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First cases of Recombinant Noroviruses in Cameroon

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ABSTRACT

Noroviruses have been reported as being a common cause of acute gastroenteritis both in children and adults worldwide. Genotyping and nomenclature of noroviruses was based on the partial capsid gene of the ORF2. Due to frequent reported recombination activities in the ORF1/ORF2 junction, a new dual nomenclature has been proposed based on genotyping of two genes – the capsid and polymerase genes. This study identified recombinant noroviruses circulating in Cameroon between 2010 and 2013. RT-PCR –based methods, next generation sequencing and phylogenetic analysis were used to genotype samples from hospitalized children. The combined RdRp/capsid dual genotype was determined for 19 GII strains including 5 RdRp genotypes (GII.P4, GII.P7, GII.P17, GII.P21, and GII.P31) and 5 capsid genotypes (GII.2, GII.3, GII.4, GII.6, GII.17). They had 17(89.5%) recombinants and 2 (112.5%) non recombinants. 17 were recombinants. The most prevalent noroviruses were GII.4 (76.5%) consisting of GII.4 Sydney [P31] (41.2%) and GII.4 Sydney [P4 New Orleans] (35.3%), followed by GII.6 [P7] (11.8%), GII.2 [P21] (5.9%) and GII.3 [P21] (5.9%). This is the first study of norovirus dual genotyping and recombinants in Cameroon. Recombination activity is high and contributes to ongoing evolution of circulating noroviruses in Cameroon.

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Introduction

Norovirus is a frequent cause of sporadic acute gastroenteritis (AGE) and is the main cause of gastroenteritis epidemics worldwide in all age groups. In children younger than 5 years, norovirus is the second cause of severe AGE after rotavirus, being the main cause in countries which have implemented rotavirus vaccination programs [1-5]. Outbreaks of AGE due to norovirus are common in semi-enclosed environments such as hospitals, nursing homes, cruise ships and army barracks and can affect large numbers of people [2]. These viruses are transmitted from person to person through the fecal-oral route, aerosolized vomit or contact with contaminated surfaces, as well as through contaminated food and water, and are the main cause of outbreaks of diarrhea caused by food [2, 4]. Noroviruses are a genus within the *Caliciviridae* family and are non-enveloped viruses whose genome has a single-stranded, positive sense, RNA that includes three open reading frames (ORF). ORF1 codifies for six non-structural proteins, including RNA-dependent RNA polymerase (RdRp). ORF2 codifies the major capsid protein (VP1) and ORF3 the minor capsid protein (VP2). The ORF1 and ORF2 sequences form the basis of the currently proposed norovirus genotyping system. Based on amino acid diversity of the complete VP1 gene and nucleotide diversity of the RNA-dependent RNA polymerase (RdRp) region of ORF1, they have been seperated into 10 genogroups (GI-GX) and 48 genotypes (9 GI, 26 GII, 3 GIII, 2 GIV, 2 GV, 2 GVI and 1 genotype each for GVII, GVIII, GIX and GX). Based on nucleotide diversity in the RdRp region, noroviruses can be divided into 60 P-types (14 GI, 37 GII, 2 GIII, 1 GIV, 2 GV, 2 GVI, 1 GVII and 1 GX), 2 tentative P- groups and 14 tentative P-types, according to the recent updated norovirus classification scheme. Nine genotypes have been proposed within genogroup I and 26 within genogroup II in the capsid region. With GII.4 being the most prevalent genotype worldwide, and 14 GI.P-groups and 37GII.P-types. The virus is highly versatile and new strains frequently arise due to antigenic drift in the VP1 and to genetic recombination between preexisting norovirus strains [6-13]. The aim of the study was to describe dual genotypes and recombinant noroviruses circulating in Cameroon between 2010 and 2013.

Materials and Methods

Ethics Statement

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. Written informed consent was obtained from the parents of the children who participated in the program, as per the WHO/AFRO rotavirus surveillance protocol. 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.

Study Population

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 theMother and Child Centre of the Chantal Biya Foundation hospital.

Norovirus Detection

The reaction was performed in a 50 µL mixture of 10 µL cDNA, 5 U Platinum®*Taq* DNA Polymerase (Life Technologies™), and 250 nM of each primer. PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. For DNA sequencing, the purified products were sent to the CDC where sequencing was done by NGS technology 1.0 µL of Enzyme mix (RT and Taq DNA polymerase (5 U/µL), 0.5 µL of Rnase inhibitor (20U/uL) and 10.5 µL of nuclease-free water. The amplification conditions were set as follows: Reverse Transcription at 42oC for 30 min, then 94°C for 15 min, followed by 40 cycles of 94°C for 30s, 50°C for 30 s, 72°C for 1 min, and an extension step at 72°C for 10 min. Resulting PCR products were visualized on a 2% agarose gel (Seakem-ME, Lonza, Allendale, NJ, USA) prepared with 10% Gel Red (Biotium, Fremont, CA, USA). Amplicons of expected size (570 bp) were gel-purified with the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced by Sanger sequencing (Eurofins MWG Operon, Louisville, KY, USA).

Genotyping

Viral RNA was extracted from 140 µL of 10% clarified stool suspensions using the QIAamp Viral RNA Mini Kit, (Qiagen, Inc., Valencia, CA USA) following the manufacturer's instructions as previously described and stored at −80°C until use. Possible recombination in noroviruses was investigated by amplifying the junction region of the polymerase gene (ORF1) and the capsid gene (ORF2), which is the recombination breakpoint in noroviruses. The primer pair Mon431 (+) : TGG ACI AGR GGI CCY AAY CA [14] and G2SKR: CCR CCN GCA TRH CCR TTR TAC AT [15] was used in a one-step RT-PCR run to amplify the OFR1/OFR2 junction region (570 bp) of suspected recombinant viruses using the Qiagen One-Step RT-PCR. The reaction was performed in a 50 µL mixture of 10 µL cDNA, 5 IU Platinum®Taq DNA Polymerase (Life Technologies™), and 250 nM of each primer. PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (table 3). For DNA sequencing, amplicons were first run on agarose gel to determine the positive cases and the gel bands (purified products) were cut out and sent to the CDC Atlanta for sequencing. The norovirus dual types and recombinants were assigned by using the online Human Calicivirus typing tool (http://www.rivm.nl/mpf/norovirus/typingtool) and the strains were named by indicating the genotype of the polymerase followed by that of the capsid [7,16].

Results

Dual Genotypes and Recombinants

From 2010 to 2013 the diversity of noroviruses in children (5 years) hospitalized with gastroenteritis in Cameroon was investigated. Five RdRp (P) genotypes (P4, P7, P17, P21 and P31) and five capsid genotypes (GII.2, GII.3, GII.4, GII.6 and GII.17) were identified. The dual genotypes were obtained from the two genes (polymerase and capsid) in 19 specimens out of which 17 (89.5%) were recombinants. The most prevalent noroviruses were GII.4 (76.5%) consisting of GII.4 Sydney [P31] (41.2%) and GII.4 Sydney [P4 New Orleans] (35.3%), followed by GII.6 [P7] (11.8%), GII.2 [P21] (5.9%) and GII.3 [P21] (5.9%) (Table 1).

Table 1: Norovirus Dual Genotypes and Recombinants Detected In Cameroon during the Period of 2010-2013

	Name		length	Genus	B-region		Type	C-region		Plot
1	15Junc	Details	527	norovirus	98 %		GII.4 Sydney[P4 New Orleans]		98 %	
2	254Junc	Details	527	norovirus	95 %		GII.3[P21]		99 %	
3	322Junc	Details	527	norovirus	97 %		GII.4 Sydney[P4]		98 %	
4	344Junc	Details	527	norovirus	97 %		GII.4 Sydney[P4]		98 %	
5	426Junc	Details	527	norovirus	96 %		GII.17[P17]		96 %	
6	442Junc	Details	527	norovirus	96 %		GII.17[P17]		97 %	
7	786Junc	Details	527	norovirus	97 %		GII.4 Sydney[P4]		98 %	
8	1057Junc	Details	527	norovirus	97 %		GII.4 Sydney[P4]		98 %	
9	1149Junc	Details	527	norovirus	96 %		GII.4 Sydney[P4]		98 %	
10	1348Junc	Details	527	norovirus	98 %		GII.4 Sydney[P31]		98 %	
11	1404Junc	Details	527	norovirus	98 %		GII.4 Sydney[P31]		98 %	
12	1458Junc	Details	527	norovirus	97 %		GII.4 Sydney[P31]		98 %	
13	1524Junc	Details	527	norovirus	97 %		GII.4 Sydney[P31]		98 %	
14	1581Junc	Details	527	norovirus	97 %		GII.4 Sydney[P31]		98 %	
15	1890Junc	Details	527	norovirus	99 %		GII.6[P7]		99 %	
16	1942Junc	Details	527	norovirus	98 %		GII.4 Sydney[P31]		98 %	
17	2176Junc	Details	527	norovirus	99 %		GII.6[P7]		100 %	
18	2178Junc	Details	527	norovirus	98 %		GII.4 Sydney[P31]		98 %	
19	2403Junc	Details	527	norovirus	98 %		GII.2[P31]		96 %	

Recombinants

The investigation of recombinants was done on all the 19 dual genotypes that were successfully amplified and sequenced for the ORF1/ORF2 junction region. Seventeen (89.47%) confirmed recombinant norovirus strains circulated during the 2010-2013 study period. The majority (n=11; 64.7%) were intergenotype recombinants and six (35.3%). Norovirus GII.P31/GII.4 (41.2%) and GII.P4/GII.4 (35.3%) predominated, followed by GII.P7/GII.6 (11.8%), GII.P21/GII.2 (5.9%) and GII.P21/GII.3 (5.9%) as shown on table 2. The recombinant types GII.4 Sydney [P4 New Orleans] and GII.3 [P21] viruses showed a high level of nucleotide identity (98%) and were detected between 2010 and 2012, and GII.4 Sydney [P31], GII.2 [P21] and GII.6 [P7] were all detected between 2012 and 2013.

Table 2: Prevalence of Recombinant Genotypes

Type of Recombinant	Recombinant	Number of samples with genotype	Percentage among recombinants
Intragenotype	GII.4 [P4]	6	35.3%
	GII.4 [P31]	7	41.2%
Inter genotype	GII.6 [P7]	2	11.8%
	GII.2 [P21]	1	5.9%)
	GII.3 [P21]	1	5.9%

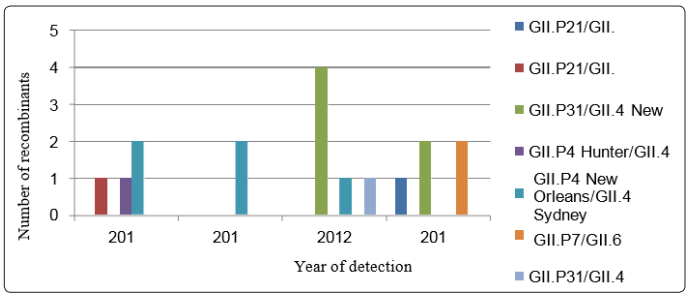

Figure 1: Number of Recombinants by Year of Detection

Discussion

Recombination frequently occurs within and between noroviruses genotypes and recombinants have been implicated in sporadic cases, outbreaks and pandemics of noroviruses. Recombination allows the virus to increase its genetic fitness, to evolve, and to spread in the host populations by escaping the host immune response. Norovirus recombinants have been widely reported in the world and more than 20 norovirus recombinant types have been identified so far. The occurrence of a variety of different noroviruses circulating within a population is a risk factor for recombination events, which results from mixed infections. These events strongly impact molecular epidemiological studies and viral control programs [17-21]. The co-circulation of two potential parental strains may facilitate recombination when the nucleic acid sequences of the strains physically interact in infected cells during copy-choice recombination. RNA recombination is responsible for a large proportion of viral diversity, leading to the production of viable recombinants. In 1999, Jiang and colleagues were the first to report a naturally occurring norovirus recombinant, in which the recombinant site was found between the RdRp and capsid genes in the RNA region. Since then, several recombinant strains have been sporadically reported around the globe.

This is the first report of dual norovirus genotypes and recombinants in Cameroon. Most genogroup II norovirus strains detected in this study belonged to the capsid genotypes GII.4 (68.4%), which are the most globally prevalent strains in children with acute gastroenteritis. We found GII.4 (capsid) combining with GII.P4 (polymerase) in most cases detected between 2010 and 2012. However, in late 2012 this combinant was rapidly replaced by GII.4New Orleans [P31], while the pandemic Sydney-2012 variant was circulating in other localities [19-28].

Dual genotyping of the polymerase and capsid regions of noroviruses has increased awareness and detection of norovirus recombinants around the world. This has provided more evidence that intergenotype and intragenotype recombination has enhanced the genetic diversity of noroviruses. Recombination events contribute to the emergence of new viral strains. Therefore, understanding the recombination events occurring in norovirus strain is important for tracking the emergence of new norovirus variants.

The most frequently detected norovirus polymerase/capsid genotype in this study was classified as GII.4 Sydney [P31], followed by the GII.4 Sydney [P4 New Orleans]. The GII.4 Sydney 2012 capsid genotype, first reported in Australia in 2012, replaced the GII.4 New Orleans 2009 strain and caused the global outbreak of 2012-2013. The GII.P31 polymerase genotype was first detected in the norovirus outbreak of 2008 in Victoria, Australia, and then at lower frequencies in 2009 and 2010 before becoming the predominant genotype in 2012 [28,29].

GII.P31 was linked to the GII.3, GII.4, and GII.12 capsid genotypes. Thus, GII.4 Sydney [GP31] is a recombinant of GII.P31 polymerase and GII.4 capsid gene variant sharing sequences with Apeldoorn 2007 and New Orleans 2009. We detected the GII.P31 in this study from the second half of 2012 through 2013, and in both years they were associated with the GII.4 capsid. As the GII.4 Sydney [P31] was the predominant strain circulating in Korea, Taiwan, it was equally the predominant strain in Cameroon [29-30]. We report here that GII.4 Sydney 2012[P4NewOrleans 2009] was the predominant norovirus recombinant circulating in Cameroon since 2010, 2011 and 2012. One variant form was detected in France during the seasons 2012/13, 2013/14 and 2014/15, at a time when the variant Sydney 2012 largely predominated. However, in the 2012/2013 season, the GII.4 Sydney [P31] recombinant strain was strangely the dominant GII.4 norovirus genotypes co-circulating in Cameroon with GII.4 Sydney [P31], while the GII.4 Sydney [P4 New Orleans] continued to circulate elsewhere and in later seasons.

The GII.4 Sydney [P4 New Orleans] variant was also described in South Africa, Denmark and Italy during the season 2012-2013 [11, 31] and more recently in Australia in August 2015 and as an altered version in June 2016 [29]. In 2016, the recombinant strains GII.4 Sydney [P31] and GII.4 2012[P4 2009] were co-circulating, but less frequently detected in that season. In a recent classification of norovirus cases detected in the USA by CaliciNet, GII.16/GII.4 Sydney is the leading cause of norovirus outbreaks (51%), followed by GI.3 [P3] (11%). GII.6 [P7] has a prevalence of 6% while GII.4 [P31] and GII.4 Sydney [P4 New Orleans] represent one percent each.

Out of the four previous studies that have reported norovirus recombinants in Africa - Burkina Faso, Madagascar, Ghana, and South Africa [32-35], this study found GII.6 [P7] that was reported in Burkina Faso in 2013 and by Mans et al. in South Africa in 2014, and GII.3 [P21], GII.4 Sydney 2012[P31], GII.4 New Orleans 2009[P31] and GII.4 Sydney 2012[P4 New Orleans] that were all reported by Mans in 2014.

The recombinant strain GII.3 [P21] which was also identified in this study (MN294757) has been widely reported. The South African strain is related to a subgroup of GII.3 [P21] recombinants, which have been reported in China, India and Korea [36-38]. This Cameroon genotype (MN294757) compared to the other genotypes from other countries shares between 92% similarity with the South African strain (KC962657), 97% in the Hong Kong strain (JX846924) and up to 98% similarity with the South Korean strain (JX439784). This intergenotype recombination is one of the main mechanisms of norovirus GII.3 evolutions. There have been reports of the common association of GII.3 (capsid) with polymerases of several genotypes, two of which—GII.P21 and GII.P12. It has been one of the most reported recombinant as a major cause of childhood gastroenteritis since 2002. The Cameroon case was detected in 2010, the same year that it was detected in South Africa and had been previously reported in China, India and Japan. It was highly prevalent in Australia and New Zealand up to May 2015, when its prevalence rapidly declined [38,39]. Reports of intense norovirus GII.3 circulations in the last decades of the 20th century [25, 36], together with high cross-reactivity and limited evolution of GII.3 epitopes could explain why the incidence of norovirus infection is currently scarce in the adult population [40].

The other intergenotype recombinant detected in this study was the GII.6 [P7] recombinant (MN294772). It was described for the first time in Burkina Faso in 2013, and later detected in South Africa in 2014 (KJ407072). The Cameroon strain shares 90% identity over 98% of the nucleotide sequence from South African strain [33], and only 90% nucleotide identity in the capsid region. The capsid region of the Cameroon recombinant is more closely related (96% identity over 92% of Region C) to a Kenyan GII.6 strain (KF279386) reported in 2013 [38] and an identity of 95% to a USA strain (AF414410). Genotype GII.6 [P7] recombinants have also been reported in Finland [41, 42], Japan (AB818397-400) and Sweden (KF768487). Inter- and intragenotypic recombination is one of the main mechanisms of norovirus evolution [12, 15, 43]. In the present study, the proportion of intergenotypic recombinant strains was high (64.7%). A review that analyzed 803 norovirus strains corresponding to 11 studies reported that 26.5% of the detected strains were intergenotypic recombinants. In 2012 and 2013, we detected a rare recombinant strain, GII.4 New Orleans [P31] in addition to the more commonly detected GII.4 Sydney [P31]. This GII.4 New Orleans [P31] recombinant strain to our knowledge has not previously been reported in literature. Intra-GII recombinant strains include GII.4 Sydney [P4 New Orleans] detected between 2010 and 2012. Since the end of the 20th century, several GII.4 variants have emerged, some of which have spread worldwide or over large geographical regions, increasing the number of sporadic episodes and outbreaks of AGE [11, 12, 25]. The Den Haag 2006b variant, which was globally dominant in 2007-mid-2009, the emergence of the New Orleans-2009 variant in 2009 which predominated in 2010 and 2011, but was replaced by the new Sydney-2012 variant in the second half of 2012 are examples. Sydney 2012 was detected in Cameroon since 2010, long before it became associated with epidemic activity 2 years later. From late 2011 and early 2012 and after the global emergence of the Sydney 2012 variant, and before its epidemic spread, we detected recombinant New Orleans 2009/Sydney 2012 strains, which have equally been reported in other areas.

The GII.17 [P17] was also detected polymerase/capsid genotype in this study in December 2010. BLAST searches revealed that GII.17 [P17] strains in this study were closest to norovirus SD153-4/GII.17/2014/CHN isolated in China in June 2014, GII Hu/JP/2014/GII.P17/GII.17/OC14032 isolated in Japan in 2014 and to Alberta SG001/CA/2014 in the USA in 2014. Thus, similar GII.17 [P17] strains were active in Korea, China, and Japan and USA in 2013 to 2015. The sequences of these strains were slightly different from those of a novel GII.17 [P17] norovirus strain (GII.17 Kawasaki 2014), which emerged as a major cause of gastroenteritis outbreaks in China and Japan in the winter of 2014/2015 [44]. From these findings, we can assume that a novel escape mutant derived from the 2013 GII.17 [P17] strains in this study emerged and caused gastroenteritis outbreaks in China and Japan in the winter of 2014/2015.

In summary, the most prevalent GII norovirus recombinant detected in Cameroon was GII.4 New Orleans [P31], followed by GII.4 Sydney [P4 New Orleans], then GII.6 [P7], and the least prevalent were GII.4 Sydney [P31], GII.2 [P21] and GII.3 [P21]. Contrary results were found in Australia, where for the same virus genotypes detected, recombinant GII.4 Sydney 2012[P4 NewOrleans] occupied the second position, followed by GII.6 [P7]. However, in New Zealand, GII.2 [P16] [45] was found to be the second most predominant GII virus identified (9.1%, n = 17/187), after the most dominant GII.P16/GII.4 Sydney 2012, both of which were not detected in this study. This was followed by GII.4 Sydney 2012[P31], GII.4 Sydney 2012[P4 NewOrleans 2009] and GII.6 [P7] [40]. Genotypes GII.2 [P21] and GII.3 [P21] were not found neither in Australia nor in New Zealand.

Conclusion

Recombination activity is high and contributes to ongoing evolution of circulating noroviruses of all types with changes in genome characteristics and function from one year to another. Combined characterisation of the polymerase and capsid regions of norovirus has increased awareness and detection of norovirus recombinants. Dual typing of both RdRp and capsid genes in a surveillance program is important for monitoring emerging strains in an effort to reduce the overall burden of norovirus disease.

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