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Sequencing of Norovirus in Southern, Nigeria: Prevalent Genotypes and Putative GII.4 Novel Recombinants among Children

Favour Osazuwa

Abstract

Norovirus is now known to be the leading cause of gastroenteritis among children worldwide. This present report highlights the genetic diversity of norovirus among children less than 5 years in Southern, Nigeria. Stool specimens were collected from 300 children with diarrhea and analyzed for norovirus using conventional reverse transcriptase-Polymerase Chain Reaction. Sequencing of the capsid region was performed to genotype the strains. Norovirus was detected in 45 (11.1%) of children with diarrhea. Genogroup II norovirus was detected in 38/45 (84.4%) patients, while genogroup I (GI) noroviruses were identified in 7/38 (15.6%) patients. Genotype diversity was large, as demonstrated by the nine identified genotypes (2 GI and 7 GII). GII.4 was the most predominant genotype. Two norovirus GII.4 variants, New Orleans_2009 and Sydney_2012 were identified in this study. A putative novel GII.4 recombinant was also detected. This study report for the first time the detection of norovirus GII.17 Kawasaki strain in South-South, region of Nigeria.

Keywords: norovirus, genetic diversity, children, RT-PCR

1. Introduction (Virology of Norovirus)

Norovirus has been identified to constitute a key biological cause of gastroenteritis worldwide [1]. It is also the most common cause of gastroenteritis world over [1]. Norovirus causes an estimated 1.1 million hospitalizations and up to 218,000 deaths among children less than 5 years annually [1]. Clinical manifestations of norovirus infection are characterized by non-bloody diarrhea, vomiting and stomach pain [2]. Incubation period is usually within 12 to 48 hrs after infection with the virus [3]. Complications are uncommon, but may include dehydration, especially in the young, the old, and those with other health problems [3]. Norovirus is typically spread by faeco-oral route, through contaminated food or water or from person to person [4]. Potential risk factors are poor environmental hygiene and overcrowded living quarters [5]. Vomiting, in particular, transmits infection effectively and appears to allow airborne transmission [5].

2. Norovirus genome and genetic diversity

Taxonomically, norovirus is a positive sense RNA, non-enveloped virus in the family *caliciviridae* [6]. The genus has one species, known as Norwalk virus [6]. Norovirus are highly genetically diverse, Phylogenetically, they can be classified into 10 different genogroups (GI-GX) and several genotypes in this order; 60 P-types (14 GI, 37 GII, 2 GIII, 1 GIV, 2 GV, 2 GVI, 1 GVII and 1 GX), with each of these genotypes having several genetic clusters and sub groups [7].

The genome is organized into three open reading frames (ORF) [8]. ORF 1 encoded six non-structural proteins (NS1-NS6) and the RNA dependent RNA Polymerase RdRP, while ORF 2 encodes the capsid proteins-the major structural proteins VP1 containing the shell (S) and protruding (P) domains. The S domain surrounds the viral RNA and the P domain, which consists of the P2 domain, is linked to the S domain through a flexible hinge [9]. ORF 3 encodes a minor structural protein [9]. In spite of the large genetic diversity of norovirus, it is noteworthy that norovirus of the genotype GII.4 are responsible for a majority of infections [10, 11].

3. Norovirus recombinants

Recombination are mechanism in the evolution of RNA viruses, this creates changes in virus genomes by exchanging sequences, thereby generating genetic variation and producing new viruses [12]. RNA recombination is very common among RNA viruses belonging to the family *picornaviridae*, *coronaviridae*, *retroviridae* and *caliciviridae* [13]. Recombination commonly occurs at the ORF1-ORF2 junction [14] although other recombination sites have been reported. Recombination event is high among the GII.4 noroviruses [14]. Norovirus recombination has been recognized to be a major tool it uses to evade host immune recognition [15]. Monitoring the incidence of rate of generation of new norovirus recombinants is a vital tool in the understanding of norovirus evolution and continuous global spread. Norovirus recombination has been linked to increased rate of generation of new norovirus genotypes and subtypes [16], this has also hampered the possibility of a possible vaccine.

4. Justification for this study

The introduction of rotavirus vaccines throughout the world has made norovirus the most common aetiologic agent of gastroenteritis world over [17]. An update on the predominant norovirus genotype in a given population is needed for the development of effective vaccine. There are no data on the genetic diversity of norovirus among children in South-South, Nigeria. Also, there are no data on the prevalence of norovirus in Nigeria. Against this background, this study was conducted to determine the prevalent norovirus genotypes and existence of possible GII.4 recombinants among children under 5 years with diarrhea in South-South, Nigeria.

5. Method

5.1 Study area and study population

This cross-sectional study was conducted in the period, March, 2018 to February, 2019. 405 children with clinical symptoms of diarrhea/gastroenteritis

from a pool of 2813, attending outpatient clinics of four secondary health facilities (Central Hospital, Warri, Central Hospital, Benin, Primary Health Centre Pessu and Federal Medical Centre, Yenagoa) in Delta, Edo and Bayelsa States, Niger-Delta region, Nigeria were randomly included. Inclusion criteria where at least 3 clinical episodes of diarrhea- with an onset of 1 to 7 days whose parents or guardians consented for their ward/children to participate were included in this study. One hundred (100) asymptomatic apparently healthy age and sex matched children who served as controls (**Figure 1**).

5.2 Sample collection and processing

Stool specimens were collected into clean universal containers. Supernatant obtained from stool suspension of 50% in 1 ml sterile phosphate buffered saline were stored at -20°C for RT-PCR analysis of norovirus.

5.2.1 RNA extraction

RNA extraction was from thawed frozen samples were performed using AccuPrep® Viral RNA Extraction Kit (Bioneer, Daejon South Korea), following manufacturers instruction.

5.2.2 cDNA synthesis

cDNA synthesis was carried out on a 20 µl reverse transcription reaction of 1.0ug of extracted RNA on 0.2 ml tubes of Accupower Cycle script RT Premix (Bioneer Corporation, South Korea). Standard protocols as recommended by manufactures were followed for cDNA synthesis.

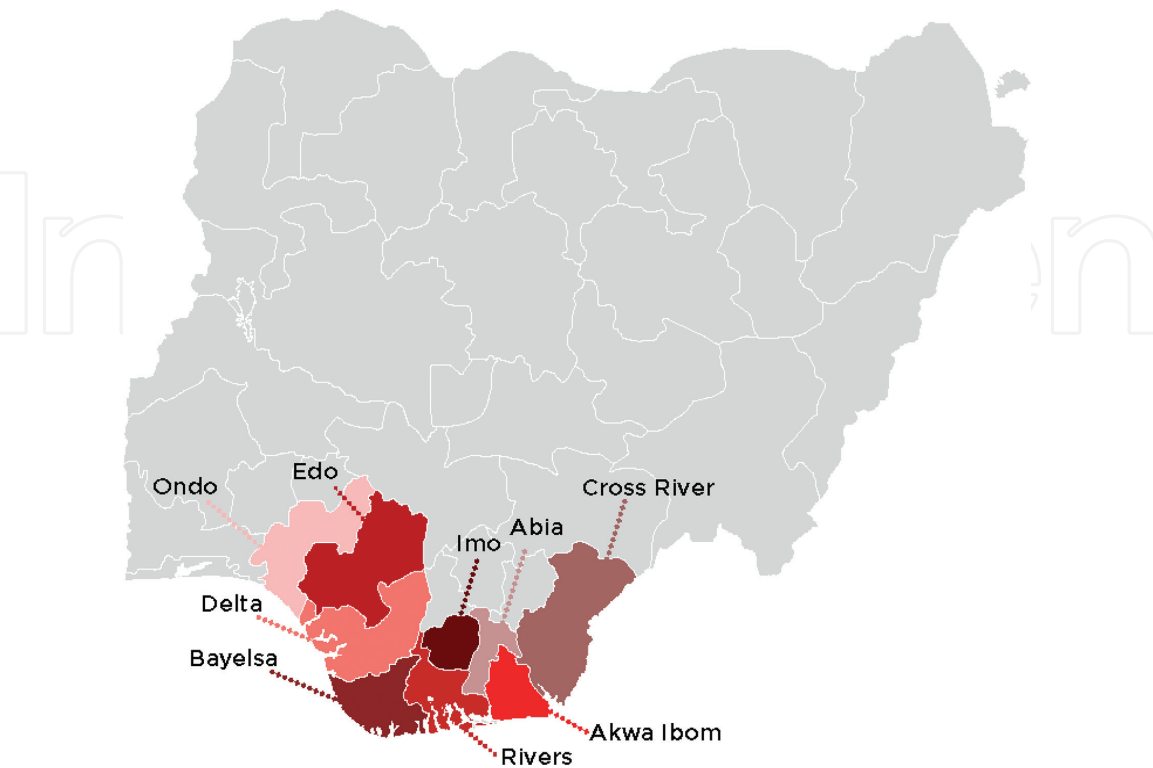


Figure 1.
Map of Southern Nigeria. States included in this study were Bayelsa, Delta and Edo.

5.2.3 Polymerase chain reaction

The cDNA generated was then amplified by PCR in a 45 µL reaction mixture as described in a previous study [18]. Specific primers (G1SKRCAACCCARCCA TTRTACA) and G1FFN (GGAGATCGCAATCTCCTGCCC) were used for GI genotyping, while for genotyping GII noroviruses, primers GIIFBN (TGGGAGGGCGATCGCAATCT) and GIISKR (CCRCCNGCATRHCCRTRTACAT), respectively, were used in an RT-PCR analysis. The products were visualized on UV illuminator and photographed using Polaroid camera [19]. The RT-PCR used is a very sensitive method, it can detect as few as 5×10^6 copies per gram of stool sample. U-Taq DNA polymerase (SBS genetech, Beijing, China), a high-fidelity thermostable enzyme that can withstand prolonged incubation at high temperature up to 95°C without significant loss of activity was used for this RT-PCR protocol.

5.2.4 Norovirus sequencing

The amplicons from the partial gene regions of the viral capsid genes were purified using QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Nucleotide sequencing was done using Big Dye ® Terminator v 3.1 Cycle sequencing kit (Applied Biosystems, Carlsbad, CA) on 3130 DNA genetic analyzer (Applied Biosystems, Carlsbad, CA). Sequences were edited using sequencher® Version 5.4.6 DNA sequence analysis software (Gene codes Corporation, Ann Arbor, MI, USA). Norovirus genotypes were determined by comparison of corresponding sequences of norovirus strains using the online norovirus genotyping tool version 1.0. available at (www.rivm.nl/mpf/norovirus/typing tool).

5.2.5 Phylogenetic analysis

For confirmation of genotyping, nucleotide sequences obtained were aligned with reference sequences using MUSCLE [20]. The evolutionary history was inferred by using Maximum Likelihood method and Kimura 2-Parameter model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale with the branch lengths measured in the number of substitutions per site. Accession Number KF307755, KF307756, MN271357, MN271380 were used as outgroup.

6. Results

Out of the 405 children enrolled, 45 (11.1%) were positive for norovirus using RT-PCR (**Table 1**). Only norovirus genogroup I and II were recovered in this study, norovirus genogroup IV were not detected. Norovirus genogroup II was the most prevalent (84.4%) among the children (**Table 2**). GII noroviruses were also most commonly encountered among all centers included in this study (**Table 3**). Based on capsid gene sequences recovered from the 45 norovirus positive samples, two GI noroviruses genotype was detected in this study GI.3, 71.4% and genotype G1.5, 28.6% respectively. Seven genotypes of GII noroviruses were detected, genotypes GII.4 (63%), GII.12 (7.9%), GII.17 (7.9%), GII.6 (5.3%), GII.7 (5.3%), GII.14 (5.3%) and GII.2 (5.3%) (**Table 4**). GII.4 was found to be the most prevalent genotypes in all States studied, Delta (53.4%), Bayelsa (75.0%) and Edo State (46.2%) (**Table 5**).

Subtyping analysis with the norovirus genotyping tool demonstrated that the majority of the norovirus GII.4 recovered in this study were homologous to the

Location	No. Tested	No. Infected (%)	P value
Central Hospital, Warri	83	13 (15.7)	0.0032
PHC, Pessu/ Ugbuwangwe	68	15 (22.1)	
FMC Yenagoa	80	4 (5.0)	
Central Hospital, Benin	99	3 (3.0)	
Stella Obasanjo Hospital	75	10 (13.3)	
<i>P</i> < 0.05.			

Table 1.
Norovirus infection detected by RT-PCR among children under 5 years from different health facilities in south–south region of Nigeria.

Norovirus genogroup	Frequency (%)
GI	7 (15.6)
GII	38 (84.4)
Total	45

Table 2.
Frequency of occurrence of genogroup of norovirus in study population.

Study Centre	No. Tested	Nov GI	Nov GII
Central Hospital, Warri	13	2 (15.4)	11 (84.6)
PHC, Pessu/Uguwangwe	15	2 (13.3)	13 (86.7)
FMC, Yenagoa	4	1 (25.0)	3 (75.0)
Central Hospital, Benin	3	1 (33.3)	2 (66.7)
Stella Obasanjo CWH, Benin	10	1 (10.0)	9 (90.0)

Table 3.
Distribution of norovirus genogroups among the health facilities in the study.

Genotypes	Frequency
GI	
GI.3	5 (71.4%)
GI. 5	2 (28.6%)
GII	
GII.2	2 (5.3%)
GII.4	24 (63.0%)
GII.6	2 (5.3%)
GII.7	2 (5.3%)
GII. 12	3 (7.9%)
GII. 14	2 (5.3%)
GII.17	3 (7.9%)

Table 4.
Distribution of genotypes of norovirus in study population.

Genotypes	Delta	Bayelsa	Edo
GI.3	3 (10.7)	0	2 (15.4)
GI.5	1 (3.6)	1 (25.0)	0
GII.2	1 (3.6)	0	1 (7.7)
GII.4	15 (53.4)	3 (75.0)	6 (46.2)
GII.6	1 (3.6)	0	1 (7.7)
GII.7	2 (7.1)	0	0
GII.12	2 (7.1)	0	0
GII.14	1 (3.6)	0	1 (7.7)
GII.17	1 (3.6)0	1 (7.7)	
Total	28	4	13

Table 5.
Distribution of norovirus genotypes among study centers.

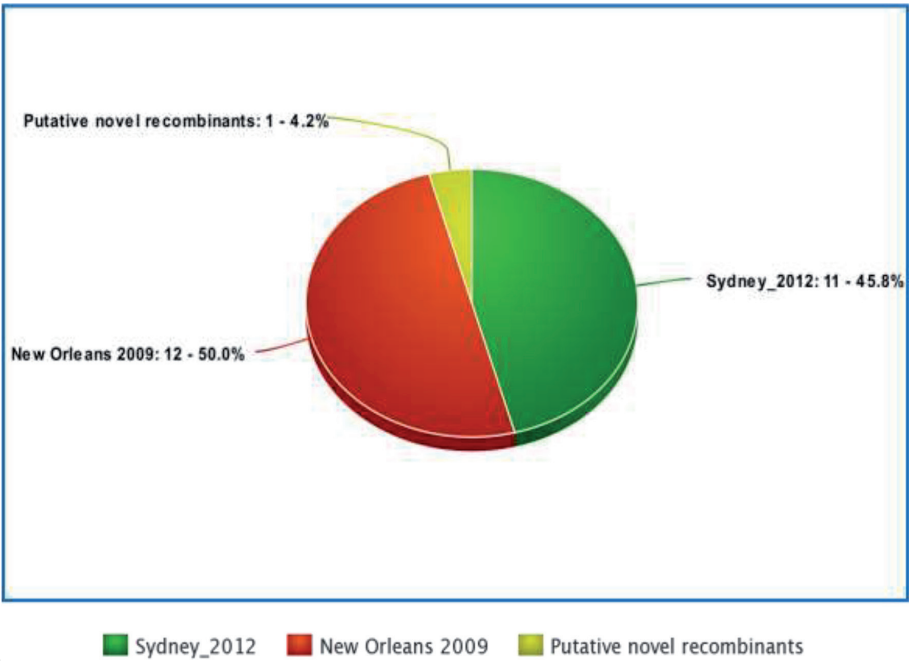


Figure 2.
Norovirus GII.4 variants among patients in this study.

New Orleans 2009 variant 12 (50.0%), while 10 (41.7%) isolates showed homology to the Sydney 2012 strains. Two GII.4 sequences were unassigned. All sequences were further subjected to phylogenetic analysis, genotypic assignment of all sequences was based on bootstrap cut off values >70%. The result showed that norovirus GII.4 were more genetically diverse (**Figure 2**).

Three genetic clusters of Sydney 2009 variants were found to circulate among study participants, with varying sequence identity (range 74–100%). The two sequences that were unassigned using the genotyping tool were assigned following phylogenetic analysis. One GII.4 isolate (MN271364) did not cluster with either New Orleans or Sydney 2009 reference strains despite being assigned to be GII.4 Sydney strain by the genotyping tool, but had genetic sequence similarity to a norovirus strain isolated in 1993 in Bristol (X76716), which itself is a recombinant strain which was neither New Orleans nor Sydney 2009 strain (**Figure 3**). This strain is however deemed a putative novel recombinant.

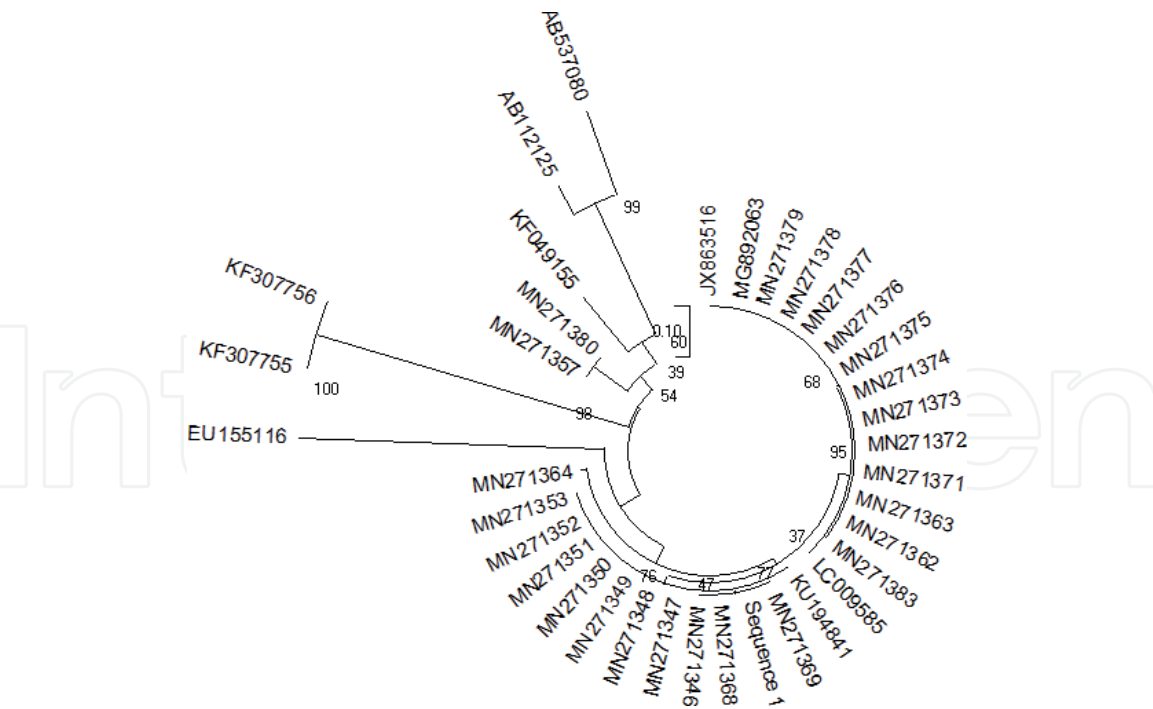


Figure 3.
Phylogenetic tree of norovirus GII.4 identified in this study: There were a total of 1487 positions in the final dataset. Sequences in this study are represented with filled triangles, reference sequences are not marked. MN271364 had low bootstrap values, as such classified to be a putative novel recombinant.

7. Discussion

A prevalence of 11.1% RT-PCR confirmed norovirus infection among children under 5 years of age was observed. This prevalence report is higher than the 6.7% in Ile –Ife, Osun State [21], but less than the 37.3% among children in Lagos, Nigeria [22]. This report supports the hypothesis that the prevalence of norovirus within Nigeria is not homogenous across communities and health centers. Hence, community and hospital-based surveillance are needed to provide an estimate of norovirus burden in Nigeria. The finding of this study might imply that norovirus may be one of the major etiologic agents of diarrhea among children less than 5 years in South–South region of Nigeria.

Only norovirus belonging to GI and GII genogroups were identified in this study, this concurs with findings from previous norovirus molecular epidemiologic studies [23, 24]. Norovirus genogroup II was the most commonly recovered norovirus in this study. This report concurs with findings from other parts of the World [25, 26]. This study further highlights the superior role of norovirus genogroup II in cases of norovirus induced gastroenteritis among children. In this study, GII.4 norovirus strains were most commonly detected. It has been well established that GII. 4 noroviruses are responsible for the majority of outbreaks worldwide [27].

Phylogenetic analysis revealed two GII.4 sequence variants, two Sydney 2012 clusters and one cluster for the New Orleans 2009 strain and a putative recombinant GII.4 virus. It is established that new variants of GII.4 norovirus emerge every 1–2 years, due to genetic recombination and point mutations resulting in the generation of new genetic clusters, recombinants/genotypes allowing increasing genetic fitness and continuous spread in populations by evading host immune responses [28]. GII.4 Possess the largest number of intra-genotypic variants and recombinants [29]. It is also possible for two or more variants to co-circulate at the same time in a geographical location [29]. Notable GII.4 variants causing majority

of pandemic diseases are the Sydney 2012, New Orleans 2009, Farmington Hills 2002, US95/961995, Hunter 2004 and Den Haag 2009 [30]. Certain variants cause localized epidemics, Cairo 2007, Japan 2008 and Asia 2003 [30].

This study reports on the finding of norovirus GII.17 among children in Southern, Nigeria for the first time, this genotype is known to be very virulent [17]. First documented evidence of emergence of GII.17 occurred in the winter of 2014–2015 in Asia [31].

The finding of this study provides evidence of the existence of diverse genetic subtypes of norovirus in our locality. This data has illuminated the epidemiological profile of norovirus induced diarrhea/gastroenteritis in our locality.

7.1 Study limitations

Whole genome sequencing of both the major capsid protein and polymerase protein (RDrP) of the putative novel recombinant identified could not be performed because of lack of funds. This would have helped to inform on the proper assignment of the putative novel recombinants as a novel genotype. Analysis of recombination breakpoints sites, homology model, evolutionary and phylogeographic relationships with reference will help provide greater information on the novel recombinants identified in this study.

7.2 In conclusion

Prevalence of RT-PCR confirmed norovirus infection among children with gastroenteritis in our locality was 11.1%. Norovirus genogroups I and II were the norovirus recovered in this study. GII.4 noroviruses were more prevalent, rare norovirus genotypes GII.2 and GII.17 were also encountered. The prevailing GII.4 variants in our study area belong to the New Orleans 2009 and Sydney 2012 strain, a putative novel GII.4 recombinant was encountered among study participants.

8. Published articles related to this research

Get more details on my research from these publications

1. Osazuwa, F., Grobler, H.S. & Johnson, W. Phylogenetic lineage of GII.17 norovirus identified among children in South–South, Nigeria. *BMC Res Notes* **13**, 347 (2020). <https://doi.org/10.1186/s13104-020-05185-0> (SNIP: 0.864)
2. Osazuwa F, Johnson, W, Grobler HS. Genetic lineage of genogroup I norovirus identified among children with diarrhea in Niger-Delta region, Nigeria. *Infectious Dis* 2019, 3: 213–215. (Impact Factor: 2.494)
3. Osazuwa F, Okojie R, Akinbo FO, Johnson W, Grobler HS. Genetic diversity of norovirus among children under 5 years in the South–South region of Nigeria. *New Zealand J Medical Laboratory Sciences* 2020, 74:(1) 39–43.

Conflict of interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This study was approved by the Ethics Committees of the Ministry of Health of Delta, Bayelsa and Edo States with reference numbers CHW/VOL14/130, FMCY/REC/ECC/2017/OC/046 and 732/T/89 respectively.

Footnote

The author is a doctoral student in Medical Molecular Biology at University of Benin.

Abbreviations

MUSCLE	Multiple Sequence Comparison by Log- Expectation
dNTPS	Dideoxynucleotide triphosphates
RNA	Ribonucleic Acid

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