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Editorial

Viral agents of gastroenteritis and their correlation with clinical symptoms in rotavirus-vaccinated children

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ABSTRACT

Background and objectives: Enteric viral infections are among the leading causes of gastroenteritis in children up to five years of age worldwide. This study was aimed to determine the disease severity, incidence, and molecular genotyping of rotaviruses, noroviruses, astroviruses, and enteric adenoviruses as gastroenteritis agents among children up to five years old.

Materials and methods: Gastroenteritis severity was determined by using the Ruuska and Vesikari score, whereas the incidence of enteric infections and their genotyping were determined by reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis.

Results: Rotaviruses were observed to possess the highest incidence with 10% (18/179) of the cases positives; nevertheless, noroviruses had the highest severe gastroenteritis score (13 ± 3 points). Results indicated that 56% (10/18) of the detected rotavirus strains were genotype G12P[8], 50% (4/8) of noroviruses were GII.4 and 25% (2/8) were genotype GI.8. Out of the sapovirus positive samples, 30% (2/6) were genotyped as GI·I and GII·I. Sixty percent of the astrovirus strains (3/5) were genotype HAstV-2, and 20% (1/5) were genotype HAstV-6. Additionally, one of the adenovirus strains was identified as human mastadenovirus C type 6 specie.

Conclusions: The diarrhea severity reduction in children provides evidence that the rotavirus vaccination program in the northwest of Mexico has been successful, even among children infected by the rotavirus emergent strain G12, however, norovirus resulted as the leading severe gastroenteritis-causing agent in children with rotavirus vaccine.

1. Introduction

Diarrheic diseases are the second leading cause of death in children; there are about 1.7 billion cases of acute gastroenteritis and 525,000 deaths in children up to five years of age worldwide each year (Organization, 2017). In children, most cases are associated with viral infections involving rotaviruses, noroviruses, sapoviruses, astroviruses, and enteric adenoviruses (Elliott, 2007). Rotavirus and norovirus infections worldwide are associated with moderate to severe gastroenteritis in children, resulting in about 215,000 and 218,000 deaths every year, respectively (Koo et al., 2010; Tate et al., 2016). Sapoviruses, astroviruses, and enteric adenoviruses cause mild to moderate gastroenteritis in children up to five years of age (Finkbeiner et al., 2009a; Rezaei et al., 2012; Sdiri-Loulizi et al., 2011).

Rotaviruses belong to the Reoviridae family with species A rotavirus being mostly associated with childhood infections. Rotaviruses classification is based on the VP4 and VP7 gene sequence identity, which represents the basis for the rotavirus binomial G (VP7, Glycoprotein) and P (VP4, Protease sensitive) genotypes (Estes and Greenberg, 2013). Rotaviruses most reported genotypes worldwide are G1P[8], G4P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8]; some uncommon genotype combinations are also reported in outbreaks, but with limited

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Table 1
Primers sequences and references.

Virus	Target	Primers (forward/ reverse)	Sequence 5′-3′ (forward/reverse)	Amplicon size (pb)	Reference
ROTAVIRUS (dsRNA)	VP7	Beg9/End9	GGCTTTAAAAGAGAGATTTCCGTCTGG/ GGTCACATCATACAATTCTAATCTAAG	1062	(Gouvea et al., 1990)
		9CON1/9CON2	GTATAAAATACTTGCCACCA/TAGCTCCTTTTAATGTATGG	904	(Das et al., 1994)
		VP7F/VP7R	ATGTATGGTATTGAATATACCAC/AACTTGCCACCATTTTTTCC	881	(Iturriza-Gómara et al., 2004)
	VP4	CON2/CON3	TGGCTTCGCCATTTTATAGACA/ATTTCGGACCATTTATAACC	876	(Gentsch et al., 1992)
		VP4F/VP4R	TATGCTCCAGTNATTTGG/ATTGCATTTCTTTCCATAATG	663	(Simmonds et al., 2008)
ASTROVIRUS (ssRNA)	ORF2	MON269/MON270	CAACTCAGGAAACAGGGTGT/TCAGATGCATTGTCATTGGT	449	(Noel et al., 1995)
	ORF1b	SF0073/SF0076	GATTGGACTCGATTTGATGG/CTGGCTTAACCCACATTCC	409	(Finkbeiner et al., 2009b)
NOROVIRUS (ssRNA)	ORF2	G1SKF/G1SKR Genogroup GI	CTGCCCGAATTYGTAAATGA/CCAACCCARCCATTRTACA	330	(Kojima et al., 2002)
		COG1F/G1SKR Genogroup GI	CGYTGGATGCGNTTYCATGA/CCAACCCARCCATTRTACA	380	(Kageyama et al., 2004)
		G2SKF/G2SKR Genogroup GII	CNTGGGAGGGCGATCGCAA/CCRCCNGCATRHCCRTTRTACAT	340	(Kojima et al., 2002)
		COG2F/G2SKR Genogroup GII	CARGARBCNATGTTYAGRTGGATGAG/ CCRCCNGCATRHCCRTTRTACAT	390	(Kageyama et al., 2004)
SAPOVIRUS (ssRNA)	ORF1	SLV5749/SLV5317	CTCGCCACCTACRAWGCBTGGTT/CGGRCYTCAAAVSTACCBCCCCA	434	(Yan et al., 2003)
ADENOVIRUS (dsDNA)	Gen Hexon	Ad1/Ad2	TTCCCCATGGCTCACAACAC/CCCTGGTAGCCGATGTTGTA	482	(Rezaei et al., 2012)

incidence (Argüelles et al., 2000; Arista et al., 1997; Gonzalez-Ochoa et al., 2016; Iturriza-Gómara et al., 2004; Rahman et al., 2007). Children between three months and two years of age are the most susceptible to rotavirus infections (Desselberger, 2014). Rotaviruses gastroenteritis is characterized by watery diarrhea, vomit, fever, and dehydration; before the vaccination program, rotaviruses were associated with the most severe gastroenteritis cases, compared with disease caused by other enteric viruses (Ruuska and Vesikari, 1990).

Noroviruses and sapoviruses are members of the *Caliciviridae* family of which GI, GII, and GIV viruses are classified by the major capsid protein VP1 gene. There are seven genogroups (GI-GVII), where GI, GII, and GIV are associated with human infections causing symptoms such as severe vomiting, watery diarrhea, nausea, abdominal cramps and fever (Hutson et al., 2004). The genogroup GII genotype 4 (GII.4) is the most reported, and is related to the most severe gastroenteritis cases, followed by GII and GIV (Patel et al., 2009; Vinjé, 2015). Sapoviruses are classified upon the major capsid protein VP1 gene. Based on the full sequence of VP1, five genogroups have been described (GI-V), but only GI, GII, GIV, and GV were found to be associated with human infections; GI and GII are the most detected genogroups in children gastroenteritis (Oka et al., 2012; Oka et al., 2015).

Astroviruses belong to the *Astroviridae* family; these viruses are classified further into the genera *Mamastrovirus* (MAstV) and *Avastrovirus* (AAstV), which infect several mammals and birds, respectively. MAstV-1, MAstV-6, MAstV-8, and MAstV-9 species have been identified in humans, where MAstV-1 includes the classic human pathogenic genotypes 1 to 8 (HAstV 1–8) (De Benedictis et al., 2011). Human astroviruses mainly affect children up to two years of age, with gastroenteritis, and are associated with 0.5–15% of pediatric outbreaks of diarrhea [21].

Human adenoviruses are part of the *Adenoviridae* family, genus *Mastadenovirus*. To date, 52 human adenovirus serotypes have been identified and classified into one of the A to G species (Harrach et al., 2011). Particularly, the 40 and 41 genotypes of F species are known to cause gastroenteritis and are designated as enteric adenoviruses (Harrach et al., 2011). Enteric adenoviruses have been detected worldwide in sporadic and outbreak cases of gastroenteritis in day-care centers, kindergartens, and hospitals (Verma et al., 2009), causing watery diarrhea lasting from 8 to 12 days, vomiting, respiratory symptoms, and low-grade fever (Banerjee et al., 2017). Due to the diarrheic diseases impact in children health globally, and the role of enteric viruses as the gastroenteritis leading agents; this study was undertaken to determine the incidence and carry out molecular genotyping of rotavirus, norovirus, astrovirus, and enteric adenovirus infections in children up to five years of age, with mild to severe gastroenteritis symptoms. Furthermore, the severity of disease was analyzed during the time of period after widespread use of rotavirus vaccination.

2. Material and methods

2.1. Sample collection

Fecal samples were collected from August 2012 to December 2014 from children with acute gastroenteritis admitted to Hospital del Niño y la Mujer in Obregón and Hospital General in Navojoa, located south of Sonora, México. The inclusion criteria included children up to five years of age, vaccinated against rotavirus and with gastroenteritis not related to bacteria or parasites.

2.2. Gastroenteritis severity

Upon admission to hospital or fecal sample reception, with parents agreement and pediatric assistance, the following information was requested: age, sex, diarrhea duration, number of stools per day, stool consistency, vomiting episodes, body temperature, and hydration status. Symptoms such as diarrhea, vomiting, fever, and dehydration level were used to calculate the gastroenteritis severity and formed the basis of the Ruuska score; scores ≤ 10 were considered mild, ≥ 11 moderate, and ≥ 15 severe gastroenteritis (Ruuska and Vesikari, 1990).

2.3. Viruses detection

Fecal samples were tested for the presence of rotaviruses, adenoviruses, noroviruses, sapoviruses, and astroviruses presence. Rotavirus and adenovirus antigens detection was performed with a rapid test, according to manufacturer's instructions (One Step Rota Adenovirus Antigen Test, Standard Diagnostics, Inc., Republic of Korea). Astroviruses, noroviruses, and sapoviruses detection were done by nucleic acid extraction and RT-PCR, using different primers and according to protocols previously described (Table 1).

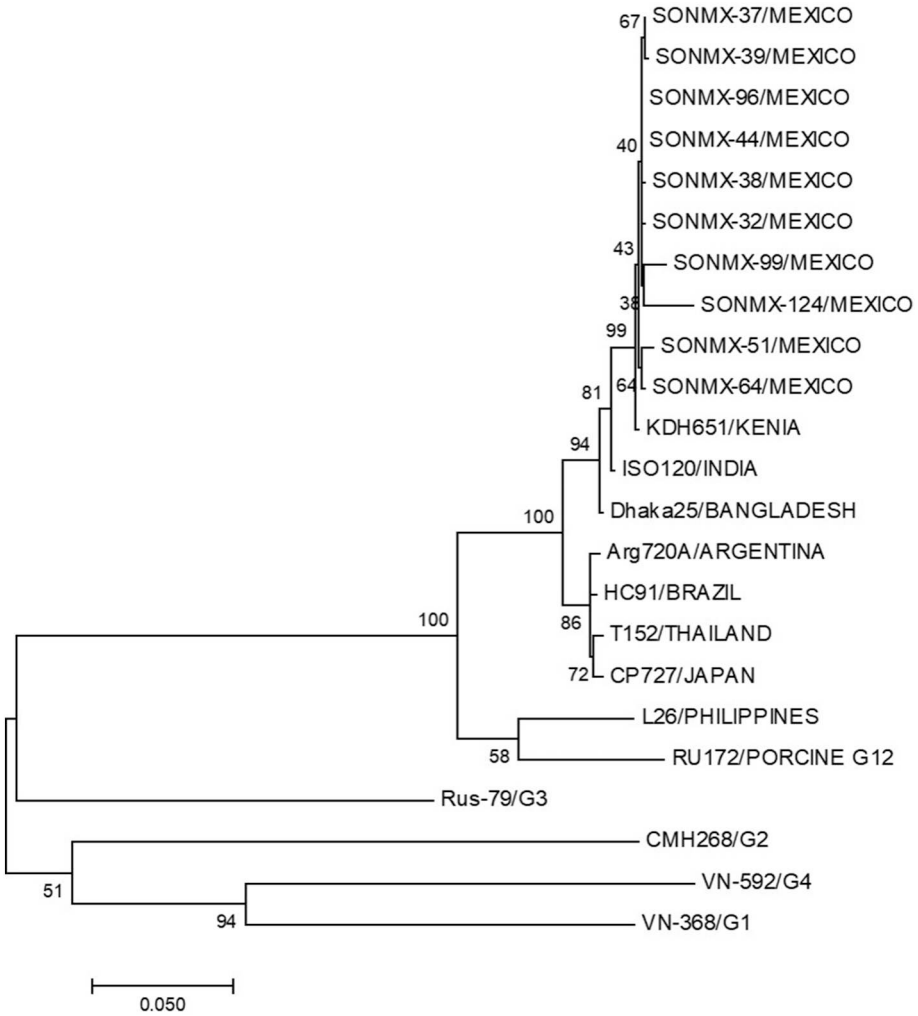

Fig. 1. Molecular phylogenetic analysis of rotavirus VP7 gene genotype G12. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model, bootstrap of 1000 replicates. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

2.4. DNA/RNA extraction and RT-PCR

RNA and DNA purification in fecal samples was performed by QIAamp® DSP Viral RNA Mini Kit (QIAGEN, Germany) and PureLink® Genomic DNA Mini Kit (ThermoFisher Scientific, Invitrogen) respectively, according to supplier's instructions. Purified RNA was used as a template for cDNA synthesis, followed by PCR amplification of specific gene segments of rotaviruses, noroviruses, sapoviruses, and astroviruses, whereas purified DNA was used for adenovirus hexon gene partial amplification (Table 1).

2.5. Sequencing and phylogenetic analysis

PCR amplicons were purified with Wizard® SV Gel and PCR Clean-Up System (PROMEGA®), and sequenced twice by the dideoxynucleotide chain termination method, using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, USA); sequences were analyzed using the MEGA 7.0 software (Kumar et al., 2016) and were compared with other sequences reported in GenBank database. Phylogenetic relationships between strains were reconstructed using the maximum likelihood method with 1000 replicates to test phylogeny (Kumar et al., 2016). The accession numbers for rotavirus VP4 and/or VP7 sequences reported in this study are SONMX-38 (MG557590), SONMX-39 (MG557585), SONMX-44 (MG557582, MG557588), SONMX-51(MG557589), SONMX-64 (MG557584, MG557587) SONMX-96 (MG557583, MG557586), SONMX-99 (KP119466), SONMX-100 (MG557591), SONMX-124 (KP119467). Norovirus: SONMX-112/HNoV (MG557592), SONMX-72/HNoV (MG557593), SONMX-74/HNoV (MG557594), SONMX-162/HNoV (MG557595), SONMX-36/HNoV (MG557596), SONMX-73/HNoV (MG557597). Astrovirus: SONMX-17/HAstV (MG557598), SONMX-3/HAstV (MG557599), SONMX-03/HAstV (MG557600), SONMX-62/Astrovirus (MG557601). Sapovirus: SONMX-55/SAPOVIRUS (MG557602), SONMX-58/SAPOVIRUS (MG557603). Adenovirus: SONMX-53/ADENOVIRUS (MG581931). The accession numbers for rotavirus VP4 and/or VP7 sequences reported previously (Gonzalez-Ochoa et al., 2016) are SONMX32 (KP119460, KP119464,), SONMX-37 (KP119461, KP119465), SONMX-38 (KT852964), SONMX-39 (KT852962), SONMX-51 (KT852965), SONMX-62 (KT852963), SONMX-99 (KP119462, KP119466), SONMX-124 (KP119463, KP119467).

2.6. Statistic

The statistical analysis of the collected data was performed by using SPSS v.11.5 software. The diarrheal severity scores were analyzed using the Mann-Whitney test ‘s rank correlation test Mann-Whitney *U* test. To determine differences between viral and non-viral infection and for viral infections (rotaviruses, noroviruses, and sapoviruses), one-way ANOVA and the Tukey test were used. The Fisher exact test was used to determine differences in severity scores.

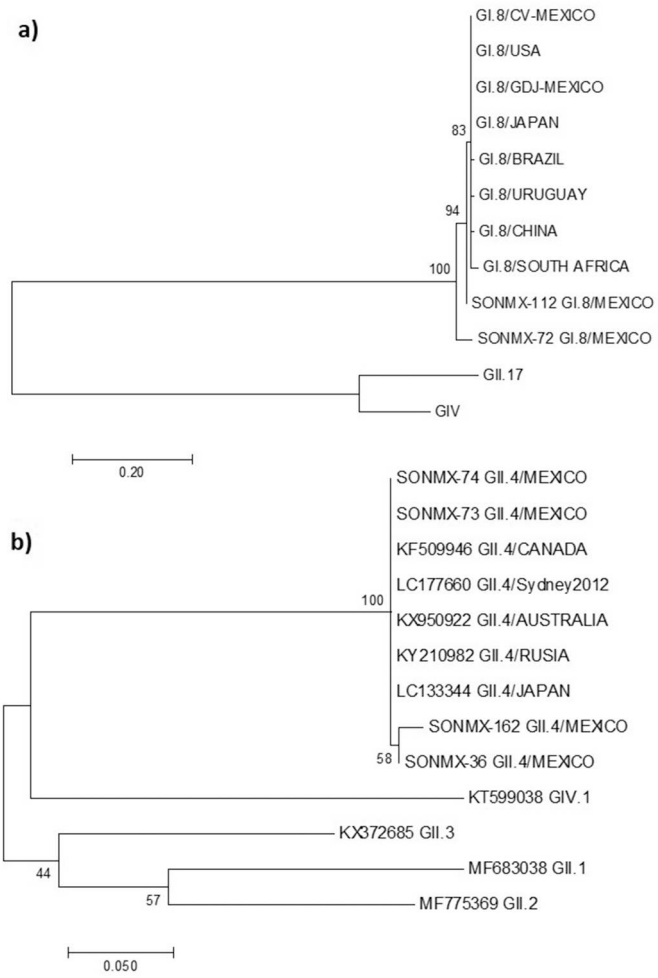

Fig. 2. Molecular phylogenetic analysis of noroviruses genotypes a) GI.8 and b) GII.4. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model, bootstrap of 1000 replicates. The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

3. Results

A total of 179 stool samples from children with gastroenteritis were analyzed to detect enteric viruses. Ten percent (18/179) of the analyzed samples were positive for rotaviruses, two of them were coinfections with astrovirus and adenovirus. Out of the 18 rotavirus positive samples, VP4 and VP7 sequence analysis revealed that 56% (10/18) of the strains were genotype G12P[8], and 11% (2/18) genotype G not typed P[8]. The phylogenetic analysis showed that the genotype G12 fell in the same rotavirus G12 clade previously reported in Kenya (AB861961), India (EU016453), and Sri Lanka (AB306268) (Fig. 1). The VP4 sequence shared 99% identity with rotavirus strains from Kenya (AB861959), Uganda (KJ753730), South Africa (KJ752364) and Buthan (AB905370).

Four percent (8/179) of the analyzed samples were positive to noroviruses. Out of them, 25% (2/8) were genogroup GI and 75% (6/8) GII. Sequence identity characterized two of the GI genotype as GI.8 (Fig. 2a), whereas four of the norovirus genotype GII were GII.4. The GII.4 partial sequences fell in the same clade of norovirus strains GII.4 variant Sydney 2012 (Fig. 2b). On the other hand, 3% (6/179) of the samples were positive to sapoviruses. Two of the six positive samples to sapoviruses were genotyped by sequence analysis as GI·I and GII·I genotypes. The sequence analysis showed that the sapovirus GI.1 strain reported in this study shared 100% identity with strains reported in Japan (FJ823082) and Venezuela (GU296663), whereas the sapovirus GII.1 shared 97% identity with a sapovirus strain previously reported in India (KU317449).

Two percent (4/179) of samples were positive for astroviruses, and one sample was positive for astroviruses and rotaviruses. Sequence analysis genotyped four of the five strains of astroviruses, three of the astrovirus strains were genotype HAstV-2, and one was genotype HAstV-6. The astrovirus strains HAstV-2 reported in this study shared identity of 95 and 98% with strains reported in Russia (CQ495608) and China (GQ495608) respectively. On the other hand, the strain genotype HAstV-6 shared identity of 96–98% with astrovirus reported in Italy (JX087964) and Canada (KU973899). Additionally, two of the analyzed samples (2/179) were positive to adenoviruses by rapid test, one of them was also positive to rotaviruses. Only one sample was successfully amplified and sequenced. This sample sequence analysis revealed an identity of 99% with human adenovirus subgenera C type 6, previously reported in Japan (LC068720).

Furthermore, severity of gastroenteritis related to viral infection indicated for rotaviruses 9 ± 4, noroviruses 13 ± 3, sapoviruses 7.2 ± 3, astroviruses 6.3 ± 1, and adenoviruses 7 points in the scale (Table 2). In the co-infection cases, rotavirus/astrovirus and rotavirus/adenovirus infections were associated to mild (3 points in Ruuska score) and moderate severity (12 points) gastroenteritis respectively (Table 2). The statistical analysis of gastroenteritis severity scores of viral infections by rotaviruses, noroviruses, and sapoviruses indicated a significant difference between noroviruses and sapoviruses severity ($p < .05$), but not with rotaviruses (Fig. 3). Nevertheless, the analysis of the symptoms such as episodes of diarrhea by day and duration showed a significant severity by norovirus in comparison with rotavirus and sapovirus infections ($p < .05$), as shown in Table 3.

Table 2
Viral infections incidence and gastroenteritis severity.

Virus	Incidence	ND[a]	Age[b] (months)	Symptoms				Hospitalization	Gastroenteritis Severity (Ruuska score)			
				Diarrhea episodes[b]/24 h	Days with diarrhea[b]	Vomiting episodes[b]/24 h	Days with vomiting[b]		≤ 10	≥ 11	≥ 15	Average
Rotavirus	9% (16/179)	6	13	4	3	2.5	1.5	2/10	6	4	0	9 ± 4
Norovirus	4% (8/179)	2	14	5.1	3.6	3.16	2.5	4/6	1	3	2	13 ± 3
Sapovirus	3% (6/179)	1	27	3.2	4.8	1.2	0.4	0/5	4	1	0	7 ± 3
Astrovirus	2% (4/179)	1	15	4.2	4	1	0.75	0/3	3	0	0	6 ± 3
Adenovirus	0.5% (1/179)	–	9	4	6	0	0	0/2	1	0	0	7
Rota/Astro	0.5%(1/179)	0	4	3	3	3	4	0/1	0	1	0	12
Rota/Adeno	0.5% (1/179)	0	14	3	4	0	0	0/1	1	0	0	3

Rota/Astro: co-infection rotaviruses and astroviruses; Rota/Adeno: co-infection rotaviruses and adenoviruses.
[a] ND No Data: No complete data was available.
[b] Average data.

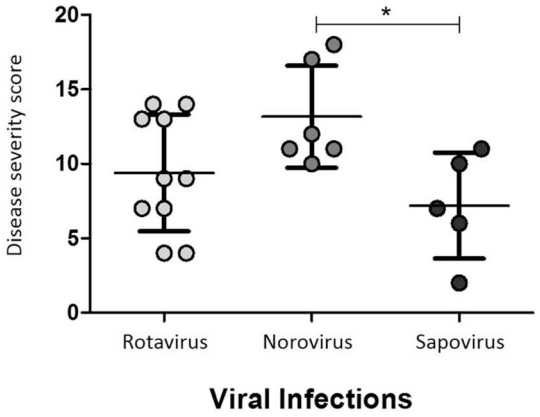

Fig. 3. Score analysis of gastroenteritis severity between rotavirus, norovirus, and sapovirus. Symptoms such as diarrhea, vomiting, fever, and dehydration level were used to calculate the gastroenteritis severity and formed the basis of the Ruuska score; scores ≤10 were considered mild, ≥11 moderate, and ≥ 15 severe gastroenteritis (Ruuska and Vesikari, 1990).

Table 3
Analysis of the severity of symptoms of rotavirus, norovirus, and sapovirus infections.

Symptoms (Ruuska score components)		Rotavirus (%) $n = 10$	Norovirus (%) $n = 6$	Sapovirus (%) $n = 5$	p value
Duration of diarrhea, days	< 3	44.4	12.5	50.0	0.0001**
	≥ 3	22.2	62.5	33.3	0.0001**
Total episodes of diarrhea/24 h	< 4	33.3	12.5	16.7	0.0005
	≥ 4	33.3	75.0	66.7	0.0001**
Duration of vomiting (days)	< 3	33.3	75.0	33.3	–
	≥ 3	5.6	0.0	0.0	
Total episodes of vomiting/24 h	< 4	5.6	26.0	16.7	0.0245
	≥ 4	33.3	50.0	16.7	
Fever	≥ 37.5 °C	27.8	50.0	16.7	–
Dehydration	Mild	33.3	50.0	50.0	–
	Moderate-to-severe	5.6	0.0	0.0	
Treatment	Hospitalization	20.0	66.0	0.0	–

significant difference **($p < 0.05$)

4. Discussion

Most children with viral gastroenteritis included in this study were one year of age or younger, which is in agreement with previous reports (Anwari et al., 2018). Before the rotaviruses vaccination program was implemented in Mexico, the most reported rotaviruses genotype was G1P[8] (Castello et al., 2004); in our study, after the vaccine introduction, 56% of the rotavirus strains were G12P[8]. In agreement with this, the prevalent rotavirus circulating strains in the pre-vaccine era in Ghana were G1P[8], and during the post-vaccine period, the strains G12P[8] were the most reported ones (Lartey et al., 2018). The rotavirus phylogenetic analysis showed that the genotype G12 fell in the same clade of rotavirus G12 previously reported in Kenya (AB861961) and associated with G12-lineage III (Fig. 1); whereas the

VP4 sequences shared an identity of 99% with rotavirus strains from Kenya (AB861959), in the phylogenetic analysis the VP4 sequences genotype P[8] fell in a different clade of the P[8] strain, previously reported in México (data not shown) (Gonzalez-Ochoa et al., 2016).

Noroviruses followed rotavirus in incidence with 4%, dropping within the reports range by noroviruses infection in acute gastroenteritis cases (0.09–44.7%), but in all age groups (Kumthip et al., 2018). The norovirus genogroups detected in this study were GI and GII; two of the GI genotypes were characterized by sequence identity as GI.8. Within the genogroup GII, four strains were identified as GII.4. The GII.4 strain fell in the same clade of norovirus strains GII.4 variant Sydney 2012. (Fig. 2a). The predominance of the norovirus GII over GI has been previously reported (Biscaro et al., 2018; da Silva Poló et al., 2016). The norovirus strains GII.4 are the most reported in acute gastroenteritis outbreaks worldwide, and they are transmitted from person to person, whereas the GI.8 strain, which has not been previously reported in Mexico, is commonly associated with acquired infections by eating seafood contaminated with noroviruses (da Silva Poló et al., 2016; Koo et al., 2010). Sapoviruses were detected in six (5%) of all the analyzed samples; previous studies showed sapoviruses incidence of up to 15% (Kumthip et al., 2018). Only two sapovirus strains were successfully genotyped and identified as GI.1 and GII.1. To our best knowledge, this is the first report of sapovirus GI.1 in Mexico.

On the other hand, the incidence of astrovirus infections reported in this study agreed with the range of 0.5–15% gastroenteritis cases usually associated with this virus; most of them in 1–4 years-old children (Biscaro et al., 2018; De Benedictis et al., 2011; Jacobsen et al., 2018). Three astrovirus strains reported here were characterized as classic genotype HAstV-2, and one as genotype HAstV-6. The astrovirus strains HAstV-2 are the most reported genotype in Mexico, whereas the genotype HAstV-1 is the most reported worldwide. Indeed, the HAstV-6 genotype is not commonly reported and has been associated with sporadic outbreaks (Guo et al., 2010). The adenovirus detection was positive in two of the analyzed samples, one of the positive samples was characterized by sequence analysis, as adenovirus group C, the adenovirus group C is often related to infections in the respiratory tract; however, this adenovirus has also been associated with mesenteric adenitis and occasionally to intussusception in young children (Bines et al., 2006). In this case, the child infected with adenovirus C serotype 6 received medical attention due to mild dehydration caused by the diarrhea episodes.

The gastroenteritis severity analysis showed that noroviruses had the highest score in gastroenteritis severity, followed by rotaviruses; the statistically significant data was mostly related to diarrhea episodes and duration (Fig. 3). Before the rotaviruses vaccine introduction in México (2004–2005), most cases were associated with severe gastroenteritis ($\geq$ 15 points) (González-Ochoa et al., 2013); in the present study, after rotavirus vaccination program implementation, since 2006, a lower incidence was detected, and we did not find rotavirus gastroenteritis associated with severe symptoms cases. Some reports indicated that noroviruses were the most important gastroenteritis infective agents in children, in comparison with rotaviruses in the post vaccination era (Bucardo et al., 2014; Koo et al., 2013).

5. Conclusion

In this study, most viral gastroenteritis cases were related to rotavirus genotype G12P[8]. Nevertheless, the severity of diarrheic episodes was associated with noroviruses, compared with other enteric viruses such as sapoviruses, astroviruses, and enteric adenoviruses (Fig.3). These results indicated that noroviruses would be the leading pathogen of severe gastroenteritis in children in the evaluated region. This study results provided evidence of the rotaviruses vaccination program success, which resulted in diarrhea severity reduction among children in the northwest of Mexico, even by rotavirus emergent strain G12 infections. Genotypes variability by the enteric viruses suggests the importance of viral gastroenteritis agents continuous surveillance in children, in order to correlate their genotypic variability with infection severity, the vaccination program efficiency, and new strategies development or design for prevention purposes.

Author contributions

Conceptualization, writing and validation: Guadalupe González Ochoa, Lilian Flores-Mendoza, Patricia Tamez-Guerra. Methodology: Guadalupe Quintero-Ochoa, Ricardo Romero-Argüelles, Armando Aviles-Hernández, Michel Cejudo-Flores, Patricia Calleja-García, Servando Cantú-Bernal, Ramona Icedo-García, José Soñanez-Organis, Jesús Rosas-Rodríguez, César Romo-Saenz. Writing-review and editing: All the authors.

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Conflicts of interest

The authors declare no conflict of interest.

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