ORIGINAL ARTICLE VIROLOGY

Large increase of rotavirus diarrhoea in the hospital setting associated with emergence of G12 genotype in a highly vaccinated population in Nicaragua

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Abstract

Rotaviruses (RVs) are a major cause of severe diarrhoea in young children. Nicaragua introduced routine immunization with the pentavalent RV vaccine (RV5) in 2006, which greatly reduced the incidence of diarrhoea. A remaining concern has been the possible emergence of new RV strains to which the vaccination has less effect. In this study, 837 children with diarrhoea in hospital settings were investigated for RV between May 2011 and July 2013. RVs were subsequently typed by multiplex PCR and/or sequencing. Fecal anti-RV IgA titres for a subset of RV-infected (n = 137) and noninfected children (n = 52) were determined with an in-house enzyme-linked immunosorbent assay. The RV detection rate was 8% in 2011, followed by a sharp increase to 29% in 2012 and 19% in 2013. This was associated with emergence and predominance of genotype G12 RV, from 0% in 2011 to 66% in 2012 and 82% in 2013, infecting children from 1 month to 10 years of age. Two sequenced G12 strains showed a Wa-like genome with genotype G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1, similar to the globally emerging G12 strains. Fecal anti-RV IgA analysis showed that most G12-infected and noninfected children had been in contact with either vaccine or wild RV strains, but such antibodies did not prevent symptomatic G12 infection. A marked increase of RV was evident in the hospital setting associated with a nationwide emergence and predominance of RV G12 genotype in a population with high RV5 vaccine coverage.

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Introduction

Rotavirus (RV) is an important pathogen of severe diarrhoea in infants and young children globally [1]. A major reduction of severe RV diarrhoea has been observed in countries with high RV vaccine coverage, but several clinical trials have shown the vaccine efficacy to be lower in countries with high RV mortality [2]. In countries where universal RV vaccination has been established, noroviruses have replaced RVs as the most common cause of children hospitalization for acute gastroenteritis [3,4]. In October 2006, the Nicaraguan Expanded Program of Immunization initiated universal RV vaccination with the pentavalent RV vaccine (G1/G2/G3/G4/P[8]) from Merck (RV5). Vaccine coverage rapidly reached over 90% and has been maintained since [5]. A study in 2007–2008 in Nicaragua showed that the vaccine had an efficacy against severe diarrhoea of only 58% [6]. A remaining concern has been the possible emergence of new RV strains against which the RV vaccine may be less effective.

RV infecting human are distributed in 12 different G genotypes and 15 P genotypes, of which five genotype

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combinations—G1P[8], G2P[4], G3P[8], G4P[8] and G9P [8]—accounted for 88% of all RVs strains circulating globally during the pre-RV vaccine era, with G1P[8] being the globally dominant strain and with a higher presence in developed (70%) compared to developing countries (40%) [7].

Several studies in different continents have reported the emergence and high detection rates of the previously uncommon G12 strain, mainly in combination with P[8]. Detection rates in a hospital setting between 2005 and 2013 ranged from 10% to 86% in India, Nepal, Niger, Spain, Argentina and Rochester, Minnesota, USA [8–14].

In the Americas, the G12 genotype in combination with P[9] were reported in Argentina (1999), Brazil (2003) and Paraguay (2006) at low frequencies [15–17]. G12P[6] was reported to circulate in the United States between 1999 and 2006 and in Brazil between 2009 and 2010, also at low frequencies [18,19]. In contrast, G12P[8] emerged in Argentina during 2008–2009 at high detection rates (24%), and same genotype was associated with a gastroenteritis outbreaks in Rochester, Minnesota, USA [11,12]. The G12 genotype strains is classified in four discrete genetic groups designated lineage I to IV (LI to LIV) [20]. The majority (>90%) of the currently known and worldwide detected G12P[6] and G12P[8] strains belong to LIII [20].

The current study reports a marked increase of RV in the hospital setting associated with emergence and persistence of G12 RV in two consecutive diarrhoea season in Nicaragua in spite of high vaccine coverage (>90%) and presence of fecal anti-RV IgA during acute phase.

Material and methods

Study sites

Through a hospital based surveillance, a total of 837 children up to 10 years of age with diarrhoea from Nicaragua were investigated for RV infection. Samples were collected between May 2011 and December 2013 at five hospitals participating in the National Rotavirus Surveillance Network of the Health Ministry of Nicaragua, including two pediatric hospital of Managua, the capital city, 'Fernando Velez Paiz' (n = 138) and 'Manuel de Jesus Rivera' (n = 320), and the general hospitals of Masaya 'Humberto Alvarado' (n = 75), Carazo 'Santiago de Jinotepe' (n = 203) and Matagalpa 'Cesar Amador Molina' (n = 101).

Sample collection and RV screening

Liquid stool samples were collected in sterile plastic containers and transported to the National Center for Diagnostic and Reference (NCDR). Aliquots of nondiluted samples were prepared and kept at −20°C until RV screening by enzyme-linked immunosorbent assay (ELISA; Oxoid ProSpecT R240396). Sensitivity and specificity of the ELISA used was reported to be 75% and 100%, respectively, when compared with reverse transcription (RT)-PCR [21]. A form containing general information regarding children age, gender and place and date of sample collection was submitted to the NCDR together with the sample. RV5 vaccination status and information about liquid stools during the past 24 hours was limited to a few cases.

Determination of fecal IgA titre

Acute-phase titres of fecal IgA anti-RV were determined by ELISA using a modification of method previously described [22]. In brief, microtitre plates of 96 wells (Greiner Bio-One) were coated with guinea pig antibody to RV (SBL), diluted 1:500 in carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After 2 hours' blocking with phosphate-buffered saline (PBS)–bovine serum albumin 3%, RV5 vaccine (Merck) diluted 1:100 was added and incubated at 37°C for 1 hour. A total of 100 µL of serially diluted stool (1:20, 1:40, 1:80 and 1:160) was added and incubated at 37°C for 1 hour, followed by addition of peroxidase-conjugated anti-human IgA (P0216; DakoCytomation) diluted 1:2000, and incubated at 37°C for 1 hour. The reaction was developed for 10 minutes with tetramethylbenzidine (Thermo Fisher Scientific) and stopped with 2M H_2SO_4. Optical density (OD) was determined at 450 nm in each well. The reciprocal of the highest stool dilution with OD $\geq$0.100 was considered sample titre. All experiments included negative control (neonate stool) and PBS for background monitoring.

RNA extraction and RT

Viral RNA was extracted from 200 µL 1:10 stool suspensions using High Pure Viral RNA Kit (Roche Diagnostics) following the manufacturer's instructions. A total of 50 µL of RNA was collected and stored at −20°C until RT. RT was carried out as described previously [23].

G-multiplex genotyping

The G genotypes were determined by PCR using generic and genotype-specific primers used for detecting *VP7* genotypes G1, G2, G3, G4, G8, G9, G10 and G12, as described previously [24,25].

VP7 and *VP4* sequencing

A subset of 15 RV-positive samples, representing different G genotypes and different time and place of collection were sequenced in the *VP7* and *VP4* genes. The 881 bp and 876 bp amplicons obtained from *VP7* and *VP4,* respectively, were sequenced by Macrogen using VP7-F, VP7-R, Con-3 and Con-2 as sequencing primers [24]. GenBank accession numbers for *VP7* are KM668708 to KM668722 and for *VP4,* KM668723 to KM668736.

Full genome sequencing of two G12 strains

Sequencing was extended to the remaining nine genes of the RV genome from two G12 strains detected in 2012 and 2013. Partial genes encoding *VP6* (1000 bp), *NSP1* (740 bp), *NSP2* (935 bp), *NSP3* (852 bp), *NSP4* (649 bp) and *NSP5* (552 bp), together with partial genes encoding *VP1* (564 bp), *VP2* (665 bp) and *VP3* (676 bp), were sequenced using the methods and primers described elsewhere [26,27]. GenBank accession numbers for genes of the strains 203VN12 and 86VN13 are KM668737 to KM668754 (Supplementary Table 1).

Phylogenetic analysis

Sequence alignment was performed by using the ClustalW algorithm, version 1.83. Phylogenetic analysis and pairwise nucleotide identities were performed by the Mega 6.0 software package, and the phylogenetic trees were constructed as previously described [23].

Results

Sharp increase of RV detection in hospital settings

A total of 185 (22%) of 837 children with diarrhoea were RV-positive. Median age of RV-positive children was 12 months (range 1 month to 10 years), with 66% in the group of 6 to 24 months of age, with boys more commonly found in the RV-infected group than girls (61% vs. 39%). Peaks of RV detection occurred in May 2012 and April 2013 (Fig. 1A). The RV detection rate increased significantly in 2012, with 114 (29%) of 385 being RV positive compared to only 8 (6%) of 124 in 2011 (Table 1). Following this unusual RV increase, prevalence of RV was slightly lower (19%) in 2013. RV detection rates varied between hospitals; for instance, in some of the hospital settings the RV prevalence was >37% in 2012 (Table 1).

RV G12 emergence and predominance in 2012 and 2013

A subset of 156 (84%) of 185 RV-positive samples with sufficient material was analyzed by G genotyping (Table 2). The predominant genotype was G12, accounting for 104 (69%) of the RV-positive samples tested, followed by G4 (7%), G3 (6%), G1 (4%) and G2 (0.6%), in addition to mixed infections of G4:G3 (n = 4) and G4:G2 (n = 3) (Table 2). In 10% of these samples, a genotype could not be assigned. Although G12 was not detected at all in 2011, it accounted for 65% and 81% of the RV-positive samples collected in 2012 and 2013, respectively (Table 2). Notably, G12 was found in children of all ages (Fig. 1B).

Nationwide spreading of G12P[8] of LIII in Nicaragua

Nucleotide sequencing showed that 11 (73%) of the selected RV positive samples (n = 15), representing different hospitals of Nicaragua and different years, carried *VP7* genes with ≥99% nucleotide homology to G12 strains recently detected in several countries, including India, Kenya, Argentina and the United States (Supplementary Fig. 1). Moreover, all Nicaraguan G12 strains belonged to the globally emerging LIII lineage. The remaining four sequenced strains had *VP7* genes with G3 (n = 3) and G1 (n = 1) specificities and nucleotide sequences with ≥99% homology to Nicaraguan G3 and Cuban G1 strains (Supplementary Fig. 1). All successfully sequenced *VP4* genes (n = 14) carried P[8] specificity and clustered into LIII (Supplementary Fig. 2). All P[8] of G12 strains subclustered with P[8] derived from Kenyan, US and Argentinean strains, but

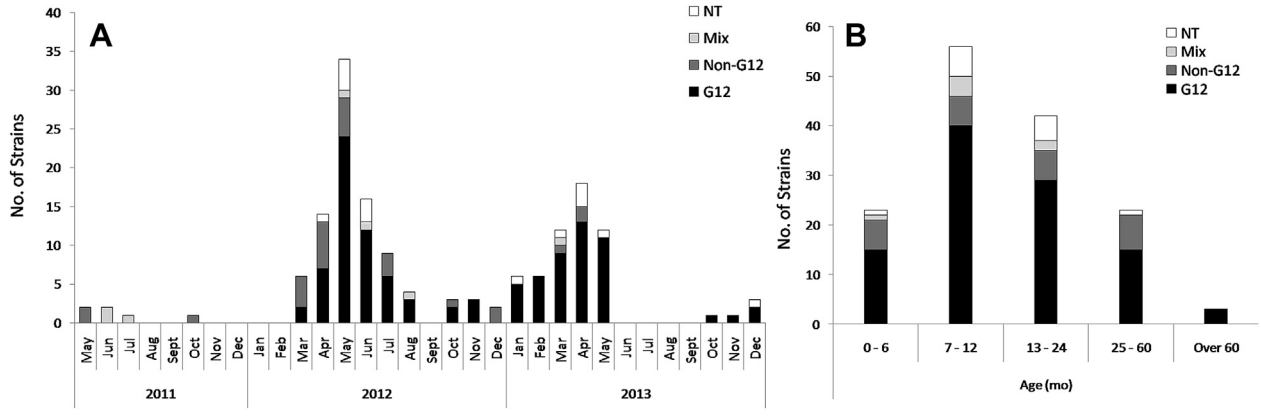

FIG. 1. (A) Temporal distribution of rotavirus (RV) strains circulating among children in a hospital setting in Nicaragua from 2011 to 2013. G12 genotype was predominant, and peaks occurred during the late dry season. Non-G12 refers to G4, G3, G1 and G2. (B) Frequency of RV detection among different age groups during 2011 to 2013 in Nicaragua. G12 genotype infected all age groups with increased frequency in children 7 to 24 months of age. Non-G12 refers to G4, G3, G1 and G2.

TABLE 1. Rotavirus (RV) detection rates in the hospital setting among children ≤10 years of age from Nicaragua in 2011–2013

City	Hospital name	2011		2012		2013		2011–2013	
		All cases	RV, *n* (%)	All cases	RV, *n* (%)	All cases	RV, *n* (%)	All cases	RV, *n* (%)
Managua	Fernando Velez Paiz	36	5 (13)	71	29 (40)	31	11 (35)	138	45 (32)
	Manuel de Jesus Rivera	0	0	169	34 (20)	151	34 (22)	320	68 (21)
Masaya	Humberto Alvarado Vasquez	15	2 (13)	24	9 (37)	36	4 (11)	75	15 (20)
Carazo	Santiago Jinotepe	54	1 (1)	74	31 (41)	75	11 (14)	203	43 (21)
Matagalpa	Cesar Amador Molina	19	0 (0)	47	11 (23)	35	3 (8)	101	14 (13)
All		124	8 (6)	385	114 (29)	328	63 (19)	837	185 (22)

not with P[8] from strains previously circulating in Nicaragua. In contrast, the P[8] of G3 strains reported in this study sub-clustered with P[8] circulating previously in Nicaraguan. The P[8] gene from G1 clustered with P[8] from G12 RVs and was identical to the *VP4* gene of the Ro4426 strain from the United States (Supplementary Fig. 2).

The Nicaraguan G12 RV genome was similar to the globally emerging G12 strain. Both G12 RV strains selected for genome sequencing analysis and circulating in 2012 (203VN12) and 2013 (86VN13) displayed a Wa-like genome: G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1. Nucleotide identities ranged from 99.9% to 99.2% between all 11 genes of strains 203VN12 and 86VN13 (Supplementary Table 1). Most genes of the 203VN12 strain were more similar to homologous genes of the strain from Kenya than to the Argentinian strain and several other global circulating G12 strains. Of interest, the *NSP4* gene from strains 203VN12 and 86VN13 were rather different (3% to 5.4%) compared to the *NSP4* gene of other global G12 strains (Supplementary Table 1).

G12-infected children with fecal IgA anti-RV

To investigate whether G12 RV infections correlated with the absence of fecal IgA anti-RV, IgA was measured in a subset of 137 acute stool samples from RV-positive children (Table 3). The results showed that 106 (77%) of 137 RV-positive children had fecal IgA anti-RV titres of ≥1:160. Most RV-negative children (79%, *n* = 52, Table 3) also had IgA anti-RV titres of ≥1:160. Very low titres (≤1:20) were observed in only 4 (3%) RV-positive and 2 (4%) RV-negative subjects (Table 3). After stratifying by genotype, it was observed that 75% of the G12 infections had IgA anti-RV titres of ≥1:160.

TABLE 2. Predominance and persistence of G12 during two consecutive years in Nicaragua. 2012–2013

Genotype[a]	All years, *n* (%)	2011, *n* (%)	2012, *n* (%)	2013, *n* (%)
G12	107 (69)	0 (0)	59 (65)	48 (81)
G4	11 (7)	2 (33)	8 (9)	1 (2)
G3	9 (6)	1 (17)	8 (9)	0 (0)
G1	6 (4)	0 (0)	5 (5)	1 (2)
G2	1 (0.6)	0	0	1 (2)
Mix[b]	7 (4)	3 (50)	3 (3)	1 (2)
NT[c]	15 (10)	0 (0)	8 (9)	7 (12)
Total	156 (100)	6 (100)	91 (100)	59 (100)

[a]Stool sample was not sufficient in 29 rotavirus-positive samples for genotyping.
[b]Coinfection with more than one genotype, of which four were cases of G4:G3 infection and three of G4:G2 infection.
[c]NT indicates not typeable with primers and methods used.

Discussion

The emergence and predominance of G12 RV in Nicaragua during 2012 and 2013 is a distinctive event. The G12 genotype has not previously been detected in this country—neither during the prevaccine or postvaccine periods [4,23,28]. This emergence was further associated with a more than twofold increase in hospitalizations due to diarrhoea among children ≤5 years of age (2011, *n* = 1418; 2012, *n* = 3300; 2013, *n* = 3439) (http://www.paho.org/hq/index.php?option=com_content&view=article&id=1892:rotavirus-surveillance&Itemid=1623&lang=en). Taken together, these results show that emergence of G12 can have a major and persisting impact on hospitalization rates over more than two seasons in RV5-vaccinated populations. The capacity of G12 RV to infect and persist in populations vaccinated with the monovalent RV vaccine (RV1) remains to be elucidated.

Available data demonstrate that temporal and regional fluctuations of globally common RV genotypes occurred before the introduction of RV5 vaccine into Nicaragua [23,29,30]. Studies performed after vaccine introduction suggested that RV5 vaccination has not yet altered the historical pattern of RV genotype fluctuation [6,28]. Therefore, it is also possible that after two seasons of G12 predominance, another genotype will emerge and replace it. Nevertheless, it is possible that G12 will become a common genotype in Nicaragua and the region, as has occurred with other globally dominant genotypes (i.e. G1, G2, G3 G4 and G9).

The finding that G12 strains in this study could to a high degree symptomatically infect a population with a reported RV5 immunization rate of over 90% must be interpreted with caution and in terms of vaccine uptake or vaccine-related immune pressure [4]. The RV5 vaccine is based on the concept of serotype-specific immunity (G1 to G4) [31]. Thus, one major concern is whether the RV5 vaccine used in Nicaragua can protect against new emerging genotypes, such as G12. Most of the countries reporting increasing detection rates of G12 strains

TABLE 3. Fecal anti-RV5 IgA titres among Nicaraguan children with acute RV diarrhoea mainly associated with G12 genotypes, 2011–2013, and children with non-RV diarrhoea

	Diarrhoea, *n* (%)		G-type infection, *n* (%)					
Acute anti-RV5 IgA titre[a]	Non-RV	RV	G12	G4	G3	G1	G2	Mix
≥1:160	41 (79)	106 (77)	77 (75)	9 (82)	9 (100)	4 (66)	1 (100)	6 (86)
1:80	7 (13)	23 (17)	19 (18)	1 (9)	0	2 (33)	0	1 (14)
1:40	2 (4)	4 (3)	3 (3)	1 (9)	0	0	0	0
≤1:20	2 (4)	4 (3)	4 (3)	0	0	0	0	0
All tested	52 (100)	137 (100)	103 (100)	11 (100)	9 (100)	6 (100)	1 (100)	7 (100)

OD, optical density; PBS, phosphate-buffered saline; RV, rotavirus.
[a]Reciprocal of the highest stool dilution with OD ≥0.100 was considered sample titre. For negative control, media and standard deviation of the OD for all dilutions intra- and interassay were 0.06 and 0.017. Media of the OD of PBS readings was 0.037 (range 0.035–0.039, SD = 0.009).

do not have RV vaccination in their national immunization schedule, and vaccine-related selective pressure is therefore unlikely [8–11]. It is more likely that G12 emergency and persistence in Nicaragua was due the presence of a susceptible population immunological naive to this globally emerging strain. Because of logistic constraints, vaccination status was only documented for 26 of the RV-positive children in this study, 20 of whom had received all recommended vaccine doses. The RV genotypes observed in these children were G12 (*n* = 10), G4 (*n* = 2), G2 (*n* = 1), G1 (*n* = 1), mixed infections (*n* = 1) and nontypeable strains (*n* = 5).

The specific immune component of protection induced by the RV5 vaccine is still unclear, and a good marker of protection remains to be established [32]. In natural RV infections, Matson *et al.* [33] found that higher fecal IgA titres were associated with protection against infection and illness. In contrast, an adult challenge study found that fecal anti-RV IgA titres could not be correlated to either infection or illness [34]. Although a clear correlation of protection has not been established, some studies have suggested serum and fecal anti-RV IgA to be infection markers, with antibody levels peaking at days 14 to 17 after inoculation [22,32,35]. A challenge study using the quadrivalent precursor of the RV5 vaccine found that no placebo recipient had a threefold or more increase in fecal anti-RV IgA, whereas 83% of vaccine recipients had an increase after one dose [36]. Anti-RV IgA titres were also found to be a poor correlate of protection in other studies [37]. In the current study, 75% of the G12-positive children had a fecal anti-RV IgA titre of ≥160, which was similar as RV-negative children (79%), indicating that most children infected with G12 viruses had previously been in contact with either RV of vaccine origin or wild-type strains; thus, these fecal IgA antibody titres were not protective. Indeed, vaccination failure was documented in 10 children (age range 6 to 24 months, median 12 months) symptomatically infected with G12.

The G12 virus found in Nicaragua displayed a Wa-like genome (G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1), which is markedly different to the vaccine genome (G1/2/3/4/6-P[8]/P[5]-I2-R2-C2-M1/M2-A3-N2-T6-E2-H3), with the exception of VP4. Additionally, nucleotide identity data suggest that the *NSP4* gene in the Nicaraguan G12P[8] RV strains may have been acquired by a reassortment event (Supplementary Table 1). However, RV5 vaccine has been shown to exert similar effectiveness against homotypic and heterotypic RV strains [38]. For instance, in Nicaragua, RV5 vaccination has exhibited large protection to many other Wa-like strains, such as G1P[8], G3P[8], G4P[8] and G2P[4], the detection rates of which have considerably decreased in the last few years, as observed in the current study and in other studies [4,5,28]. Nevertheless, the emergence and high prevalence of G12 RV in a vaccinated population with RV5 (G1/2/3/4:P[8]) vaccine suggest that G-type-specific immune protection may be required to prevent disease and that new strains with *VP7* genotypes not included in the vaccine may lead to extensive vaccine failures. However, there may be several additional factors contributing to RV vaccine protection, including immune and nutritional components, as well as host histo–blood group antigens that mediate susceptibility to RV infection [39]. More studies are needed to clarify the risk factors behind RV vaccine failures.

Transparency declaration

All authors report no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.cmi.2015.01.022.

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