Timing and genotype distribution of symptomatic and asymptomatic sapovirus infections and re-infections in a Nicaraguan birth cohort

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ABSTRACT

Objectives: To characterize the timing and genotype distribution of symptomatic and asymptomatic sapovirus infections and re-infections in a Nicaraguan birth cohort.
Methods: Infants ($N = 444$) were enrolled at 10–14 days of life and observed weekly until 2 years of age. Stool samples were collected for each acute gastroenteritis (AGE) episode, and routine stool samples were collected monthly. Stool samples were tested for sapovirus using RT-qPCR, and positive samples were genotyped.
Results: A total of 348 children completed 2 years of AGE weekly surveillance; 93 (26.7%) of them experienced sapovirus AGE. Most infections occurred after 5 months of age and mainly during the second year of life (62.4%, 58/93) and early in the rainy season. Sapovirus screening in all stools from a subset of 67 children who consistently provided samples showed sapovirus infections in 91 of 330 (27.6%) AGE episodes and in 39 of 1350 (2.9%) routine stools. In this subset, the median age at the first sapovirus AGE was 11.2 months (95% CI, 9.3–15.9 months); 38 of 67 (57%) children experienced re-infections, 19 symptomatic and 19 asymptomatic. On average, sapovirus re-infections were reported 7.2 months after symptomatic and 5.3 months after asymptomatic infections. Genogroup GI (64%, 69/108) was the most common detected. Sapovirus GI.1 was more frequently detected in AGE stool samples than in routine stool samples (47.2%, 43/91 vs. 25.6%, 10/39; p 0.005), and re-infection with the same genotype was uncommon.
Discussion: The first sapovirus infections occurred at approximately 11 months of age, whereas the median time to symptomatic re-infection was 7.2 months. Re-infections with the same sapovirus genotype were rare during 2 years of life suggesting genotype-specific protection after natural infection.
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Introduction

Acute gastroenteritis (AGE) is an important cause of global mortality accounting for 9% of all deaths in children under 5 years of age [1]. The global burden of rotavirus-associated AGE declined after the widespread introduction of rotavirus vaccines, making caliciviruses, such as norovirus and sapovirus, the leading cause of

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paediatric AGE [2]. A multi-site international birth cohort study found that among all enteric pathogens, sapovirus had the third highest attributable incidence of diarrhoea among children younger than 12 months and the second highest among children 12–24 months of age [3]. In another birth cohort study in Peru, 64% of the children had experienced at least one sapovirus AGE episode by the age of 2 years [4]. In Central America, the prevalence of sapovirus ranges from 7% to 17% [5–7]. Despite this high disease burden, little is known about the natural history of sapovirus infections, including the dynamics of re-infection and naturally induced protection.

Sapoviruses are genetically diverse single-stranded RNA viruses belonging to the genogroups GI, GII, GIV and GV that infect humans [8,9]. Viruses in these four genogroups can be further divided into 18 genotypes (GI.1–7, GII.1–8, GIV.1 and GV.1–2), with GI.1 viruses reported most frequently globally [10]. To date, few studies have examined and genotyped sapovirus in stool samples obtained from asymptomatic children [11]. In the Peruvian birth cohort previously mentioned, GI sapoviruses were found to be more common in symptomatic children, whereas GII sapoviruses were more common in asymptomatic children [4]. There is limited knowledge about the patterns of sapovirus re-infections [4] and whether the first infection provides protection against re-infections.

We previously reported the risk factors and clinical characteristics during the first 2 years of life in a Nicaraguan community birth cohort [12]. Here, we characterized the timing of symptomatic and asymptomatic sapovirus infections, determined the sapovirus genotypes and characterized the patterns of re-infection. The data presented in this study could be used to help guide the timing of targeted interventions to prevent sapovirus and increase our understanding of protection after natural infection.

Materials and methods

Study design

The Sapovirus-Associated Gastro-Enteritis study is a population-based birth cohort study conducted in León, Nicaragua, which has been described previously [12].

The present study was conducted in the Perla Maria Norori (PMN) Health Sector, one of the three Health Sectors in León. PMN includes both urban and peri-urban areas. Pregnant women living in the PMN sector were invited to participate in this study in the third trimester of their pregnancy, and new-born infants were enrolled within 14 days of delivery. Before the enrolment, all mothers signed an informed consent. The enrolled children were monitored weekly for AGE symptoms until 2 years of age. AGE was defined as an increase in stool frequency to at $\geq$3 stools per 24-hour period or a substantial change in the stool consistency (bloody, very loose, watery) and/or vomiting. A new AGE episode was defined when a child had experienced at least 3 days without diarrhoea or vomiting before the onset of AGE symptoms [13,14]. Stool samples and clinical data were collected for each AGE episode, and routine stool samples were collected monthly.

Study procedures

Stool samples from AGE episodes were collected within 4 days after symptom onset and tested for sapovirus. A subset of children who completed 2 years of weekly AGE monitoring, consistently contributed stool samples from AGE episodes and monthly routine stools and experienced at least one sapovirus AGE episode were selected to investigate the timing of infections, frequency of symptomatic and asymptomatic re-infections and genotype distribution. Specimens from the AGE episodes and from the monthly routine stools were transported on ice packs to the Microbiology Department of UNAN-León where a 10% (wt/vol) clarified stool suspension in phosphate-buffered saline (pH = 7.2) was prepared and stored frozen at −20 °C.

Real-time RT-qPCR

Sapovirus screening in the AGE episodes was performed in Nicaragua, and sapovirus detection in routine stools was performed at CDC; both laboratories follow the method described by Oka et al. [15]. In brief, viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Sapovirus was detected using RT-qPCR as described previously [15], with bacteriophage MS2 (ATCC 15597-B1) included as an internal amplification control. RT-qPCR was performed using the AgPath-ID kit (Thermo Fisher Scientific, Waltham, MA) on a Roche Lightcycler 96. A sample was considered positive for sapovirus if the Ct value was $\leq$35.

Sapovirus genotyping

Sapovirus RT-qPCR–positive samples were amplified by a hemi-nested conventional RT-PCR [16] that targeted the N-terminal and shell (NS) region of the capsid gene. The RT-PCR products were purified using ExoSAP-IT (Affymetrix, USB, Cleveland, OH) or QIAquick PCR purification kit (Qiagen) and were submitted for Sanger sequencing (Eurofins MWG Operon, Louisville, KY). Sequences were typed using the online human calicivirus typing tool [17]. The nucleotide sequences homology from the children experiencing consecutive infections with the same genotype was calculated using the BioEdit sequence alignment editor, version 7.2.

Ethics statement

The study was approved by the institutional review boards of the UNAN-León (acta No. 4, 2017), UNC-CH (Study #: 16-2079) and CDC (project ID: 0900f3eb81c526a7). Each mother provided written informed consent for her infant's participation.

Measures

In addition to quantifying the number of symptomatic and asymptomatic stool samples that were infected with sapovirus, we assessed the child's age at each sapovirus infection, the season when the infection occurred, the time elapsed between infections and the genogroup and genotype of each sapovirus infection.

Statistical analysis

Descriptive statistics for continuous variables are presented as median plus 95% CI. For categorical variables, we quantified the number and percentage in each category and compared the differences between asymptomatic and symptomatic sapovirus AGE episodes using the Fisher's exact tests. The non-parametric Wilcoxon Mann-Whitney test was used to compare the median age between the first symptomatic and first asymptomatic infections. Differences were statistically significant when the level of two-tailed test was $p < 0.05$. We used multiple imputations to estimate sapovirus incidence, including symptomatic stools that were not collected from approximately 10% of AGE episodes, assuming that infection data were missing completely at random. SPSS (Statistical Program for Social Science version 21.0 for Windows; Chicago, IL) was used for statistical analyses, and Graph Prism 9.0 (GraphPad Software 2365 Northside Dr. Suite 560 San Diego, CA 92108) was used for figures.

Results

Epidemiological profile of sapovirus AGE in children aged ≤2 years

Sapovirus AGE was observed in 93 of 348 (26.7%) children who completed 2 years of household weekly surveillance (Fig. 1). More infections occurred in the second year of life (62.4%, 58/93) than in the first year, with high frequencies of detection between 6–9 and 18–23 months of age (Fig. 1b). Sapovirus infection was more common during June–July and September–October, the rainy season in Nicaragua.

Symptomatic and asymptomatic sapovirus infections

To investigate the burden of symptomatic and asymptomatic infection during 2 years of life, a total of 330 AGE episode stools and 1350 routine stool specimens from a subset of 67 children who experienced at least one sapovirus AGE episode during the two-year study period were tested for sapovirus (Fig. 2). Sapovirus was detected in 91 of 330 (27.6%) specimens collected during AGE episodes and in 39 of 1350 (2.9%) routine stool specimens (Fig. 3). Among the 67 children, 38 (56.7%) experienced symptomatic ($n = 19$) or asymptomatic ($n = 19$) re-infections (Fig. 4a). Monthly routine stool specimens collected from 9 (13.4%) children tested positive before their first sapovirus AGE episode (Fig. 4a). Of note, although 29 (43.2%) children experienced only one symptomatic infection during 2 years of life, 18 (26.9%) experienced three or more infections, among which one child experienced five sapovirus AGE episodes; all infections were of a different genotype (Fig. 4a).

Timing of sapovirus infections

The median age at which the children had their first symptomatic sapovirus episode was 11.2 months ($n = 67$; 95% CI, 9.3–15.9 months) and 18.4 months ($n = 19$; 95% CI, 12–19.4 months, p 0.001) for their second symptomatic infection (Fig. 4b). Three children (subjects 14, 19 and 28; Fig. 4b) experienced a third symptomatic sapovirus episode at a median age of 20.8 months. The median age at which children experienced their first sapovirus infection (either symptomatic or asymptomatic) and the time elapsed to symptomatic re-infection was investigated in 38 of 67 children with ≥2 infections (Fig. 4b). There was no difference between the age at which the children had their first symptomatic or asymptomatic infection (median, 11.2 vs. 11.5; $p > 0.05$) (Fig. 4b). The time elapsed between an asymptomatic infection followed by the first sapovirus AGE episode was 6.2 months, similar to the observed time between two consecutive symptomatic infections (first and second sapovirus AGE, 7.2 months).

Genotype diversity in symptomatic and asymptomatic sapovirus infections

Of the 130 sapovirus infections (91 samples from AGE episodes and 39 from routine stool examination), 108 (83%) were successfully genotyped into four genogroups—GI (64%), GII (27%), GIV (2%) and GV (7%)—and eight genotypes (Fig. 3). GI viruses, primarily GI.1, were more commonly detected in AGE stool samples than in the routine stool samples (47.2%, 43/91 vs. 25.6%, 10/39; p 0.005), whereas GII.1 viruses were more frequently found in the asymptomatic monthly stool specimens (10.2% vs. 2.2%, $p \geq 0.05$) (Fig. 3). Of the 19 symptomatic re-infections, 17 (89.5%) had a different genotype than the first infection (Table 1). The most common genotype during re-infection was GI.2 (7/19) (Table 1 and Fig. 4a). No asymptomatic sapovirus infection was detected in 29 of 67 (43.2%) children with at least sapovirus AGE episode (Fig. 4a). Of note, in 27% of the sapovirus-positive routine stools, no genotype could be determined because of the poor sequence quality or low viral load. Co-infections with multiple genotypes were not observed in this study. Pairwise alignment of the nucleotide sequences from children experiencing consecutive infections with the same genotype within 42 days showed 100% nucleotide homology, with two exceptions (subjects 5 and 29).

Discussion

We used clinical data and stool specimens from a birth cohort study (Sapovirus-Associated Gastro-Enteritis cohort) conducted at a community level in Nicaragua to determine the timing of symptomatic and asymptomatic sapovirus infections in the first 2 years of life and described the genotype distribution and patterns of re-infections. Our findings extend knowledge on the importance and natural history of sapovirus infections in early childhood. After the introduction of rotavirus vaccine, sapovirus has been increasingly acknowledged as a leading cause of AGE in children in Nicaragua and other countries [6,18–20]. Thus, the incidence of norovirus, sapovirus and rotavirus was 21.9, 13.3 and 5.9 cases per 100 child-years after the rotavirus vaccine implementation in Nicaragua, respectively [5,12,21].

We found that the first symptomatic or asymptomatic sapovirus infections occurred at 11 months of age on average. The median time between the first infection (whether symptomatic or asymptomatic) and re-infection was approximately 6 months, suggesting that children may develop broad short-lived immunity against sapovirus. These observations may have implications for future vaccine strategies.

Comparable to the data from previous studies, sapovirus infections were rarely observed during the first 5 months of life

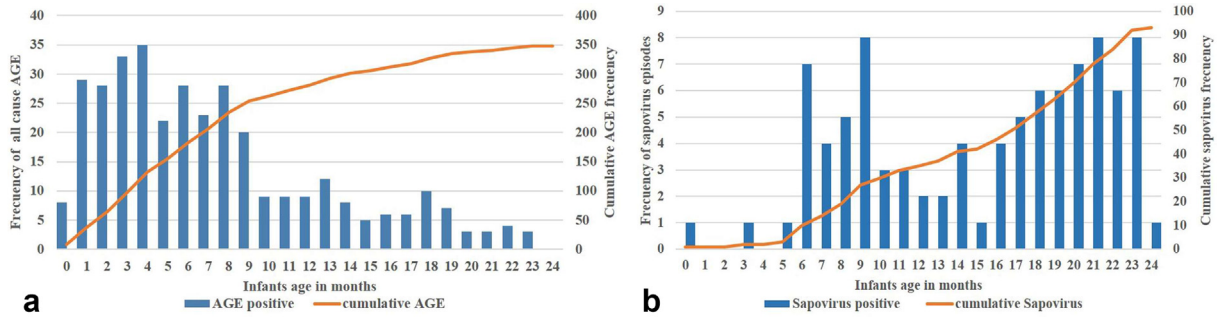

Fig. 1. Cumulative incidence of the first acute gastroenteritis (AGE) episode ($n = 348$) and first sapovirus AGE episode per month in children aged up to 2 years ($n = 93$) enrolled in the Sapovirus-Associated Gastro-Enteritis birth cohort in Nicaragua. The bar scale indicates the number of (a) AGE and (b) sapovirus AGE episodes. The secondary Y axis represents the monthly cumulative incidence of (a) AGE and (b) sapovirus AGE episodes.

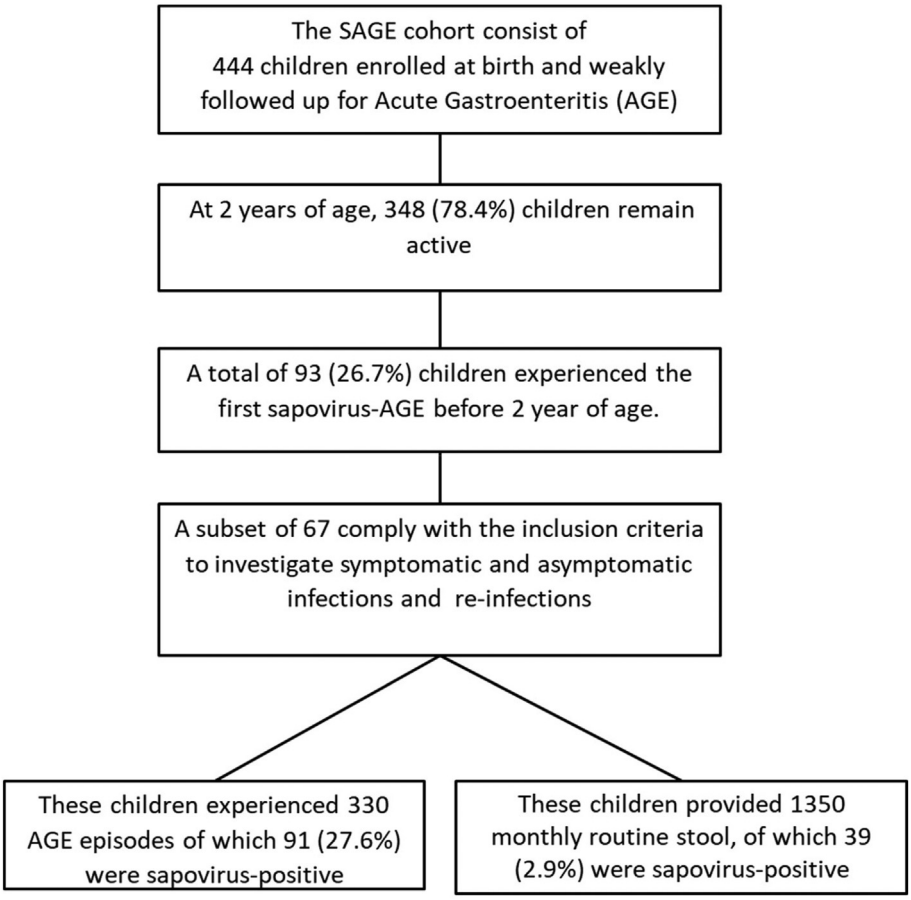

Fig. 2. Flowchart of the subset from the Sapovirus-Associated Gastro-Enteritis (SAGE) cohort and samples analysed in this study. AGE, acute gastroenteritis.

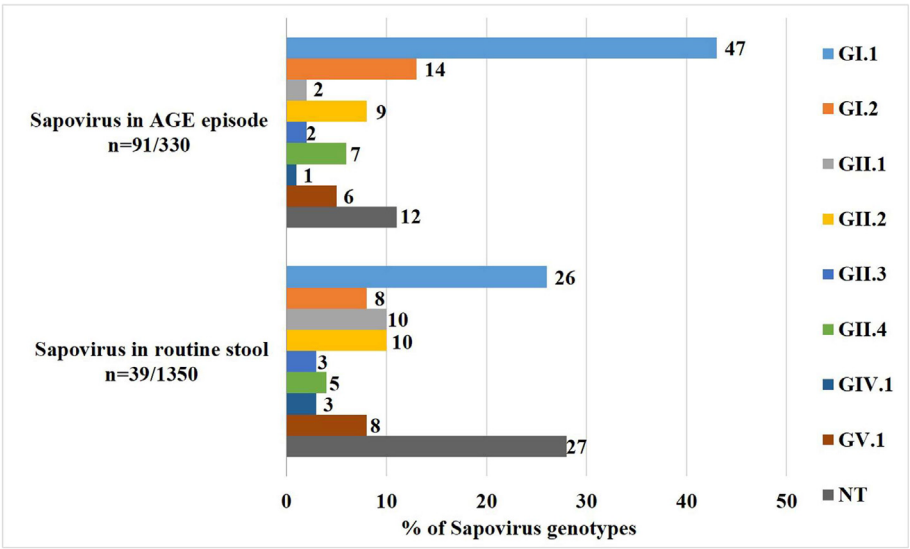

Fig. 3. Distribution of sapovirus genotypes in acute gastroenteritis (AGE) episodes and routine stool samples from 67 sapovirus-positive children from the Sapovirus-Associated Gastro-Enteritis birth cohort followed from birth up to 2 years of age. NT, not typed.

[4,22,23], probably because of the protection conferred by transplacental antibodies in the first months of life [24,25]. Other factors contributing to protection in early childhood may include adaptive and innate immune factors transferred by oligosaccharides or the microbiome in breast milk [26] or high IgA titres as has been reported for norovirus [27,28]. Finally, during early infancy, exposure to sapovirus may be prevented by caregivers, and when children start crawling and exploring their environment, the risk for exposure may increase [29].

Understanding which sapovirus genotypes infants are exposed to may help inform which strains to include in a future vaccine. The most common genogroups found in this study were GI (64%) and

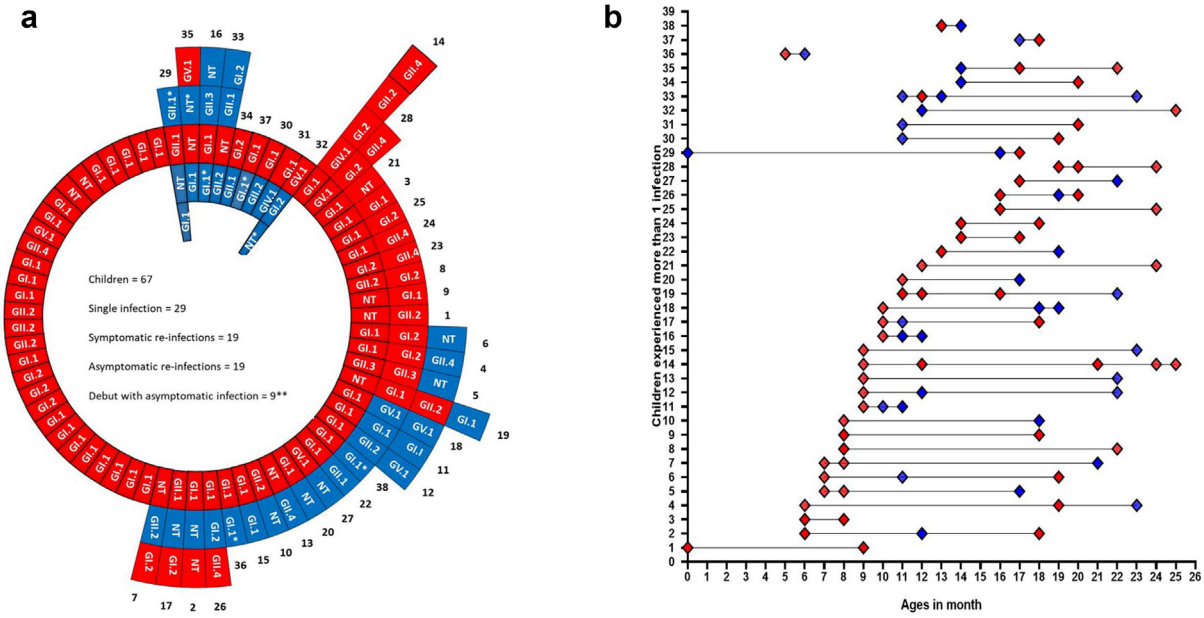

Fig. 4. Chronological order and timing of sapovirus genotypes infections until 2 years of age. (a) Sapovirus genotypes of 130 infections in 67 children. Circles order is chronological, with the inner circle representing debuting infection and external circle representing consecutive infections. Red and blue squares represent the symptomatic and asymptomatic infections, respectively, and numbers represent the subject IDs. *Consecutive samples were collected <23 days. **Children ($n = 9$) debuting with an asymptomatic infection are also included in the re-infection group. (b) Red and blue dots represent the symptomatic and asymptomatic infections ($n = 38$), respectively. The Y- and X-axes show children who experienced more than one infection and children's age in months, respectively. Asymptomatic infections occurring within 23 days (ID 16, 29, 32 and 35) were excluded. NT, not typed.

GII (27%), which aligns with the percentages reported in a meta-analysis that included data from 35 countries [30]. GI sapoviruses were more commonly detected in symptomatic infections than in asymptomatic infections (62% vs. 34%), as previously observed in studies conducted in Nicaragua, Peru, Burkina Faso and South Africa [4,6,31,32]. Of note, the most prevalent genotype (GI.1) in symptomatic children was less commonly detected in asymptomatic infections (47% vs. 26%), suggesting that some genotypes might be less pathogenic or that infection with GI.1 might confer protective genotype-specific immunity. After infection with any given genotype, some children seem to remain susceptible to subclinical infection with uncommon genotypes, which might result in broadening of the immune response.

There are limited data about the correlation between sapovirus antigenicity and genetic diversity [33,34]. There is some evidence that the four human sapovirus genogroups are antigenically different and that different genotypes within the same genogroup have distinct antigenicity based on data from binding virus-like particles to rabbit hyperimmune sera [35]. Herein, repeated AGE episodes of the same genotype were very rare, suggesting genotype-specific immunity.

Our data also contribute to a better understanding of asymptomatic sapovirus infections in children for which there are limited data. In this cohort, asymptomatic sapovirus infections were detected in 2.9% of the 1350 monthly stools from a subgroup of 67 children. A Canadian cross-sectional study reported almost identical findings [36], and in the United States, sapovirus was detected in 3.0% of stools from 272 healthy children under 2 years of age [37]. Children in a day-care centre in Denmark experienced sapovirus symptomatic and asymptomatic infections year-round [38]. Altogether, asymptomatic sapovirus infections in children may contribute to household transmission, serve as a possible reservoir and potentially increase the immune responses.

Our study has several limitations. First, we focused our study on samples from 67 children who experienced at least one symptomatic sapovirus infection AGE during the first 2 years of life and

Table 1
Distribution of sapovirus genotypes in the first and secondary symptomatic infections in children until 2 years of life

First symptomatic sapovirus infection		Genotype of secondary symptomatic sapovirus re-infections ($n = 19$)[a]									
Genotype	Frequency	GI.1	GI.2	GII.1	GII.2	GII.3	GII.4	GIV.1	GV.1	NT	All re-infections
GI.1	40	1	3				2	1		2	9
GI.2	5						1				1
GII.1	2		1								1
GII.2	5		1								1
GII.3	1					1					1
GII.4	1										0
GIV.1	0										0
GV.1	4		1								1
NT	9	2	1		1				1		5
All genotypes	67	3	7	0	1	1	3	1	1	2	19

NT, not typed.
[a] Three children experienced >2 re-infections.

who had consistently contributed stools throughout the study period. Therefore, we cannot extrapolate our data to children who did not experience symptomatic sapovirus infections. However, this approach allowed us to understand the frequency and genotype make-up of re-infections. Second, because of the low viral load and poor nucleotide sequence quality, we were unable to determine the genotype in 27% of asymptomatic sapovirus infections. Furthermore, we may have missed asymptomatic infections because the duration of sapovirus shedding has been found to be approximately 23 days, whereas the stool samples in our study were collected monthly [4,39]. Third, the timing to infection and re-infections and genotypes diversity analysis are limited to 2 years of surveillance. Serological studies would be needed to better understand the duration of protection against sapovirus infection and disease after natural infection and to define a possible correlate of protection. All efforts were made to retain children in surveillance, including allowing generous windows for contributing stool samples; completing visits by phone when home visits were not possible and maintaining constant contact and positive relationships with field staff.

Sapovirus is increasingly recognized as an important cause of AGE in children in both low- and high-income settings [20,40], which is supported by our study. Because sapovirus GI.1 is consistently the most common genotype and associated with symptomatic disease and infection might result in homotypic protection, inclusion of this antigen in a multivalent calicivirus vaccine may present a reasonable strategy in the future to reduce the overall burden of childhood gastroenteritis [19].

In summary, sapoviruses are a common cause of symptomatic infections in young children during the first 2 years of life in Nicaragua. Furthermore, this study showed that children become susceptible to sapovirus infections at around 6 months of age, the time to re-infection varies from 5.3 to 7.2 months and re-infections with the same genotype are rare suggesting the generation of immune protection against the infecting genotype.

Author contributions

F.B., S.B.-D. and J.V. were responsible for the conception and design of the study. F.G. performed analysis and interpretation of the data and wrote the original draft. M.D.-V. and H.B. performed and developed the experiments. Y.R., O.Z., E.C.C. and P.B. ran the laboratory analyses. C.T. and L.G. handled the data and biological samples. N.A.V., N.M.B. and S.V. assisted with critical revising and editing. S.B.-D. and F.B. are co-senior authors. All authors approved the final version of the manuscript.

Transparency declaration

The authors declare that they have no conflicts of interest. This study was supported by the National Institute of Allergy and Infectious Diseases award R01AI127845. F.G., Y.R., O.Z. and L.G. were supported by an international research capacity-building award from the Fogarty International Center D43TW010923. The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2022.11.013.

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