HHS Public Access

Author manuscript

J Med Virol. Author manuscript; available in PMC 2022 April 04.

Published in final edited form as:

J Med Virol. 2019 May ; 91(5): 738–743. doi:10.1002/jmv.25380.

Molecular epidemiology of noroviruses in children under 5 years of age with acute gastroenteritis in Yaoundé, Cameroon

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Abstract

Norovirus is a common cause of acute gastroenteritis (AGE) among children in developing countries. Limited data on the prevalence and genetic variability of norovirus are available in Cameroon, where early childhood mortality due to AGE is common. We tested 902 fecal specimens from children younger than 5 years of age hospitalized with AGE between January 2010 and December 2013. Overall, 76 (8.4%) samples tested positive for norovirus, of which 83% (63/76) were among children below 12 months old. Most of the noroviruses detected were in children infected between July and December of each year. All norovirus-positive specimens were genotyped, with 80% (61/76) being GII.4 (three variants detected). Genotypes GI.2, GI.6, GII.1, GII.2, GII.3, GII.6, GII.16, GII.17, and GII.21 were also detected. Interestingly, GII.4 Sydney and GII.17 Kawasaki viruses were found as early as 2010, years before their emergence globally. This study suggests norovirus is a significant cause of moderate to severe gastroenteritis among young children in Cameroon. The results are important to highlight appropriate prevention and control strategies for reducing the burden of norovirus disease.

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The findings and conclusions in this study are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Keywords

acute gastroenteritis (AGE); Cameroon; genotypes; norovirus

1 | INTRODUCTION

Norovirus is the leading cause of human viral gastroenteritis globally, being responsible for more than half of the gastroenteritis outbreaks that occur annually.[1] It is the second largest contributor to severe childhood gastroenteritis in low resource countries where they cause an estimated 71 000 deaths in children below 5 years of age.[2,3] Its high morbidity in developing countries makes norovirus infections an important public health issue with a substantial socioeconomic burden. In Cameroon, diarrheal disease is ranked third as a cause of mortality in young children, accounting for about 14 400 deaths per year.[4]

Noroviruses are nonenveloped positive-stranded RNA viruses that belong to the family *Caliciviridae* and are classified into at least seven genogroups (GI-GVI).[5] Most infections in humans are caused by GI and GII viruses, which can be further divided into at least 9 and 22 genotypes, respectively.[5] Genogroup II, genotype 4 (GII.4) viruses are of particular importance as they have been associated with norovirus pandemics since the mid-1990s. New GII.4 variants have emerged, causing distinct global epidemics over the last two decades including GII.4 US 1995/96 in the mid-1990s, GII.4 Farmington Hills in 2002, GII.4 Hunter in 2004, GII.4 Den Haag 2006b in late 2006, GII.4 New Orleans in 2009, and the current predominant GII.4 strain in circulation, GII.4 Sydney, in 2012. However, recently different genotypes (GII.17 in 2014/2015 and GII.2 in 2016–2017) emerged in China, Japan, and South Korea and replaced GII.4 strain predominance, at least temporarily.[6–9]

Data on norovirus prevalence and the distribution of genotypes are available for many Sub-Saharan African countries such as Ghana, Tanzania, Nigeria, Gabon, Burkina Faso, Malawi, Botswana, and South Africa.[1,10–12] In Cameroon, norovirus has been detected in stools of asymptomatic children and HIV-infected adults,[13,14] but there are no studies investigating norovirus infections among children hospitalized with acute gastroenteritis (AGE). Thus, there is a lack of information on norovirus genotypes associated with diarrhea in Cameroon. In this study, we determined the stool prevalence and molecular epidemiology of norovirus in hospitalized children under 5 years of age in Yaoundé, Cameroon between 2010 and 2013.

2 | MATERIALS AND METHODS

2.1 | Case definition, patients and sample collection

This study used samples collected as a part of the Cameroon Rotavirus Sentinel Surveillance Program which was approved by the Cameroon Ministry of Public Health and supported by WHO/AFRO as part of the WHO Rotavirus Sentinel Surveillance Program.[15] Written informed consent was obtained from the parents of the children who participated in the program, as per the WHO/AFRO rotavirus surveillance protocol.[15] The WHO case definition of gastroenteritis, the occurrence of at least three looser than normal or watery

stools in a 24 hours period and/or two or more episodes of vomiting unexplained by other reasons, was used.[15] From January 2010 through December 2013, 2831 stool specimens were collected within 48 hours of admission from children below 5 years with acute diarrhea to sentinel hospitals. Patients were recruited from ten health districts (BiyemAssi, CitéVerte, Djoungolo, Efoulan, Ebolowa, Nkolndongo, Ntui, Mfou, Obala, and Okola) in Yaoundé and stool samples were sent to the Mother and Child Centre of the Chantal Biya Foundation hospital. A total of 902 (32%) stool samples were randomly selected from all specimens based on the year of collection. Additional clinical data were also available for the specimens and included child age, sex, classification of dehydration status (mild, moderate, or severe),[16] and the presence of fever (temperature greater than 37.5°C).

2.2 | RNA extraction, detection by RT-PCR, and sequencing

Viral RNA was extracted from 140 μL of 10% clarified stool suspensions using the QIAampViral RNA Mini Kit, (Qiagen, Inc, Valencia, CA) following the manufacturer's instructions and stored at −80°C until use. Conventional RT-PCR was performed using the primers sets G1SKF/G1SKR and Ring2/G2SKR[17–19] to amplify a region of the 5′end of the ORF2 gene using the Qiagen One Step RT-PCR kit (Qiagen, Hilden, Germany). The final volume for each reaction was 25 μL. Each reaction contained 5.0 μL of 5x buffer; 1.0 μL of 10 mM dNTPs; 0.5 μL 10 μM each of mixed primers for G1SKF (CTG CCC GAA TTY GTA AAT GA), GISKR (CCA ACC CAR CCA TTR TAC A), Ring2 (TGG GAG GGC GAT CGC AAT CT), and G2SKR (CCR CCN GCA TRH CCR TTR TAC AT); 1.0 μL of Enzyme mix (RT and Taq DNA polymerase (5 U/μL), 0.5 μL of Rnase inhibitor (20 U/μL), and 10.5 μL of nuclease-free water. The amplification conditions were set as follows: reverse transcription at 42°C for 30 minutes, then 94°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute, and an extension step at 72°C for 10 minutes. Resulting PCR products were visualized on a 2% agarose gel (Seakem-ME, Lonza, Allendale, NJ) prepared with 10% Gel Red (Biotium, Fremont, CA). Amplicons of the expected size (330 bp for GI and 341 bp for GII viruses) were gel-purified with the QIAquick Gel Extraction kit (QIAGEN GmbH) and sequenced by Sanger sequencing (Eurofins MWG Operon, Louisville, KY).

2.3 | Phylogenetic analysis

Sequences were aligned and trimmed using Assembler (BioNumerics version 5.10; Applied Maths Inc, Austin, TX). The genotypes were determined using an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree and Genbank norovirus reference sequences. Nucleotide sequences were compared with sequences of prototype norovirus strains from GenBank using multiple alignments generated by ClustalW and phylogenetic trees were constructed by Neighbor-Joining using MEGA7 software.[20] A bootstrap of 1000 replications was used to determine the significant differences between strains. The evolutionary distances were computed using the Kimura 2-parameter method, reporting the number of base substitutions per site. All nucleotide sequences from this study are available upon request.

3 | RESULTS

3.1 | Clinical and demographic characteristics

Of the 902 randomly selected stool samples collected from patients with AGE in Cameroon between January 2010 and December 2013, 76 (8.4%) tested positive for norovirus (Table 1). The majority of the specimens selected (92%; 830/902) and testing positive (89.5%; 68/76) originated from four locations (BiyemAssi, CitéVerte, Djoungolo, and Nkolndongo; Table 1). Across the health districts, between 1 and 233 specimens (median 7.4) from the original specimen collection were tested and between 0% and 50% (median 8.4%) were norovirus positive. The individual positivity ratios of the districts where fewer than 150 specimens were tested did not differ significantly from the four districts with more than 150 specimens screened or the overall ratio of norovirus-positive specimens (mean 8.5%; SD 2.7%; 95% CI, 4.2%–12.8%; z-tests each with $P \geq 0.22$). Among the norovirus-stool samples, 61.8% (47/76) were from males and 38.2% (29/76) were from females (binomial test; $P = 0.09$). Vomiting was reported for 92.1% (70/76) of the children. Forty-three (69.4%) of the 62 children reported fever with a median temperature of 38.7°C (IQR 38.2°C–39.0°C). Dehydration was recorded for 76.3% (58/76) of the children with norovirus AGE and could be split into mild (60.3%; 35/58), moderate (24.1%; 14/58), and severe (15.5%, 9/58). The median age of children with norovirus-associated diarrhea was 7 months (IQR = 5–10 months), with the majority (97.4%; 74/76) under 24 months of age. Approximately 53.9% of children with a positive stool were 6 to 11 months old; 28.2% were younger (0–5 months) and 17.1% were older (12–59 months; Table 2). Coinfection with rotavirus was detected in 9 norovirus-positive samples. The median age for children with rotavirus coinfection was 8 months (IQR 4–10 months). All but one child with coinfection had a fever and over half had either moderate or severe dehydration.

3.2 | Genotyping

A genotype was determined for all 76 norovirus-positive specimens. There were two (2.6%) GI and 74 (97.4%) GII viruses detected; none of the samples tested positive for both GI and GII. A total of 10 genotypes and three GII.4 variants were detected in the four years of the study (Figure 1A and 1B and Figure 2). GII.4 was most common, representing 60% (9/15) of strains in 2010, 90% (9/10) in 2011, 86.2% (25/29) in 2012, and 82% (18/22) in 2013. In 2010, three GII.4 variants were detected and included Apeldoorn (11%, 1/9), New Orleans (11%, 1/9) and Sydney (78%; 7/9). Among 2010 norovirus-positive specimens, GII.1 (7%, 1/15), GII.3 (13.3%; 2/15), GII.16 (7%; 1/15), and GII.17 (13.3%; 2/15) types were also identified. Genotype GII.4 Sydney was the only variant among GII.4 positive samples detected in subsequent study years from 2011–2013. In 2011, GI.2 (10%, 1/10) was the only other genotypes other than GII.4. Other norovirus strains detected included GI.6 (4%; 1/29) and GII.21 (12%, 3/29) in 2012, GII.2 (4.5%, 1/22), GII.6 (4.5%, 1/22), GII.16 (4.5%; 1/22), and GII.21 (4.5%; 1/22) in 2013.

3.3 | Seasonality

Noroviruses were detected in every month of the year except March (Figure 2). Seasonality could only be examined for GII.4 viruses since other genotypes were detected infrequently. There was no difference between the yearly percentage of GII.4 positive samples collected

during the yearly wet (April-September; mean 47.5%) and dry (October-March; mean 52.5%) seasons (*t* test of equivalence to 50% each resulted in $P > 0.45$). There were more GII.4 positive samples detected during the second half of the year from July through December (mean 73.2%; 95% CI, 65.4%–81.0%) than from January through June (mean 26.8%, 95% CI, 19.0%–34.6%; Student *t* test; $P < 0.0001$). Most GII.4 norovirus strains were detected in November (23%; 14/61), although annual fluctuations in the percentage of norovirus-positive specimens detected in November (mean 27%; 95% CI, 5%–48%) were not statistically significant when compared to a hypothetical monthly frequency of 8.3%, representing 1/12 of the calendar year (*t* test; $P = 0.07$).

4 | DISCUSSION

We detected norovirus in 8.4% of diarrheal stools from hospitalized children under five years of age with sporadic cases of AGE collected from January 2010 to December 2013 in Cameroon. These rates are in the same range as those reported in systematic reviews of noroviruses in Africa with 11% (95% CI, 8%–14%) prevalence reported across the entire African continent and 12.6% (range 4.6%–32.4%) in Sub-Saharan Africa (both symptomatic and asymptomatic cases) between 1990–2013.[10,12] A mean overall prevalence of 13.5% (range 0.8%–25.5%) was reported among children with AGE (most associated with hospitalization or outpatient treatment) in longitudinal studies conducted from 1976–1979 and 1997–2013.[11] Our data, together with the data reported in these reviews, show that norovirus prevalence in Africa is similar to the range reported among all age groups globally (18%, 95% CI, 17%–20%), and those reported among high-mortality developing countries worldwide (14%, 95% CI, 11%–16%).[1]

Globally, 70% of norovirus cases occurred among children 6–23 months of age,[21] a similar proportion (68%) of norovirus-associated stools were from children in this age category in our study. The proportion of positive stools (29%) which came from younger infants (0–5 months of age) was in range with those reported in SouthAfrica and Angola.[12,22] Similar to reports from other Sub-Saharan African countries, a substantial proportion of norovirus-positive stools (83%) were from children below 12 months old in Cameroon.[10,12,21–23]

In agreement with previous reports in Africa and worldwide, GII viruses were more frequently detected than GI viruses and there was heterogeneity of genotypes in circulation in Cameroon.[10,11] Ten norovirus genotypes and three GII.4 variants were detected in this study. Only two samples were confirmed to be GI viruses (GI.2 and GI.6), and the GI.3 viruses, thought to have a widespread distribution in Africa,[11] were not detected in this study. This may be due to the sampling strategy or the year of collection. Each year, two to four genotypes cocirculated and GII.4 viruses were detected most frequently. GII.21 viruses were found in the stools of four children over a three-month period (November 2012 to January 2013). While infrequently detected in diarrheal stools worldwide, infections by genotype GII.21 viruses have been reported with increased frequency in Bhutan in 2011–2012[24] and in South Korea during the same period of time.[25] Genotype GII.17 Kawasaki viruses, which emerged and caused pandemics in China, Japan, and Korea in 2014–2015, were detected in two samples from December 2010 in this study.[6,7,26] Apart from two samples in 2010, all GII.4 viruses detected were the Sydney 2012 variant. Interestingly, like

GII.17 Kawasaki viruses were detected in this study, GII.4 Sydney was detected two years before the emergence of this variant globally. Sporadic detection of these viruses and other GII.4 variants in stools and environmental samples from communities in Africa before the epidemic or pandemic activity has been reported previously, suggesting potential reservoirs from which these new variants could emerge.[11] Improvements in norovirus surveillance capabilities in Cameroon, as well as in other developing countries in Africa and globally will enhance our ability to investigate this possibility.

Unlike in the temperate northern hemisphere, where norovirus infections most commonly peak during winter months and are driven by GII.4 viruses,[27] in Africa, the seasonal distribution of noroviruses varies between different countries.[10,11] A previous study in Cameroon showed that norovirus seasonality (asymptomatic infections) peaked between June and August, which coincides with an increase in rainfall.[28] In this study, norovirus detection was not positively associated with the wet season (April-September). However, for unknown reasons, most of the norovirus-positive diarrheal stools were detected during the second half of the calendar year. Yaoundé has a tropical climate with constant temperatures (23°C–26°C) throughout the year and relative humidity reaching as high as 86%. Further study on norovirus seasonality in Cameroon is warranted as there may be an interaction between rainfall, temperature, and relative humidity as described in a recent study.[29]

The current study investigated norovirus prevalence in the stools of children seeking medical treatment for diarrhea, but attributing norovirus as the cause of diarrhea was not possible because coinfection by other pathogens was not tested and because noroviruses are known to be shed asymptomatically. Two studies conducted previously in Cameroon (in a different region of the country) found that approximately 30% of samples from healthy children tested positive for norovirus.[13,14] This is a greater proportion of samples testing norovirus positive than in our study. Differences in study design and years investigated, underlying differences in the populations studied, regional climatic conditions, and levels of sanitation between these regions of Cameroon may contribute to differences in asymptomatic norovirus infections. Further study including a more robust study design to better represent the population is needed to address disease attribution and report norovirus incidence estimates in Yaoundé children. In addition, genetic sequencing was only performed on the capsid region for viruses in this study, therefore the detection and characterization of recombinant strains, which can be the source of norovirus epidemics, was not assessed. Lastly, only a portion (32%; 902/2831) of the collected specimens was tested. Testing all the specimens would have provided a clearer picture of norovirus-associated diarrhea in Cameroon.

This report shows that norovirus was present in a substantial proportion of stools from children with acute pediatric gastroenteritis in Cameroon. Genotype GII.4 noroviruses prevailed, but genotypic diversity was high during the years of this study. Higher proportions of children under 1 year of age were infected, providing support for a vaccination strategy targeting infants. This is the first study to investigate noroviruses in Yaoundé, Cameroon. Larger-scale community and/or hospital-based epidemiological studies, as well as molecular surveillance, are needed to better characterize the incidence and molecular epidemiology of noroviruses in pediatric populations of the country. Results from such studies will

provide evidence to inform policy makers and health care professionals on the importance of noroviruses in diarrheal illnesses and deaths among Cameroonian children and support improving prevention and control strategies.

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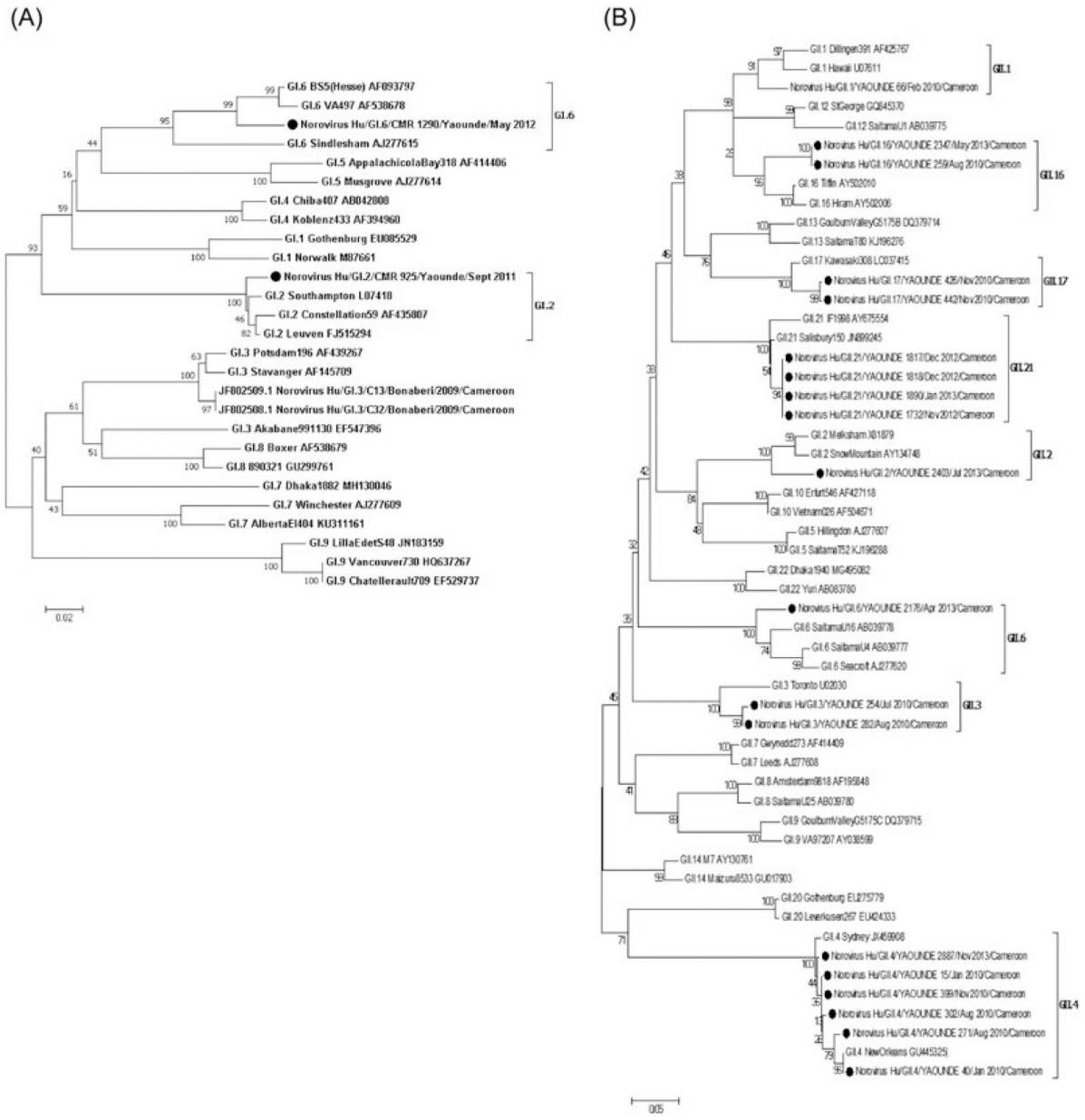

FIGURE 1.

A. Maximum likelihood phylogenic analysis of GI norovirus sequences (region C) detected from 2010 to 2013 in Yaoundé, Cameroon. Phylogenetic trees were constructed using the Neighbor-Joining method with bootstrap support (1000 replications) and evolutionary distances were computed using the Kimura 2-parameter method. Representative samples from this study are indicated with dark circles. Reference sequences were used for comparison and are indicated with GenBank accession numbers. Figure 1B. Maximum likelihood phylogenic analysis of GII norovirus sequences (region C) detected from 2010 to 2013 in Yaoundé, Cameroon. Phylogenetic trees were constructed using the Neighbor-Joining method with bootstrap support (1000 replications) and evolutionary distances were computed using the Kimura 2-parameter method. Representative samples from this study are

indicated with dark circles. Reference sequences were used for comparison and are indicated with GenBank accession numbers

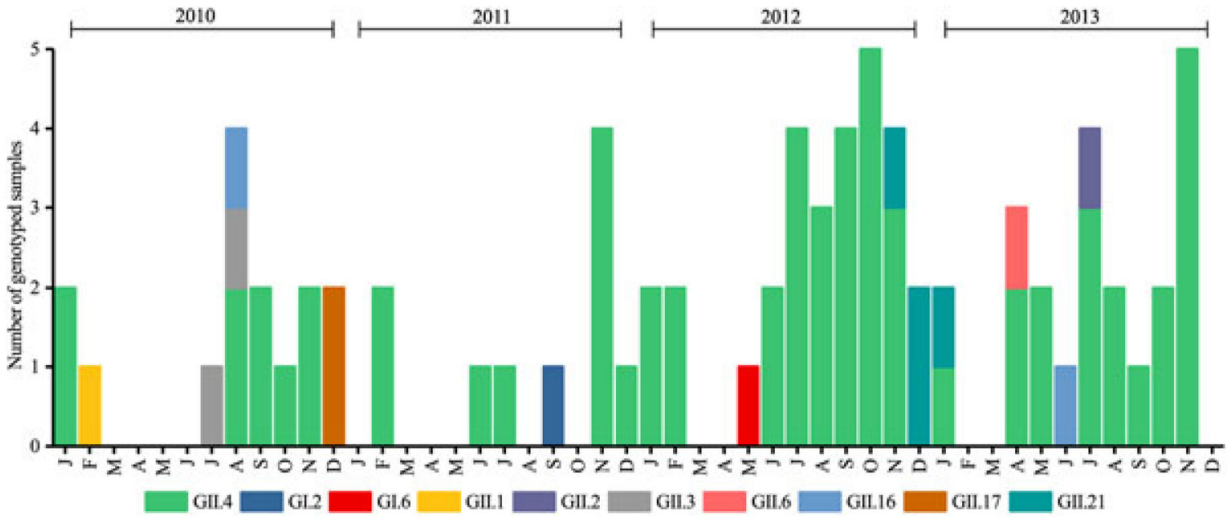

FIGURE 2.

Monthly distribution of norovirus genotypes detected from 2010–2013 in Yaoundé Cameroon

TABLE 1

Number of samples tested and proportion testing norovirus-positive in each Health District

Health district	Tested	Positive	% Positive
BiyemAssi	152	18	11.8
CitéVerte	233	13	5.6
Djoungolo	222	21	9.5
Efoulan	32	4	12.5
Ebolowa	3	1	33.3
Nkolndongo	223	16	7.2
Ntui	2	1	50.0
Mfou	8	1	12.5
Okola	7	1	14.3
Obala	11	0	0.0
Monatele	1	0	0.0
Ngaoundere	1	0	0.0
Soa	5	0	0.0
Bertoua	2	0	0.0
Total	902	76	8.4

TABLE 2

Number of samples tested, proportion testing norovirus-positive, and distribution of positive samples across age categories

Age category	0–5 mo	6–11 mo	12–23 mo	24–59 mo
Total number of samples tested	274	373	200	55
Number testing positive	22	41	11	2
Percent positive per age category	8.0	11.0	5.5	3.6
Percent positive across age categories	28.9	53.9	14.5	2.6