshold line in both replicas up to a C_t value of 40.00 and presented a characteristic sigmoid curve were regarded as positive.

Secretor status and Lewis antigen phenotyping

The HBGA phenotyping was performed using the processed saliva samples diluted 1:100 via Enzyme Immunoassay (EIA), as previously described, to detect A, B, Le^a and Le^b antigens (Moraes et al., 2019). Additionally, all saliva samples diluted 1:100 were assayed for Fucα1-2Gal-R detection using the lectin-based EIA (Nordgren et al., 2014) in order to confirm the secretor phenotype. All saliva samples were evaluated in duplicate together with control saliva samples from secretor and non-secretor adult donors (The Oswaldo Cruz Foundation Ethical Research Committee - CEP/Fiocruz number 311/2006) from a different Lewis profile storage at RRRL–LVCA.

Nucleotide sequencing of the FUT3 *(*le*) gene*

The Le (a-b-) saliva samples (did not react to either monoclonal Ab anti-Le^a or Le^b) were selected for total genomic DNA extraction using a silica-based method (Boom et al., 1990) and 200 μL of processed saliva samples. Touchdown PCR (TD-PCR) was used to amplify the entire coding region of the *FUT3* gene using Platinum SuperFi DNA Polymerase enzyme, according to the manufacturer's recommendations (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The *FUT3* gene primers were described by Nordgren et al. (2013) for the entire coding region of the *le* gene and for covering the five main single-nucleotide polymorphisms (SNPs) of the *le* gene (Soejima and Koda, 2005; Elmgren et al., 1996; Mollicone et al., 1994): rs28362459 (59 T > G), rs812936 (202 T > C), rs778986 (314 C > T), rs3745635 (508 G > A), and rs3894326 (1067 T > G). The purified amplicons were sequenced using all eight primers described by Nordgren et al. (2013) on the ABI Prism BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit and ABI Prism 3730 Genetic Analyzer (Applied Biosystems, California, USA).

Statistical analysis of data

The Statistica 12.6 software (December, 2014) was used for all statistical analysis. The statistical tests were Pearson Chi-Square (differences were considered statistically significant at $P > 0.05$) (Pearson, 1900). Odds ratio (OR) values were calculated according to Szumilas (2018).

Results

Clinical and epidemiological features of the children infected by the viruses

The children were all from the Amazon region and aged 1–5 years; 58.6% (430/734) were boys and 41.4% (304/734) girls. Most of the children in this study lived in RR, Brazilian state (91.1%, 669/734), but 9.9% (65/734) lived in the Brazilian state of Amazonas (AM) (2%, 14/734) and the countries of Guyana (1.2%, 9/734) and Venezuela (5.7%, 42/734). Some of them (4.5%, 162/734) lived in isolated areas in the Amazon rainforest without any basic sanitation or properly treated water for consumption. Table 1A shows the name of the indigenous ethnic groups that live in these areas (Moraes et al., 2019). Oral hydration was administrated to all children in this study at the time of admission to the emergency care unit. Tables 1B and 1C show the aspects detected according to what was recorded in each child's form. As shown in Table 1B, the presence of mucus in feces (94.8%, 460/485), fever of ≥38.5 °C (89.5%, 434/485) and vomit (46.8%, 227/485) were more prevalent in children with AGE compared with children with ARI. Table 1C shows the same clinical aspects as the most prevalent detected in AGE children infected with RVA, norovirus, HAdV, and SaV.

Frequencies of human rotavirus A, norovirus, adenovirus and sapovirus in children living in the Amazon region

Thirty-two percent of children were negative for all the viruses investigated here (235/734): 29.9% (145/485) of these with AGE and 36.1% (90/249) with ARI. High frequencies of RVA, norovirus, and HAdV were detected in the feces of children with AGE. The frequencies were highest for norovirus (38%, 184/485) and HAdV (28.6%, 139/485) and higher than that detected for RVA (22.7%, 110/485). High frequencies were detected for HAdV in the ARI child group, corresponding to 37.3% (93/249), and were the lowest for RVA and norovirus at 19.3% (48/249) and 21.3% (53/249), respectively. SaV frequencies that were detected were low for the AGE group of children (7.2%, 35/485), but higher than the ARI group (6.8%, 17/249). Table 2 shows the frequencies described above both without considering co-infections and considering a single virus infection or co-infection with the different viruses investigated. The frequencies for single virus infections were proportionally similar to those without considering co-infection. The GII norovirus was predominantly detected in AGE children (82.6%, 152/184). The infection frequencies of GI norovirus and GII + GI were 6.5% (12/184) and 10.9% (20/184), respectively, in AGE children (data not shown).

Figure 1 presents the frequencies of RVA, norovirus, HAdV, and SaV by Amazon region (municipality, state or country) and detected in the feces of the children enrolled in this study with AGE or ARI (Figure 1A and B, respectively). Children living in the municipalities of Boa Vista, Alto Alegre, Uiramutã in RR state, and children living in Venezuela attended HCSA in greater numbers in comparison with other municipalities or with Guyana, and infection by RVA, norovirus, and HAdV was mostly detected in children from these areas. HAdV was detected in higher numbers in regions with children with both AGE and ARI, and at frequencies similar to norovirus in children with AGE. In the group of children with ARI, both RVA and norovirus were detected less. The median C_t value detected by qRT-PCR for RVA was 36.3 (range 23, lowest value 16.5, highest value 39.5); 29.4 (range 28.2, lowest value 11.6, highest value 39.7) for norovirus; and 28.8 (range 23.9, lowest value 13.1, highest value 36.9) for SaV. For HAdV, the median C_t value detected by qPCR was 36.4 (range 27.6, lowest value 15.3, highest value 40.0). No difference in the median C_t value was detected between the groups of children with AGE and ARI for all viruses investigated in this study.

Table 1A
Clinical and epidemiological features of the children infected by the viruses investigated in this study. Data from the study population and number of children/samples collected.

Municipality name[a]	N (%)			Ethnic group
	Total	AGE	ARI	
Alto alegre	81 (11)	55	26	Macuxi, Wapixana, and Yanomami
Amajari	31 (4.2)	25	6	Macuxi, Wapixana, and Yanomami
Amazonas (AM)	14 (2)	13	1	Yanomami
Boa vista	356 (48.5)	227	129	Macuxi, Wapixana, and Taurepangue
Bonfim	31 (4.2)	19	12	Macuxi and Wapixana
Cantá	22 (3)	15	7	Wapixana
Caracarai	9 (1.2)	6	3	Yanomami
Caroebe	2 (0.3)	2	0	Wai-Wai
Guyana	9 (1.2)	4	5	Macuxi and Wapixana
Iracema	8 (1.1)	6	2	Yanomami
Mucajaí	26 (3.5)	17	9	Yanomami
Normandia	18 (2.5)	11	7	Macuxi, Wapixana, and Ingarincó
Pacaraíma	15 (2)	9	6	Macuxi, Wapixana, and Taurepangue
Rorainópolis	14 (2)	8	6	Waimirim and Afroari
São Luiz	1 (0.1)	0	1	Wai-Wai
Uiramuitã	55 (7.5)	37	18	Macuxi, Wapixana, and Ingarincó
Venezuela	42 (5.7)	31	11	Taurepangue (Pemon[b]) and Macuxi
Total	734 (100%)	485 (66%)	249 (34%)	

The municipality name is the region of origin of each child.
N, number of saliva samples collected, (%) percentage of the number of total saliva samples collected corresponding to each group of children.
Ethnic group corresponds to the group of individuals that lives in that particular region to which the children belong.
[a] All the municipalities are located in the state of Roraima (RR), except Amazonas, which corresponds to the state of Amazonas (AM) and the countries Guyana and Venezuela.
[b] The Taurepang self-designate Pemon, a term that means "people".

Table 1B
Main clinical aspects with statistical significance observed in the children in accordance with the recorded form.

Clinical	Group of children	
	AGE (N = 485) Frequency N (%)	ARI (N = 249)
Fever ≥38.5 °C	434 (89.5)	144 (57.8)
Mucus in feces	460 (94.8)	30 (12)
Vomit	227 (46.8)	24 (9.6)

Table 1C
Clinical aspects recorded in the form from the children presenting acute gastroenteritis (AGE) or acute respiratory infection (ARI) associated with detected viruses.

Group of children	Detection of viruses	Clinical		
		Fever ≥38.5 °C (%)	Mucus in feces (%)	Vomit (%)
AGE	RVA (N = 110)	99 (90)	105 (95.4)	59 (53.6)
ARI	RVA (N = 48)	31 (64.6)	2 (4.2)	3 (6.2)
AGE	Norovirus (N = 184)	169 (91.8)	177 (96.2)	81 (44.0)
ARI	Norovirus (N = 53)	32 (60.4)	6 (11.3)	6 (11.3%)
AGE	HAdV (N = 139)	119 (85.6)	125 (89.9)	78 (56.1)
ARI	HAdV (N = 93)	52 (55.9)	13 (14.0)	8 (8.6)
AGE	SaV (N = 35)	32 (91.4)	34 (97.1)	18 (51.4)
ARI	SaV (N = 17)	11 (64.7)	2 (11.8)	0 (zero)

Table 2
Summary of frequencies detected in feces from children living in the Amazon region: A. Rotavirus; B. Norovirus; C. Human Adenovirus (HAdV); D. Sapovirus (SaV). AGE and ARI are acute gastroenteritis and respiratory infections, respectively.

A

Groups of infants (n = 734)	Virus				
	RVA				
	RVA alone	RVA + norovirus	RVA + HAdV	RVA + SaV	Total RVA frequency[1]
AGE (n = 485)	7.6% (37/485)	4.9% (24/485)	4.5% (22/485)	0.2% (1/485)	22.7% (110/485)
ARI (n = 249)	8.4% (21/249)	1.2% (3/249)	6.4% (16/249)	0.4% (1/249)	19.3% (48/249)

B

Groups of infants (n = 734)	Virus			
	Norovirus			
	Norovirus alone	Norovirus +HAdV	Norovirus + SaV	Total norovirus frequency[1]
AGE (n = 485)	15.5% (73/485)	4.1% (20/485)	1.8% (9/485)	38% (184/485)
ARI (n = 249)	8.0% (20/249)	7.2% (18/249)	1.2% (3/249)	21.3% (53/249)

C

Groups of infants (n = 734)	Virus		
	HAdV		
	HAdV alone	HAdV + SaV	Total HAdV frequency[1]
AGE (n = 485)	13.2% (64/485)	0.2% (1/485)	28.6% (139/485)
ARI (n = 249)	16.9% (42/249)	Not detected	37.3% (93/249)

D

Groups of infants (n = 734)	Virus SaV SaV alone	Total SaV frequency[1]
AGE (n = 485)	1.8% (9/485)	7.2% (35/485)
ARI (n = 249)	1.2% (3/249)	6.8% (17/249)

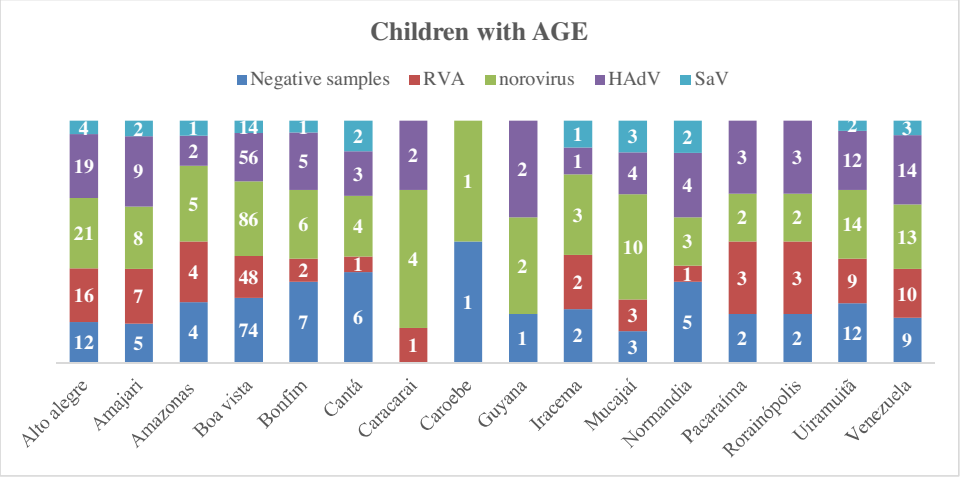
[1]Total frequency for RVA, norovirus, HAdV and SaV considering co-infections with one or more viruses.

Figure 2 shows the seasonality profile for each virus detected inthis study from fecal samples of children living in the Amazon. The seasonality was heterogeneous: the months with the highest incidence of RVA were October to December (dry months with scarce water for the season). For norovirus and SaV, the highest incidence occurred in the months of May to July (May is a dry month and June and July are rainy months of the wet season). HAdV was detected practically throughout the year.

Low Rotarix™ (RV1) vaccination coverage in the Amazon region

Rotarix™ was the vaccine received by all vaccinated young children enrolled in this study (which is given in two doses at 2

A



B

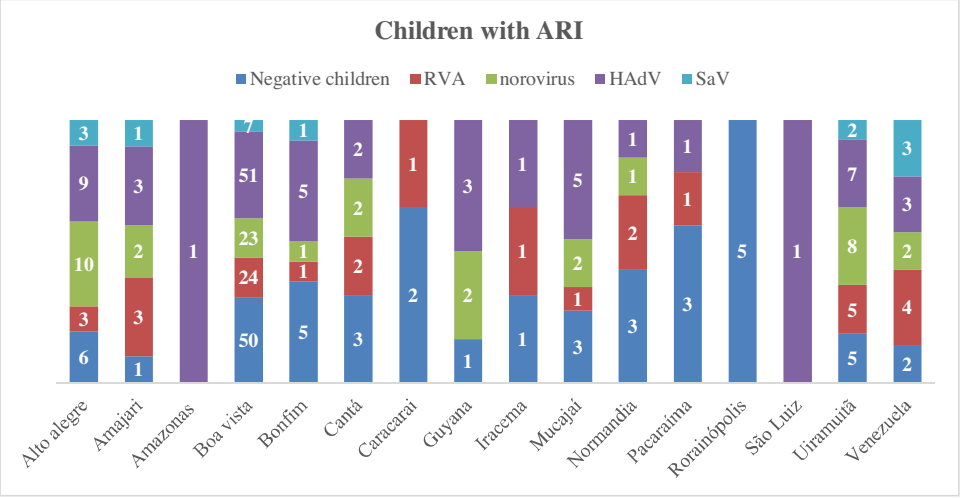


Figure 1. Frequency of viruses investigated in this study detected in the feces of children living in the Amazon region either with A. acute gastroenteritis (AGE) or B. acute respiratory infection (ARI). Rotavirus A (RVA), norovirus, and sapovirus (SaV) were detected by quantitative reverse transcription-quantitative polymerase chain reaction. Human adenovirus (HAdV) was detected by quantitative polymerase chain reaction. The numbers within each bar indicate the number of samples according to the information indicated above. Negative samples are those where none of the investigated viruses were detected. None of the investigated viruses were detected in children living in the Rorainópolis municipality presenting with ARI. The names of different Amazon regions (municipality, state or country) where the children lived are shown in the X-axis.

and4 months of age). The percentage of children that received one dose was 21.1% (155/734) and two doses 40% (293/734). Thirty-nine percent (286/734) of children in this study had not received the RV1 vaccine. The RV1 vaccination coverage calculated was 61% (448/734) and ranged from 21.4% (3/14) in the state of Amazonas (AM-Brazil) to 100% (1/1) in the São Luiz municipality. According to Figure 3, the municipalities of Amajari, Normandia, and Alto Alegre (Amazon rainforest reserve) presented RV1 vaccination coverages <50%. RV1 vaccination coverage in children from Venezuela and Amazonas (AM-Brazil) was also very low (<30%). The RV1 vaccination coverage in the other municipalities had an average of 60%–75%. Table 1A shows all the municipalities cited above. The frequency of detection of RVA in both the RV1 vaccinated and unvaccinated groups of children was similar for the group of children presenting AGE ($P = 0.09$) and the ARI group ($P = 0.07$); these frequencies were between 13.9%–25.3%.

Findings concerning ABO (H), Lewis, and secretor histo-blood group antigens in children from the Amazon region

Children from the Amazonian region with a secretor phenotype of all Lewis phenotypes, these being Le (a+b+), Le (a-b+) or Le (a-b-), corresponded to 79.5% (583/734) of children. Non-secretor children (21.5%) had either Le (a-b-) (14.4%) or Le (a+b-) phenotypes (6.1%). Blood group O was the most predominantly detected (54.5%, 400/734) followed by A (9%, 66/734). Few children were blood typed as B (2.6%, 19/734) or AB (0.4%, 3/734); therefore, they were not considered in the statistical calculations due to low power. No significant association between blood groups 0 or A was observed in regards to acquiring any of the four viral infections (RVA, norovirus, HAdV, or SaV). No significant association between secretor status and viral infection was observed for RVA, norovirus, and SaV. However, secretor children were more

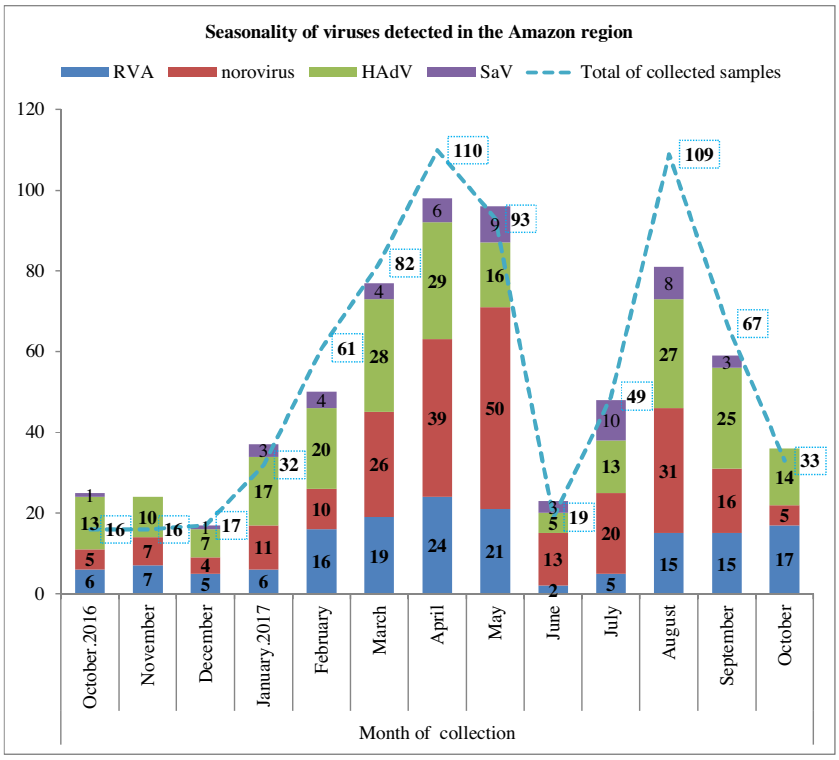


Figure 2. Seasonality profile of rotavirus A (RVA), norovirus, human adenovirus (HAdV), and sapovirus (SaV) detected in the feces of children with acute gastroenteritis (AGE) living in the Amazon region. The numbers within each bar indicate the number of samples according to the color indicated for each virus. Numbers outside indicate the total of collected samples in each month. No SaV-positive sample was detected in November 2016 and October 2017. The dry months (low water season) are December to May. The remaining months (June to November) are rainy (wet season), according to the Brazilian National Institute for Space Research (INPE) (http://climanalise.cptec.inpe.br/~rclimanl/boletim/cliesp10a/fish.html).

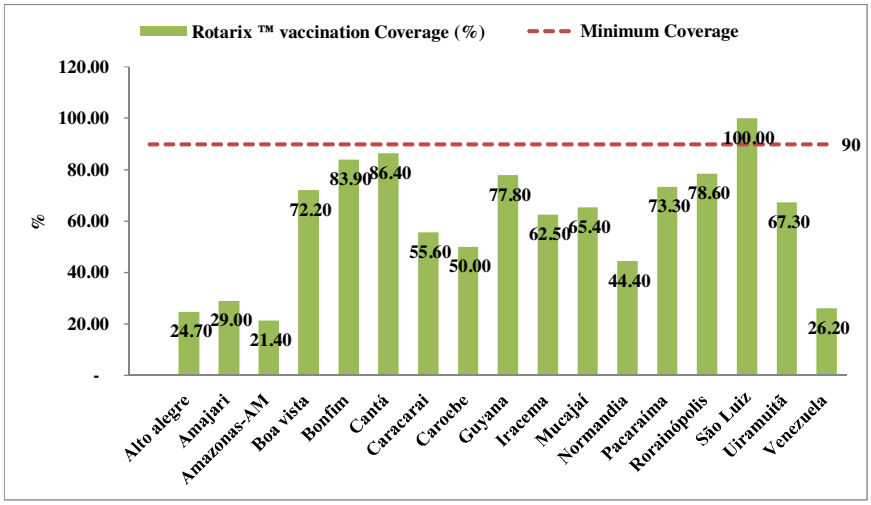


Figure 3. Rotarix ™ vaccination coverage.
The graph shows the Rotarix™ (RV1) vaccination coverage in the different municipalities of the states of Roraima, Amazonia (Brazil) and the countries of Venezuela and Guyana. The numbers within each bar indicate the % of RV1 vaccination coverage. The red line in the graph represents the expected 90% for RV1 coverage according to the Brazilian National Immunization Program (NIP).

susceptible to acquiring HAdV infection, according to the OR = 1.5 (95% CI 1.0–2.3; P = 0.04) (Table 3).

Diversity of the FUT3 genotype detected in children from the Amazon region

DNA samples from 43.4% (42/106) of Le (a-b-) children were successively amplified from saliva and the occurrence of the five main SNPs of the *le* gene was investigated. New mutations adjacent to these were also detected. The 59T > G and 508G > A SNPs were detected in high frequency – 50% (21/42) and 76.2% (32/42), respectively – as well as the rs139326855 (858A > G) SNP, and the new mutation 1011C > G, both in 31% (13/42) of children. All these SNPs have been identified in children infected with RVA, norovirus, and HAdV. However, they are also present in the *FUT3* gene of children not infected by any of the viruses investigated here

Table 3
The ABO (H), Lewis, and secretor (HBGA) susceptibility profile of the Amazonian children and rotavirus A, norovirus, and human adenovirus infections.

HBGA phenotype[a] (N = 734)	Viruses[b], N (%)			
	RVA	Norovirus	HAdV	SaV
Secretors (N = 583)	128 (22)	188 (32.2)	189 (32.4)	42 (7.2)
Non-secretors (N = 151)	30 (19.9)	49 (32.4)	36 (23.8)	10 (6.6)
OR	1.1 (95% CI 0.7–1.8) (*P* = 0.58)	1.0 (95% CI 0.7–1.4) (*P* = 0.96)	1.5 (95% CI 1.0–2.3) (*P* = 0.04)	1.1 (95% CI 0.5–2.2) (*P* = 0.80)
Blood type O (N = 641)	139 (21)	215 (33.5)	207 (32.3)	46 (7.2)
Blood type A (n = 66)	14 (21%)	15 (22.7%)	16 (24.2%)	4 (6.6%)
OR	1 (95% CI 0.5–1.9 (*P* = 0.92)	1.7 (95% CI 0.9–3.1) (*P* = 0.07)	1.5 (95% CI 0.8–2.7) (*P* = 0.18)	1.2 (95% CI 0.4–3.4) (*P* = 0.74)

[a] Only the secretor or non-secretor status (the children being either Le + or Le-) and blood type O or A were considered.
[b] The odds ratio (OR), confidence interval (CI), and *P*-values were calculated according to SZUMILAS (2010).

(Table 4). The 202T > C, 1067T > G SNPs and the rs28362463 (484G > A) SNP (Nordgren et al., 2014) were also detected, at a lower frequency, in 4.8% (2/42) of children, with the new mutations being: 34C > T and 870C > A, 7.1% (3/42); 251T > A and 292A > T, 14.3% (6/42); 254T > G, 9.5% (4/42); 305A > T, 16.7% (7/42); 512G < A, 4.8%; and 509G > A, 12% (5/42).

Discussion

The difficulty that health teams face when accessing the Amazon rain forest where children live may have an impact on their health. The low RV1 vaccination coverage detected in the Amazon region could be explained by this fact. Branco et al. (2014) reported a low RV1 vaccination coverage in the state of Acre, located in the extreme north of Brazil, eight years after the introduction of RV1 via the NIP. It might be necessary to study special immunization strategies for these regions. The most detected clinical aspects in children with AGE were the presence of mucus in feces and fever ≥38.5 °C. The presence of mucus in feces has been associated with AGE (WHO, 2005, 1990). Fevers of ≥38.5 °C are a usual clinical symptom associated with several etiological agents of infectious diseases.

To determine whether norovirus is an important causative agent of AGE in this study population, the median C_t value of 29.4

Table 4
FUT 3 gene single nucleotide polymorphisms (SNPs) of the *le* gene detected in children from the Amazon region phenotyped as Le (a-b-): 1. Children numbers 7, 12, 13, 25, 29, and 30 did not have any of the five main SNPs; 2. The five main SNPs are rs28362459 (59T > G), rs812936 (202T > C), rs778986 (314C > T), rs3745635 (508G > A, and rs3894326 (1067T > G).

Child[a]	*FUT3* gene single nucleotide polymorphisms	Virus (es) detected
1	59T > G, 508G > A, 858A > G	RVA, HAdV
2	508G > A	RVA, HAdV
3	305A > T, 508G > A, 858A > G, 1011C > G	RVA, Norovirus
4	59T > G, 508G > A	RVA, Norovirus
5	59T > G, 314C > T, 508G > A	RVA
6	59T > G, 508G > A, 1011C > G	Norovirus
8	314C > T, 484G > A	Norovirus, SaV
9	508G > A	Norovirus HAdV
10	34C > T, 59T > G, 251T > A, 292A > T, 508G > A, 858A > G	Norovirus
11	508G > A, 858A > G	Norovirus
14	870C > A	Norovirus, HAdV
15	508G > A	Norovirus
16	59T > G, 508G > A, 251T > A, 305A > T, 870C > A, 1067T > G	Norovirus
17	314C > T, 292A > T, 305A > T, 508G > A, 1067T > G, 1011C > G	Norovirus
18	202T > C, 305A > T, 314C > T, 508G > A	Norovirus
19	508G > A, 1011C > G	HAdV
20	508G > A, 509G > A, 858A > G	HAdV
21	59T > G, 251T > A, 254T > G, 508G > A	HAdV
22	59T > G, 508G > A	HAdV
23	59T > G, 508G > A	HAdV
24	509G > A, 512G < A, 870C > A	HAdV
26	59T > G, 508G > A	SaV
27	508G > A, 484G > A, 858A > G	Negative[b]
28	59T > G, 858A > G, 1011C > G	Negative
29	59T > G, 508G > A, 509G > A, 858A > G, 1011C > G	Negative
30	59T > G, 305A > T, 508G > A	Negative
31	59T > G, 254T > G, 314C > T, 508G > A	Negative
32	59T > G, 251T > A, 254T > G, 508G > A	Negative
33	59T > G, 508G > A	Negative
34	508G > A	Negative
35	34C > T, 59T > G, 251T > A, 292A > T, 508G > A	Negative
36	59T > G, 251T > A, 508G > A, 858A > G, 1011C > G	Negative
37	508G > A	Negative
38	59T > G, 305A > T, 508G > A, 512G < A, 858A > G	Negative
39	59T > G, 251T > A, 508G > A	Negative
40	59T > G	Negative
41	508G > A, 858A > G, 1011C > G	Negative
42	34C > T, 202T > C, 314C > T, 508G > A, 1011C > G	Negative

[a] Six Le *(a-b-)* children did not have any of the five main SNPs.
[b] Viruses investigated in this study not detected.

was considered. Trang et al. (2015) estimated that a C*t* < 21.36 could be assumed to represent cases for which norovirus was the causal agent of diarrhea. In addition, the frequencies detected for norovirus in the AGE and ARI groups supported this, these being 38% (184/485) and 19.3% (48/249), respectively. Phillips et al. (2009) assumed that C*t* values up to 31.0 could be considered as criteria (cut-off) to select norovirus-positive samples. Otherwise, the median C*t* value detected for RVA and HAdV in Amazonian children was high at 36.3 and 36.4, respectively. Similar frequencies for RVA and HAdV were detected in both AGE (respectively 22.7%, 110/485 and 33.6%, 163/485) and ARI (21.3%, 53/249 and 39.7%, 99/249) groups in this study, and thus not clearly associated as a cause for AGE. There is one study of children with AGE from RR (Soares et al., 2014), where 25 fecal samples were investigated and an RVA detection rate of 52% (13/25) was found. Similar studies have reported an overall frequency of up to 20% RVA in children from the Brazilian Amazon but higher frequencies have also been found, as reported in Acre (46%), Amazonas (28.5%), Amapá (38%), Pará (26.3), Rondônia (71.4%), and Roraima (52%) (Neves et al., 2016; Orlandi et al., 2006).

Different studies from the Amazon region have observed norovirus frequencies from 7.8%–35.2% in young children (Bitencurt et al., 2019; Costa et al., 2017a,b; Amaral et al., 2015). Norovirus is the most common cause of diarrheal episodes globally (Frenkel, 2018; Lopman et al., 2016) but the high frequency in this study suggests that norovirus infections can be frequently asymptomatic. Riddle and Walker (2016) mentioned that there is an increase in the frequency of norovirus as a cause of AGE in countries where the vaccine has been introduced. Therefore, the low coverage of the RVA vaccine could act as an inducer of high frequencies of norovirus in the Amazon region. However, there are probably other factors that were differentiated for norovirus, such as seasonality with periods of floods (wet season) that occur in the months of June and July, as discussed below.

Notably, no association between secretor status or O and A blood groups to infection of RVA and norovirus was observed, although secretor positivity is a susceptibility marker for common norovirus and RVA (Heggelund et al., 2017; Nordgren and Svensson, 2019). One reason for this might be that norovirus and RVA circulating during the study period were uncommon. There was also no observed association between O and A blood groups to infection with HAdV. However, the secretor Amazonian children showed a slight susceptibility to HAdV infection. HAdVs are ubiquitous pathogens depending on the type/species and display various tropisms that correlate with clinical manifestations and typically infect the respiratory, digestive and ocular tracts. HAdV is one of the most frequently detected agents in children presenting ARI (Frenkel, 2018). The high frequency of HAdV detected in this study in both the AGE and ARI groups of children could be explained by the association observed with the secretor status. The available data regarding the incidence of HAdV in Brazil is limited, especially in diarrheic diseases in the post RVA vaccine era. Portal et al. (2019) reported an HAdV positivity of 50.2% (110/219) in fecal samples collected from March 2012 to April 2015 from children hospitalized for AGE in two large pediatric hospitals in the Amazon region. HAdV was also detected in this study in high frequencies in both AGE and ARI groups. HAdV was the most frequently detected in children living in different municipalities of RR and Amazonas states (Brazil), also in the countries of Venezuela and Guyana.

The SaV detection rate was relatively low in this study (7.2%, 35/485) in children with AGE, and Costa et al. (2017) also reported a low detection rate (5.2%, 9/172). An interesting fact is concerning the seasonality that was investigated for each virus detected in this study. The dry season was the period where the children were more affected by AGE caused by all viruses; however, a peak of high norovirus infection frequency was observed in July (wet season). High norovirus frequencies in the wet season in the Amazon region have previously been reported by Vieira et al. (2017) and elsewhere in Latin America (Bucardo et al., 2014; Ahmed et al., 2013).

The five main SNPs of the *FUT3* gene in Le (a-b-) Amazonian children were verified via nucleotide sequencing of DNA from saliva. Adjacent to the five main SNPs, nine new ones were detected. These new SNPs could explain the Le (a-b-) phenotype, especially the 1011C > G, because of its high frequency and because one child phenotyped as Le (a-b-) only had this SNP. Further functional studies are necessary to confirm that the novel FUT3 polymorphisms can confer the Lewis (a-b-) phenotype of non Le (a-b-) control samples. The most common haplotype SNPs correspond to the $le^{59/508}$, which have been detected in Asian (24%) and African (19%) populations, while the $le^{202/314}$ (17%) and $le^{59/1067}$ (4%) were mainly found in European populations (Liu et al., 1999; Elmgren et al., 1996). The rs3745635 (508 G > A) and rs3894326 (1067 T > A) SNPs, which are both detected in the catalytic domain of the enzyme, inactivate the product of the *le* gene, while the rs28362459 (59T > G) SNP, in the transmembranous domain of this protein, only reduces its enzymatic activity rather than eliminating it altogether (Soejima and Koda, 2005; Elmgren et al., 1996). The profile of the *le* gene SNPs detected from children from the Amazon region was mainly the haplotype $le^{59/508}$, showing some genetic proximity between native Asian and Amazonian individuals, as previously reported (Moraes et al., 2019).

Conclusions

This study found high frequencies of RVA, norovirus, and HAdV infections in children from the Amazon region. No association was verified for HBGA susceptibility to RVA, norovirus, and SaV. Otherwise, secretor children showed a slight susceptibility to HAdV infection and the Le (a-b-) heterogeneous SNPs on the *FUT3* gene.

Conflict of interest

The authors declare there is no conflict of interest.

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