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ABSTRACT

Background: Acute diarrhea is a leading cause of morbidity and mortality among children under five worldwide. As no published data is available on the occurrence of this infection in the Republic of Congo, this study aimed at (1) determining the prevalence and (2) characterizing genotypes of norovirus strains in Brazzaville.
Methods: From June 2012 to June 2013, stool samples were collected from hospitalized young children with acute gastroenteritis. A total of 545 samples were tested for GI and GII norovirus infections using nested duplex reverse-transcription–polymerase chain reaction and sequencing.
Results: The GI and GII norovirus infection were detected in 148 samples. Males (28%) were not significantly more infected than females (25%). Norovirus infection was found exclusively in children aged under 24 months with a higher prevalence (P = 0,048) in the age group of 7–12 months, and throughout the year with a peak in August and September. Genetic diversity of norovirus strains revealed that GII was the most prevalent (87%). No risk factor was significantly associated with norovirus infection.
Conclusion: This study showed that noroviruses are important agents responsible for acute diarrhea in Congolese children and highlights the importance of continued surveillance.

Introduction

Acute gastroenteritis (AGE) is one of the common diseases among children under five years old worldwide (WHO, 2013; Mitui et al., 2014; Oh et al., 2003; Huynen et al., 2013; Qazoui et al., 2014) with a higher burden in sub-Saharan Africa, India, and Pakistan (Tate et al., 2012).

Among pathogens responsbile for diarrheal diseases (Aragão et al., 2010; Ayolabi et al., 2010; Moyo et al., 2011) enteric viruses like rotavirus, norovirus, adenovirus 40/41 and astrovirus are the most identified causing massive inflammation of the gastrointestinal tract membranes leading to frequent diarrhea and/or vomiting (De Rougemont 2011; Qazoui et al., 2014). Therefore, characterization of circulating virus strains in different geographical areas is a key component towards effective vaccine development.

Following rotavirus, norovirus infcetion is considered the second most common cause of acute gastroenteritis (De Rougemont 2011; Qazoui et al., 2014) in children under five years, with a prevalence ranging between 6 % and 39 % in sub-Saharan Africa (Munalula, 2015).

Noroviruses belong to the Caliciviridae family, non-enveloped, icosahedral capsid viruses that contain a single-stranded positive-sense RNA of about 7,500 nucleotides in length, organized into three open reading frames (ORF). The ORF1 encodes a non-structural polyprotein, including RNA-dependent RNA polymerase, a highly conserved region used for the diagnosis and identification of NoV. ORF2 and ORF3 encode the major capsid protein (VP1) and the minor structural protein (VP2) respectively. Noroviruses are composed of five genogroups (GI - GV), those detected in humans

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belong to two main genogroups, I and II, and each genogroup is further subdivided into several genotypes (Green et al., 2000; De Rougemont 2011; Qazoui et al., 2014).

The genogroups I and II respectively comprise 8 and 21 genotypes. Among them, NoV GII.4 was the most prevalent genotype worldwide. The transmission of norovirus occurs through the fecal-oral route either by direct contact with infected individuals or by contaminated surfaces, and is marked by seasonality, with a peak in colder seasons (Ayukekbong et al., 2014; Fretz et al., 2005).

In the Republic of Congo, diarrheal diseases causing 7.5% of deaths are considered as the third cause for children's consultation in healthcare facilities, after malaria and respiratory infections (WHO Africa, 2005; Liu et al., 2015). Before the introduction of rotavirus vaccine in the country in 2014, a first investigation conducted among a population of hospitalized children in Brazzaville showed a prevalence of 46.4% and 5.5% of rotavirus and adenovirus infections, respectively (Mayindou et al., 2016) but so far, no published data on norovirus infection in the country.

The aim of the present study is (1) to determine the prevalence of norovirus infection among Congolese children hospitalized for severe diarrhea; (2) to characterize the norovirus strains detected in stool samples, and (3) to identify risk factors associated with norovirus infection.

Materials and methods

Ethical approval and consent

This work was approved by the institutional ethics committee of the Fondation Congolaise pour la Recherche Médicale. The written informed consent was obtained from the parents or guardians of these children prior to the start of the patient enrollment.

Study location

This study was conducted in Brazzaville, the capital of the Republic of Congo, located in the Central Africa. The average yearly rainfall ranges from 1100 mm in the south to over 2000 mm in the central and north parts of the country. The rainy season, which lasts 9 months, has two rainfall maxima: one in March–May and another in September–November (Samba et al., 2008). The dry season is from June to August (Koukouikila-Koussounda and Ntoumi, 2016). This study was conducted within the framework of the gastroenteritis surveillance organized by the Ministry of Health in Brazzaville before the initiation of the rotavirus vaccination program. The study took place at Makelekele hospital in the Southern area of Brazzaviile.

Sample collection

The study design refers to the previous and published study conducted in the same children (Mayindou et al., 2016). From June 2012 to June 2013, stool samples were collected from children under five years old hospitalized for acute diarrhea. After obtaining informed consent from parents or guardian, socio-demographic and clinical data were collected. A stool sample was collected from each child within 48 h after admission into the hospital. Stool samples were transported at the laboratory at 4 °C in an icebox and stored at −80 °C until analyses. Children admitted I> 48 h after admission were excluded from the study.

RNA extraction and norovirus detection

Norovirus infection was assessed using nested RT-PCR with norovirus specific oligonucleotide primers, and tested for genogroups GI and GII. Every stool sample was suspended in 500 µl ultrapurified water and centrifuged at 3000 rpm for 5 min. The supernatant was moved into a new tube and the RNA extraction was carried out using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), accordingly to the manufacturer's instructions. The extracted RNA was either stored at −80 °C or immediately used for the detection analyses. Molecular detection of GI and GII Norovirus was carried out by duplex nested RT-PCR. In order to detect a broad range of sequence variants of norovirus genomes, primer systems were complemented as follows: For the first round (RT/1.PCR) of amplification, an equimolar mixture of sense primers NV1a and NV1b was used in combination with antisense primers NV7 and NV7a. The second round (2.PCR) reaction was carried out using the antisense primer mixture NV4, NV4a and NV4c and the sense primer mixture NV6 and NV6a. A complete overview of the primers used is given in Table 1. The first round was carried out with the One-step RT-PCR kit (Qiagen) and the second round with the Hot Star Taq Master Mix Kit (Qiagen) with GI and GII specifics primer systems (showed hereinbefore) targeting the RdRp region as previously described by Oh et al. (2003). All PCR products were examined by electrophoresis in 100 ml of 1.5% agarose gel stained with SYBR Green. Positive samples showed one band at 338pb.

Norovirus genotyping and phylogenetic analysis

The RT-PCR products were purified using Sephadex TM G-50 fine DNA grade (GE Healthcare, Buck-inghamshire, UK) and sequenced with the same primers used for their amplification, with the ABI Prism Big Dye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems Corporation, Foster City, CA, USA) and ABI 3100Genetic Analyzer (Applied Biosystems). DNA was sequenced in both directions using the BigDye terminator

Table 1
Sequences of Primers used for the GI and GII norovirus detection.

Assay	Primer name	Sequences	Band size	Reference
Nested RT-PCR	RT/1.PCR			Oh et al. (2003)
	NV1a (s)	5′-ATGAATATGAATGAAGATGG		
	NV1b (s)	5′-ATGAACACAATAGARGATGG		
	NV7 (as)	5′-ATTGGTCCTTCTGTTTTGTC		
	NV7a (as)	5′-GGYCCYTCAGTYTTGTC		
	2. PCR			
	NV6 (s)	5′-TACCACTATGATGCAGATTA	338 pb	Oh et al. (2003)
	NV6a (s)	5′-TATCACTATGATGCTGACTA		
	NV4 (as)	5′-GTTGACACAATCTCATCATC		
	NV4a (as)	5′-ACAATYTCATCATCICCAT		
	NV4c (as)	5′-GTGCTGACGATCTCGTCATC		

I = Inosin; Y = C/T; R = A/G.

Table 2
Characteristics of recruited Congolese children.

Characteristics	All patients N = 545	Patients with Norovirus N = 148	aOR	P value
Age (months) n (%)				
0–3	27 (5.0)	4 (14.8)		
4–6	92 (16.9)	22 (23.9)	1	
7–12	287 (52.8)	92 (32.1)	1.80 (0.56–5.79)	0.319
13–24	132 (24.3)	30 (22.7)	2.71 (0.91–8.07)	0.073
25–60	6 (1.1)	0 (0.0)	1.69 (0.54–5.27)	0.365
Missing	1			
Gender n (%)				
Female	312 (58.21)	56 (25.0)		
Male	224 (41.8)	90 (28.8)	1.21 (0.82–1.79)	0.324
Missing	9	2		
Body temperature (°C)				
<37,5	346 (64.7)	88 (25.4)		
≥37,5	189 (35.3)	58 (30.7)	1.30 (0.88–1.92)	0.193
Missing	10	2		
Number of diarrhea episodes in 24 h				
1–3	141 (27.1)	37 (26.2)		
4–6	342 (65.6)	96 (28.1)		
>6	38 (7.3)	8 (21.1)	1.09 (0.70–1.70)	0.683
Missing	24	7	0.75 (0.31–1.79)	0.514
Duration of diarrhea (days)				
1	432 (81.2)	121 (28.0)		
2–3	57 (10.7)	9 (15.8)	0.48 (0.22–1.01)	0.05
4–6	32 (6.0)	9 (28.1)	1.00 (0.45–2.23)	0.989
7–9	11 (2.1)	4 (36.4)	1.46 (0.42–5.10)	0.54
Missing	13	5		
Number of vomiting episodes in 24 h				
0–2	185 (35.8)	47 (25.4)		
3	138 (26.7)	34 (24.6)	0.96 (0.58–1.60)	0.88
≥4	194 (37.5)	60 (30.9)	1.31 (0.84–2.06)	0.23
Missing Duration of vomiting (days)	28	7		
0	16 (3.0)	4 (25.0)		
1	424 (79.7)	120 (28.3)	1.18 (0.37–3.74)	0.773
2–3	57 (10.7)	11 (19.3)	0.72 (0.19–2.66)	0.62
≥4	35 (6.6)	9 (25.7)	1.04 (0.27–4.05)	0.96
Missing	13	4		
Type of dehydration				
Slight	35 (6.9)	14 (40.0)		
Moderate	476 (93.2)	120 (25.2)	0.50 (0.25–1.03)	0.059
Missing	34	14		
Stool consistence				
Liquid	275 (53.6)	74(26.9)		
Soft	33 (6.4)	10(30.3)	1,18 (0.54–2.60)	0.68
Mixed	205 (40.0)	56(27.3)	1,02 (0.68–1.53)	0.921
Missing	32	8		

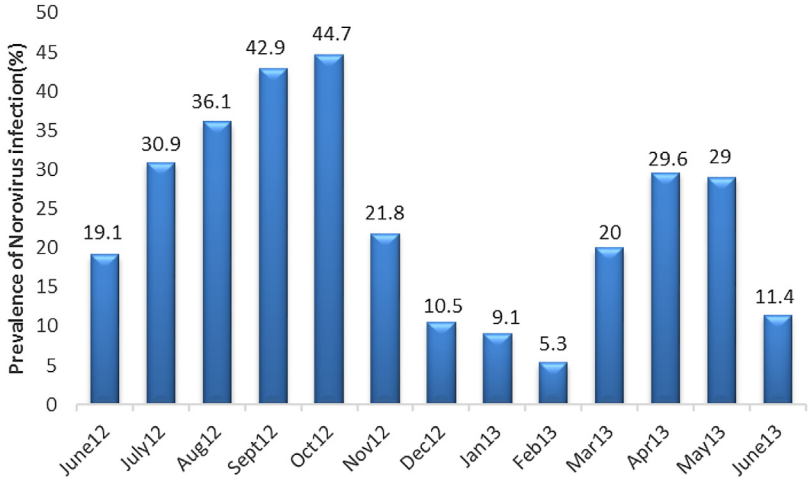

Figure 1. Monthly distribution of Norovirus infection in Congolese children under 5 years.

cycling methodology (Applied Biosystems) and an ABI 3130 Genetic Analyzer (Applied Biosystems). The sequences obtained were assembled and corrected using codon code Aligner software. The corrected sequences were analyzed with the BLAST (Basic Local Alignement Search Tool) software available on the website of the National Information Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and aligned using Clustal W included in the Bio Edit software. A phylogenetic analysis was performed with MEGA version 7.0.9 (Figure 3).

Data analysis

All analyses were performed using the SPSS software. Socio-demographic and clinical and other data such as guardian age, relationship with the child, School educational level, food-handling hygiene, number of people living in the same house, number of bedrooms in the house, number of children living in the same house, milk used for breastfeeding, type of drinking water, and the type of water treatment were analysed for identifying risk factors associated with the disease. The frequency distribution of study participant demographics, clinical data of patient and sociobehavioural data of parents were presented among PCR diagnosis of norovirus, and significant differences were determined by Chi Square or Fisher's exact test, whichever was appropriate. Bivariate logistic regression analysis was also applied to assess for risks factors of norovirus disease. Odds ratios and a corresponding 95% CI were calculated for each norovirus infected group. All tests were statistically significant for P-value < 0.05. Analyses were performed by using a statistical software package (SPSS version 24 and R version 3.0).

Results

Description of the population study

Considering only one stool sample per child, a total of 545 stool samples were analysed for the presence of norovirus infection. Children were distributed as follows, 312 and 224 were girls and boys, respectively, Table 2. The sex ratio (male/female) was 0, 71. The mean age was 10.64 months, with range between 0 and 60 months.

Norovirus infection

Of the 545 children with acute diarrhea recorded in this study, 148 (27, 14 %) were positive for norovirus infection. Most of infected children were between ages 1 and 24 months, with a mean age of 10. However, 4 infants between ages 0 and 3 months were detected with norovirus infection, and no children above 24 months were infected, Table 2. The prevalence of infection was not different (P value = 0,324) according to the sex, 85% and 25% in males and females respectively Table 2. The norovirus infection was observed throughout the year but peaked significantly in August and September ($p < 0.05$) and early in October ($p < 0.05$) corresponding to the dry season and eraly rainy season, refering to the methods section (Figure 1). Several risk factors were considered based on the literature (Table 3) and responses collected on questionnaires were analysed. No specific risk factor was significantly associated with the occurrence of the Norovirus infection excepted for the type of toilets used for children under 5 years ($p < 0.05$). It is important to report that no death case was recorded in this study.

Norovirus genotyping

The characterization of the norovirus strains detected in this study showed a circulation of various genotypes in Congolese children between June 2012 and June 2013. Among the 148 positive samples for norovirus, 100 samples were successfully characterized in region B (ORF 1) of the genome and 48 samples failed.

Norovirus genogrouping revealed that GII was the most prevalent at 87% (87/100) compared with GI 13% (13/100). The GII.P4 genotype was detected at 24.1% (21/87) followed by the GII. P12 and GII.Pg, each at 1.14% (1/87).

The GII.Pe was detected in combination with other GII. Among combined RdRp and capsid genotypes, 50% (33/64) were identified

Table 3
Risk factors associated to the Norovirus infection.

Factors	All patients N = 545	Infected by Norovirus N = 148		
		Number (%)	aOR (CI.95%)	P-value
Age's mother (years)				
<20	61 (12.55%)	21 (34.43%)	0.69 (0.39–1.23)	0.217
>20	425 (87.45%)	114 (26.82%)		
Missing	59	13		
Mother's education level				
High school level	382 (72.76%)	103 (26.96%)		
Others	143 (27.24%)	39 (27.27%)	1.01 (0.65–1.56)	0.943
Missing	20	6		
Number of people in house				
0–3	117 (22.03%)	34 (29.06%)		
Above 3	414 (77.97%)	109 (26.33%)	0.87 (0.55–1.37)	0.557
Missing	14	5		
Number of children by share				
One	412 (77.74%)	109 (26.46%)		
More than one	118 (22.26%)	34 (28.81%)	1.12 (0.71–1.77)	0.611
Missing	15	5		
Number of rooms in the house				
0–1	138 (25.99%)	41 (29.71%)	0.82 (0.53–1.27)	0.393
Several	393 (74.01%)	102 (25.95%)		
Missing	14	5		
Type of toilet used by adults				
Modern toilet	454 (87.14%)	117 (25.77%)		
Others	67 (12.86%)	23 (34.33%)	1.50 (0.87–2.60)	0.142
Missing	24	8		
Type of toilet for children under 5 years				
Modern toilet	2 (0.77%)	2 (100%)	–	
Others	258 (100%)	70 (27.13%)		0.022
Missing	285	76		
Place where child stool is thrown				
Toilet	196 (38.66%)	55 (28.06%)	0.96 (0.63–1.46	0.868
Other places	311 (61.34%)	84 (27.01%)		
Missing	38	9		
Water evacuation system				
Septic tank	34 (6.59%)	7 (20.59%)		
Others	482 (93.93%)	133 (27.59%)	1.00 (0.66–1.51)	0.992
Missing	29	8		
Washing mother's hands before child's meal				
No	371 (69.48%)	100 (26.95%)		
Yes	163 (30.52%)	44 (26.99%)	1.22 (0.70–2.13)	0.469
Missing	11	4		
Child's stool in diaper				
No	0 (15.04%)	19 (23.75%)		
Yes	452 (84.96%)	125 (27.65%)	1.38 (0.59–3.25)	0.451
Missing	13	4		
Type of water drunk by the child				
Treated	192 (86.88%)	47 (24.48%)		
Untreated	29 (13.12%)	9 (31.03%)	0.94 (0.63–1.40)	0.779
Missing	324	92		
Washing mother's hands after child's meal				
No	336 (62.92%)	92 (27.38%)	0.87 (0.56– 1.34)	0.542
Yes	198 (37.08%)	52 (26.26%)		
Missing	11	4		
Cleaning the child's eating utensils				
No	135 (25.38%)	39 (28.89%)	1,04 (0.70–1.54)	0.839
Yes	397 (74.62%)	104 (26.20%)		
Missing	13	5		
Cleaning mother's hands after baby's cleaning				
No	204 (38.20%)	54 (26.47%)		
Yes	330 (61.80%)	90 (27.27%)		
Missing	11	4		

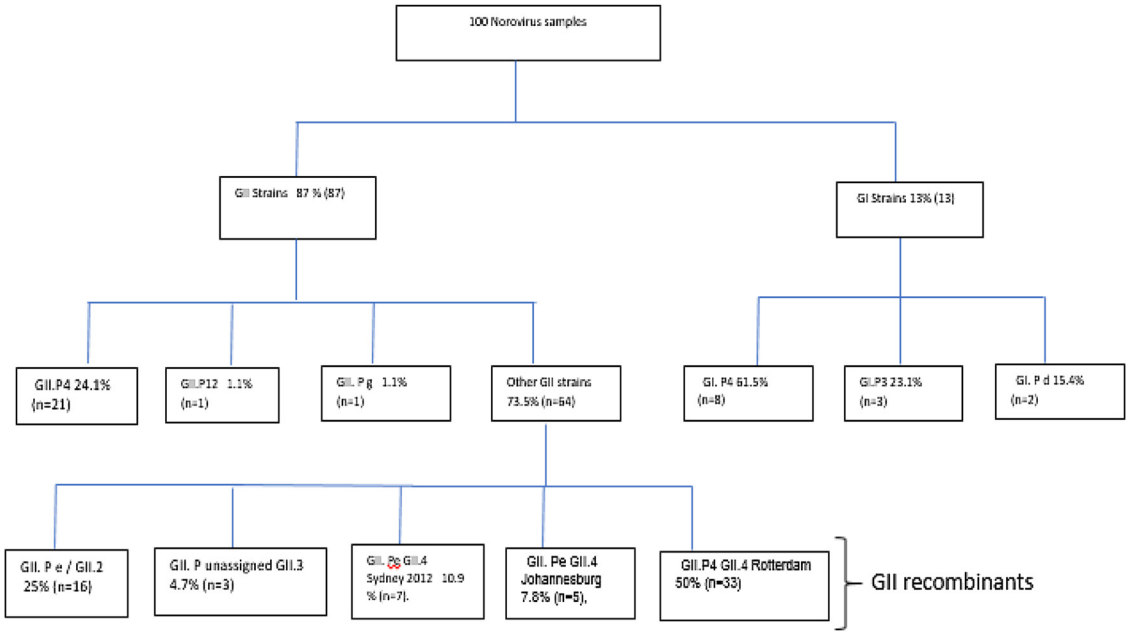

Figure 2. Distribution of genotypes associated with norovirus infection in Congolese children.

on the Norovirus Genotyping Tool as phylogenetically related to the GII.Pe GII.4 Rotterdam 2014, 10.9% (7/64) to the GIIPe GII.4 Sydney 2012 and 7.8% (5/64) to the GII.Pe GII.4 Saitama 2014. 16% (16/87) were GII.P4 GII.2 Johannesburg 2009, 5 (7.6%) and 4.7% (3/87) were unassigned such as GII.P unassigned GII.3 Bushbuckridge.

The other identified genotypes were GI.P4 at 61.53% (8/13) followed by GI.P3 at 23.07% (3/13) and GI.Pd at 15.4% (2/13). All these genotypes are represented in Figure 2. A representative phylogenetic tree based on the nucleotide sequences of the polymerase region (B region) from these strains was constructed with the neighbor-joining method (Figure 3).

Discussion

The goals of this study were to determine the prevalence of norovirus infection and to characterize infecting strains in Congolese hospitalized children under five years old with acute diarrhea. This is the first work to investigate norovirus infection in Republic of Congo, where diarrheal disease is reposnible for 7.5% of deaths in children under 5 years.

The prevalence of norovirus infection in Congolese children represented 27% and is fully in line with those reported from central Africa with 23% and 29,6% in Gabon and Cameroon respectively (Lekana-Douki et al., 2015; Ayukekbong et al., 2011). Interestingly, the prevalence of norovirus infections seems to be much higher in Central Africa compared to West and East Africa ranging around 12% (Trainor et al., 2013; Nordgren et al., 2013; Moyo et al., 2014).

In the same population of hospitatlized Congolese children, this study showed a prevalence of norovirus, rotavirus and adenovirus of 27% 46.% and (5.5%) (Mayindou et al., 2016). Therefore, this work establishes that norovirus is the second leading cause of acute gastroenteritis in Republic of Congo as reported in many countries (Qazoui et al., 2014; Lekana-Douki et al., 2015; Dove et al., 2005).

With regard to the age, norovirus infection was detected exclusively in children less than 24 months of age. These findings are consistent with reports from Africa (Ayolabi et al., 2010; Oluwatoyin et al., 2012), Asia (Trang et al., 2012; Qazoui et al., 2014), and South America (Siqueira et al., 2013; Picanço da Costa et al., 2017). This seems to suggest that acquisition of immunity against this infection occurs after the age of 2 years reflected by less severe cases of diarrhea due to norovirus (Qazoui et al., 2014). However, in this study, four children were infected between 0 and 6 months, showing that maternal antibodies during breastfeeding are not sufficient to protect against the Norovirus infection. This also has been documented previously in Vietnam and Nigeria (Oluwatoyin et al., 2012; Trang et al., 2012).

It is reported here that cases of Norovirus associated with gastroenteritis were detected throughout the year with a peak during the dry season, and early in the rainy season as seen in West Africa (Oluwatoyin et al., 2012). In fact, the seasonality of norovirus infection varies according to the country (Qazoui et al., 2014; Dove et al., 2005; Lekana-Douki et al., 2015; Sdiri-Loulizi et al., 2009; Fretz et al., 2005).

The present study did not identify a clear factor that could be associated significantly with norovirus infection. Others have reported human behavior, new strain variants, climate, host factors, the mode of transmission and the etiology of gastroenteritis (2013; Tang et al., 2013; Makhaola et al., 2018) as possible factors.

The genogroup II of norovirus was found predominantly responsible for diarrhea in Congolese hospitalized children with severe diarrhea. This finding is consistent with other reports from all parts of the world confirming its high circulation (2014; Siqueira et al., 2013; Makhaola et al., 2018; Lopes de Paula et al., 2018;). This study focused on children hospitalized with severe diarrhea and it has been shown by others that genogroup I is more common in mild cases (Siqueira et al., 2017).

The analysis of the VP1 protein fragment of the capsid is considered efficient for carrying out the genotyping of NoV, as previously described by Vinjé et al. (2004). A high genetic variability of NoV was found in this study. It is important to go further in the analysis of strains sequences for a vaccine development perspective. The establishment of a national database of Congolese strains of NoV could provide greater insight into the virus and its epidemiological impact on the population, also understanding the circulation, the emergence, and the spread of NoV disease.

Study limitations

This study has several limitations. We only detected Norovirus infection in hospitalized children with acute diarrhea

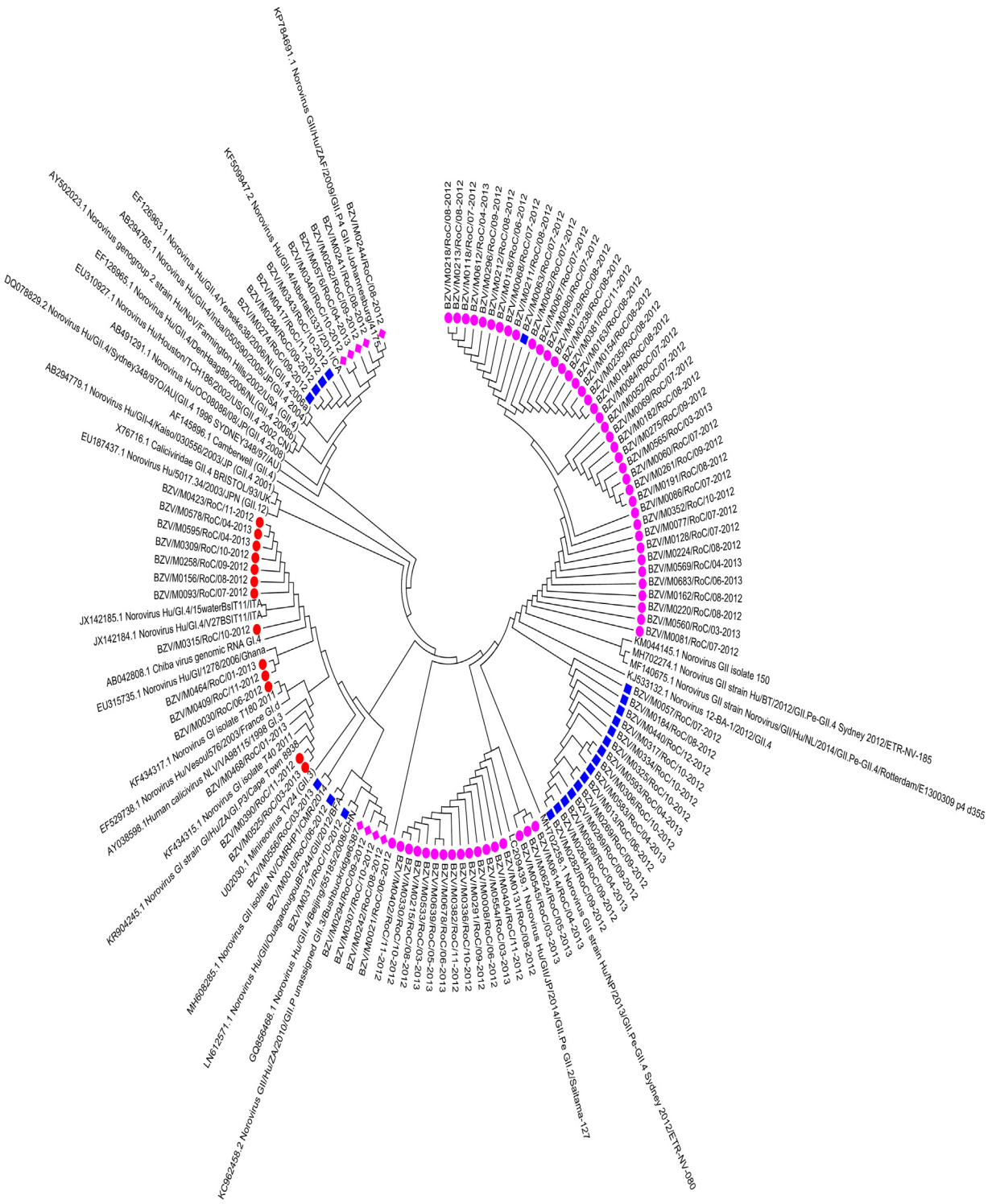

Figure 3. Evolutionary relationships of norovirus strains. Dendrogram showing the genetic classification of the samples that tested positive for NoV obtained from children with diarrhea admitted to hospital in Brazzaville, Republic of Congo, from June 2012 to June 2013: GI (Red), GII (Blue), recombinant (purple). It has been constructed based on the RdRp gene (338 bp) using a maximum likelihood method by MEGA 7.0.9. The codes representing the positive samples are organized as follows: study area (Brazzaville)/ sample number/country of collection: Republic of Congo (RoC)/month–year of collection.

(symptomatic) but not in children without acute diarrhea (asymptomatic). The stool samples were collected at a single site and it would be valuable to have a similar study at another site in Brazzavile and another city in the Republic of Congo.

The lack of some data from patients such those having pets did not allow identification of the specific risk factors associated with norovirus infection. Finally, the study has not described mixed infections and may have excluded those children hospitalised for a longer period (>48 h).

Conclusion

This is the first report of NoV infection in Brazzaville. Finally, we have found that noroviruses are important agents responsible for

gastroenteritis with a high diversity of strains and they constitute the second leading cause of gastroenteritis in this study population. These findings reinforce the need to study the molecular epidemiology of NoV infections in acute gastroenteritis. Further studies are necessary in order to identify the NoV in other parts of the country and to identify the genetic variability of this virus. The findings from these investigations will provide the first data before the norovirus vaccine introduction in the country.

Conflict of interest

All authors have no conflicts of interest.

Authors' contributions

FN and FKK designed the study. MLVE participated in the study design, performed the experiments. FKK supervised the study procedures. CV and FV analysed the data. FN was responsible of overall study. All authors participated in drafting the manuscript, read and approved the final version.

Availability of data

The sequences generated will be submitted to GeneBank and accession numbers will be attributate for all sequences. The raw data may be provided upon request.

Ethics

This work has been approved by the institutional ethics committee of the Fondation Congolaise pour la Recherche Médicale. Written informed consent was obtained from all parents and guardians of the children that were enrolled before the start of the study.

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