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# 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 followed weekly until 2 years of age. Stool were collected for each acute gastroenteritis (AGE) episode and routine stool were collected monthly. Stools were tested for sapovirus by RT-qPCR and positive samples were genotyped.

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Authors' contributions

FB, SBD and JV were responsible for the conception and design of the study. FG performed the analysis and interpretation of the data and wrote the original draft. MDV and HB performed and developed the experiments. YR, OZ, ECC and PB ran the laboratory analyses. CT and LG handled the data and biological samples. NAV, NMB and SV assisted with critical revising and editing. All authors approved the final version of the manuscript.

Transparency declaration

The authors have no conflicts of interest to disclose.

**Results.—**A total of 348 children completes 2 years of AGE weekly surveillance, 93 (26.7%) of them experienced sapovirus AGE. Most infections occurred after 5 months of age and mainly 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, that consistently provided samples, show sapovirus infections in 27.6% (91/330) of the AGE episode and in 2.9% (39/1350) of the routine stool. In this subset, the median age at the first sapovirus AGE was 11.2 month (95%CI; 9.3 – 15.9), 57% (38/67) experienced re-infections, 19 symptomatic and 19 asymptomatic; on average, sapovirus re-infections were reported 7.2 months after symptomatic and 5.3 after asymptomatic infections. Genogroups GI (64%, 69/108) was the most common detected. Sapovirus GI.1 was more frequently detected in AGE than in routine stools (47.2%, 43/91 vs 25.6%, 10/39; $p = 0.005$) and re-infection with the same genotype was uncommon.

**Conclusion.—**The first sapovirus infections occurred around 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 following natural infection.

### Keywords

Sapovirus; birth cohort; timing of infection; re-infection; genotype distribution; natural protection

## INTRODUCTION

Acute gastroenteritis (AGE) is an important cause of global mortality accounting for 9% of all deaths in children under five years of age[1]. The global burden of rotavirus-associated AGE declined following the widespread introduction of rotavirus vaccines, making caliciviruses, such as norovirus and sapovirus, the leading cause of pediatric 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 of age 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 2 years of age[4]. In Central America, the prevalence of sapovirus ranges from 7% and 17%[5–7]. Despite this high disease burden, little is known about the natural history of sapovirus infections, including the dynamic of re-infections and natural induced protection.

Sapoviruses are genetically diverse single-stranded RNA viruses belonging to genogroup I (GI), GII, GIV and GV infect humans[8,9]. Viruses in these 4 genogroups can be further divided into 18 genotypes (GI.1–7, GII.1– 8, GIV.1, GV.1–2) with GI.1 viruses reported most frequently globally[10]. To date, few studies have examined and genotyped sapovirus in stool from asymptomatic children[11]. In the Peruvian birth cohort previously mentioned, GI sapoviruses were found to be more common in symptomatic children, while 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 characterize the timing of symptomatic and asymptomatic sapovirus infections, determine the sapovirus genotypes, and characterize 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 following natural infection.

## MATERIALS AND METHODS

### Study design.

The Sapovirus-Associated Gastro-Enteritis (SAGE) 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 at the third trimester of their pregnancy and new-born infants were enrolled within 14 days after delivery. Prior to enrolment all mothers signed an informed consent. Enrolled children were monitored weekly for AGE symptoms until 2 years of age. AGE was defined as an increase in stool frequency to at ≥3 stools per 24-hour period or a substantial change in 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 prior to onset of AGE symptoms [13,14]. Stool samples and clinical data were collected for each AGE episode and routine stool were collected monthly.

### Study procedures.

Stool from AGE episodes were collected within 4 days post-symptoms 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 1 sapovirus AGE episode, were selected to investigate timing of infections, the 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 reverse transcription polymerase chain reaction (RT-qPCR).

Sapovirus screening in the AGE episodes was performed in Nicaragua and sapovirus detection in routine stools was performed at CDC, both laboratories follows the method described by Oka and co-workers [15]. In brief, viral RNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) 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 sapovirus-positive if the Ct value was ≤ 35.

### Sapovirus genotyping.

Sapovirus RT-qPCR positive samples were amplified by a hemi-nested conventional RT-PCR [16] that target the NS region of the capsid gene. The RT-PCR products were purified by ExoSAP-IT (Affymetrix, USB, Cleveland, OH, USA) or by QIAquick PCR purification kit (Qiagen) and submitted for Sanger sequencing (Eurofins MWG Operon, Louisville, KY, USA). Sequences were typed using the online human calicivirus typing tool [17]. The nucleotide (nt) sequences homology from the children experiencing consecutive infections with the same genotype was calculated by 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% confidence interval (95% CI). For categorical variables, we quantify the number and percent in each category, and compare differences between asymptomatic and symptomatic sapovirus AGE episodes using 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 was $p < 0.05$. We used multiple imputation to estimate sapovirus incidence including symptomatic stools that were not collected from approximately 10% of AGE episodes, assuming 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 was used for figures.

## RESULTS

### Epidemiological profile of sapovirus AGE in children ≤ 2 years of age.

Sapovirus AGE was observed in 93 (26.7%) of the 348 children who completed 2 years of household weekly surveillance (Figure 1A and B). More infections occurred in the second year of life (62.4%, 58/93) compared to the first year with higher frequencies of detection between 6 – 9 and 18 – 23 months of age (Figure 1B). Sapovirus 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 (Figure 2). Sapovirus was detected in 27.6% (91/330) of the specimen collection during AGE episodes and in 2.9% (39/1350) of the routine stool specimens (Figure 3). Among the 67 children, 38 (56.7%) experienced symptomatic (n = 19) or asymptomatic (n = 19) re-infections (Figure 4A). Monthly routine stool specimens collected from 9 (13.4%) children tested positive before their first sapovirus AGE episode. (Figure 4A). Of notice, while 29 (43.2%) of children experienced only one symptomatic infection during 2 years of life, 18 (26.9%) experienced 3 or more infections, among which one child experienced 5 sapovirus AGE episodes all infections were of a different genotype (Figure 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) and 18.4 months (n = 19; 95% CI: 12 – 19.4, $p = 0.001$) for their second symptomatic infection (Figure 4B). Three children (subjects 14, 19 and 28 in figure 4B) experienced a 3$^{rd}$ 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 the 67 children with ≥2 infections (Figure 4B). There was no difference between the age at which children had their first symptomatic or asymptomatic infection (median of 11.2 vs 11.5, p ≥ 0.05) (Figure 3B). The time elapsed between an asymptomatic infection followed by the 1st sapovirus AGE episode was 6.2 months, similar to the observed time between 2 consecutive symptomatic infections (1$^{st}$ and the 2$^{nd}$ sapovirus-AGE, 7.2 months).

### Genotype diversity in symptomatic and asymptomatic sapovirus infections.

Of the 130 sapovirus infections (91 from AGE episodes and 39 from routine stool), 108 (83%) were successfully genotyped into 4 genogroups (GI (64%), GII (27%), GIV (2%) and GV (7%)) and 8 genotypes (Figure 3). GI viruses, primarily GI.1, were more commonly detected in AGE stools compared to the routine stool (47.2%, 43/91 vs 25.6%, 10/39; $p = 0.005$), while GII.1 viruses were more frequently found in asymptomatic monthly stool specimens (10.2% vs 2.2%, $p \geq 0.05$) (Figure 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, Figure 4A). No asymptomatic sapovirus infection was detected in 29 (43.2%) of the 67 children with at least sapovirus AGE episode (Figure 4A). Of note, in 27% of the sapovirus-positive routine stool no genotype could be determined due to poor sequence quality or low viral load. Co-infections with multiple genotypes was not observed in this study. Pairwise alignment of the nt sequences from children experiencing consecutive infections with the same genotype within 42 days showed 100% nt homology, with 2 exceptions (Subjects 5 and 29).

## DISCUSSION

We used clinical data and stool specimens from a birth cohort study (SAGE cohort) carried out at the community level in Nicaragua to determine the timing of symptomatic and asymptomatic sapovirus infections in the first two 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. Following rotavirus vaccine introduction, 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 following the rotavirus vaccine implementation in Nicaragua, respectively [21] [12,5].

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 about 6 months, suggesting that children may develop broad short-live immunity against sapovirus. These observations may have implications for future vaccine strategies.

Comparable to data from previous studies, sapovirus infections were rarely observed during the first 5 months of life [4,22,23], probably due to 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 titers as has been reported for norovirus [27,28]. Finally, in 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 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 as compared to 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 4 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]. In here, repeated AGE episodes of the same genotype were very rare, suggesting genotype-specific immunity.

Our data also contributes 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 1,350 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 two years of age[37]. Children in a day care centre in Denmark experienced sapovirus symptomatic and asymptomatic infections year-round [38]. Altogether suggesting that 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 two years of life, and 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 reinfections. Second, due to low viral load and poor nt 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 about 23 days, while 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 following 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 acute gastroenteritis in children in both low- and high-income settings[20, 40], which is supported by our study. As sapovirus GI.1 is consistently the most common genotype and associated with symptomatic disease and infection might results 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[41].

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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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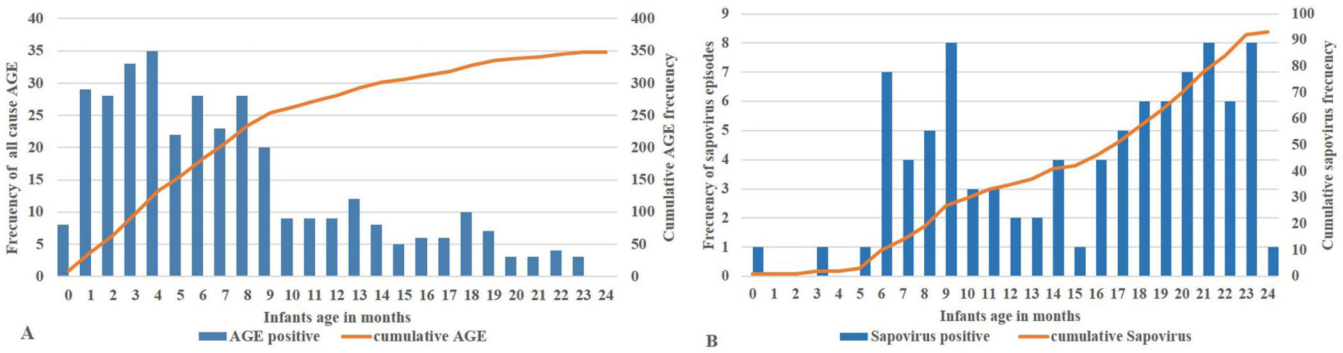


**Figure 1.**

Cumulative incidence of the 1st acute gastroenteritis (AGE) episode (n=348) and 1st sapovirus-AGE episode per month in children up to 2 years of age (n = 93) enrolled in the SAGE birth cohort in Nicaragua. The bar scale indicates the number of AGE (A) and sapovirus-AGE episodes (B). The secondary Y axis represents the monthly cumulative incidence of AGE (A) and sapovirus-AGE episodes (B).

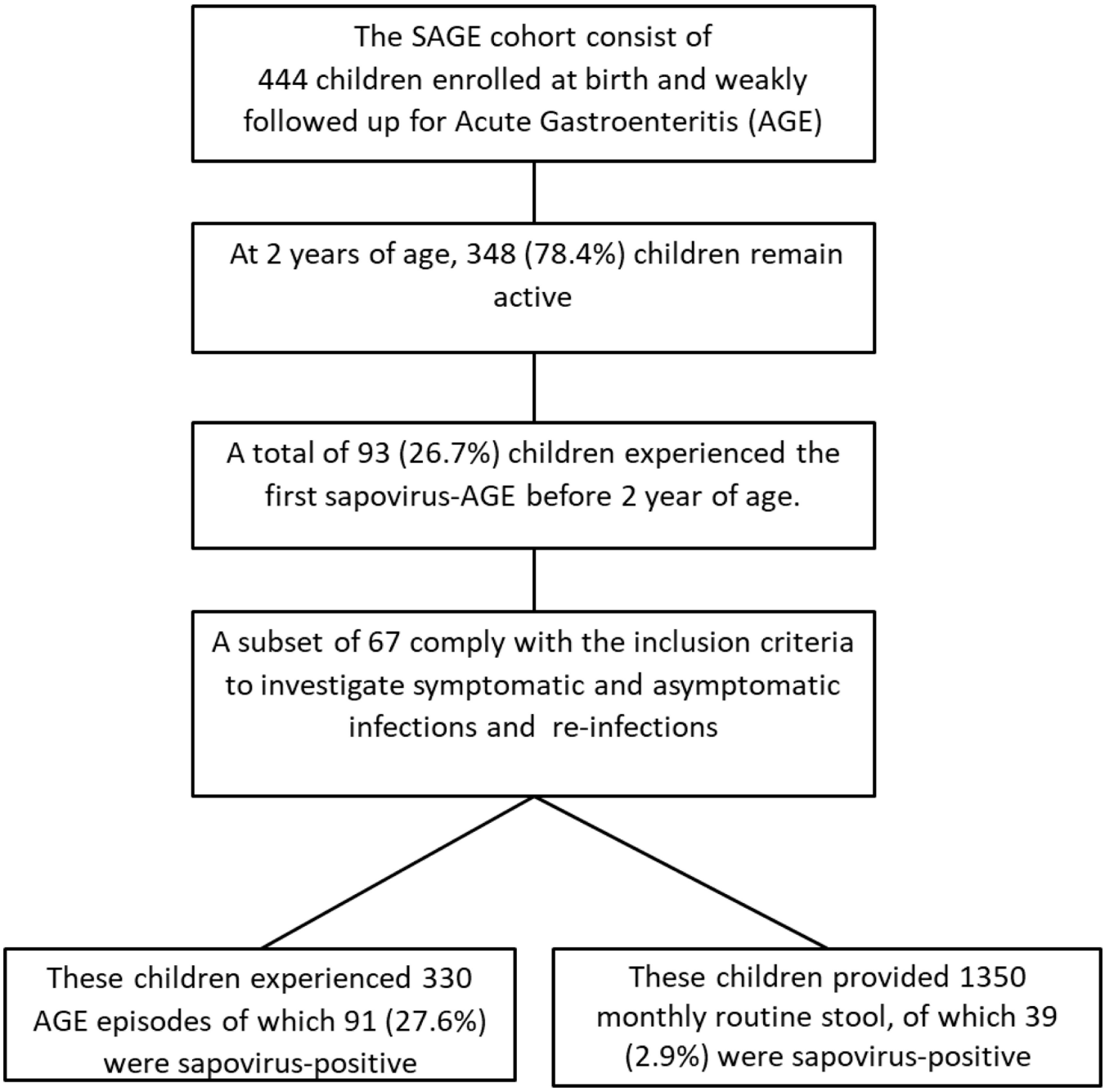


**Figure 2.**
Flowchart of the subset from the SAGE birth cohort and samples analyzed in this study.

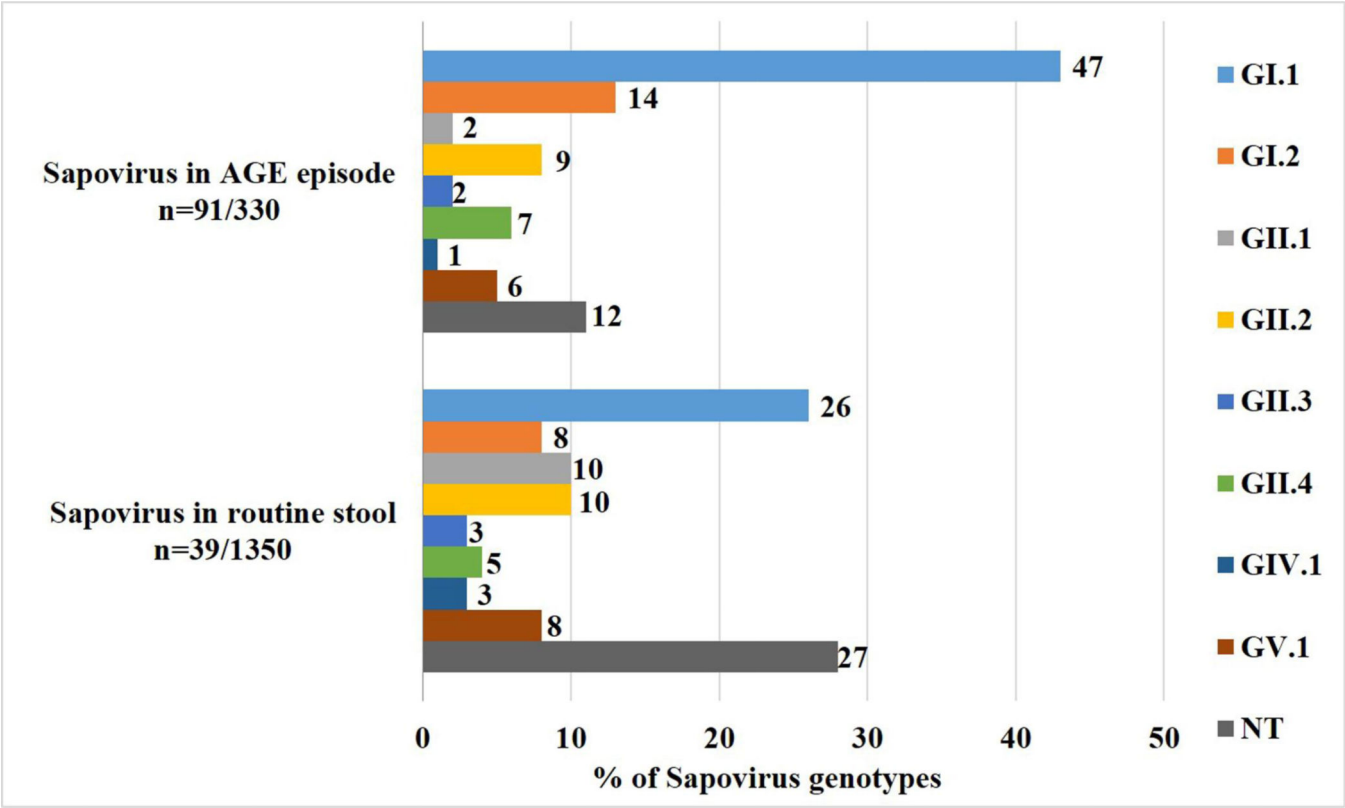


**Figure 3.**

Distribution of sapovirus genotypes in AGE episodes and routine stool samples from 67 sapovirus-positive children from the SAGE birth cohort followed from birth up to 2 years of age. NT refers to ‘not typed’.

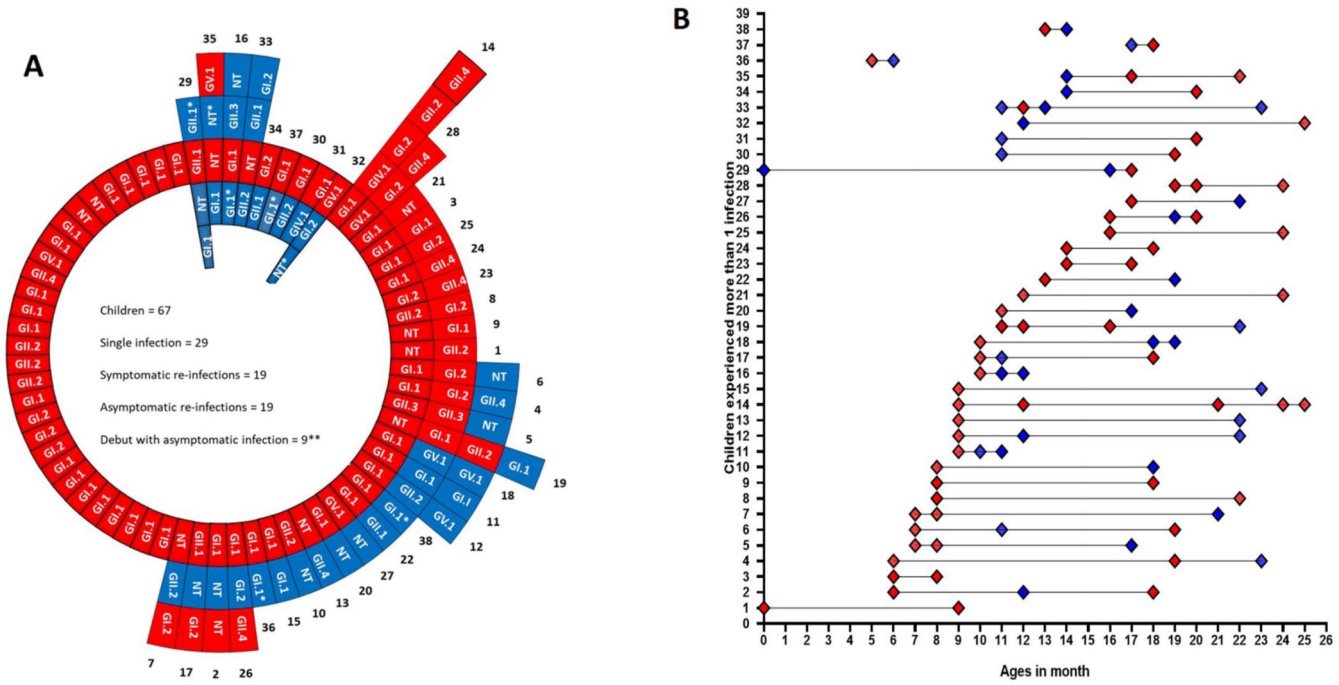


**Figure 4.**

Chronological order and timing of sapovirus genotypes infections until 2 years of age. Figure 4A. Sapovirus genotypes of 130 infections in 67 children. Circles order is chronological, with inner circle representing debuting infection and external circle consecutive infections. Red and blue squares represent symptomatic and asymptomatic infections, respectively, and numbers represent the subjects ID. *Indicate that consecutive samples were collected <23 days, and NT refers to 'not typed'. **Children (n = 9) debuting with asymptomatic infection are also included in the re-infection group. Figure 4B (n=38): Red and blue dots represents symptomatic and asymptomatic infections. Y and X axes show children that 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 in Figure 4B.

**Table 1.**

Distribution of sapovirus genotypes in the first and secondary symptomatic infections in children until two 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 | | | | | 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]3 children experienced more than 2 re-infections