Genetic characterisation of Norovirus strains in outpatient children from rural communities of Vhembe district/South Africa, 2014–2015

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ABSTRACT

Background: Norovirus (NoV) is now the most common cause of both outbreaks and sporadic non-bacterial gastroenteritis worldwide. However, data supporting the role of NoV in diarrheal disease are limited in the African continent.

Objectives: This study investigates the distribution of NoV genotypes circulating in outpatient children from rural communities of Vhembe district/South Africa.

Study design: Stool specimens were collected from children under five years of age with diarrhea, and controls without diarrhea, between July 2014 and April 2015. NoV-positive samples, detected previously by Realtime PCR, were analysed using conventional RT-PCR targeting the partial capsid and polymerase genes. Nucleotide sequencing methods were performed to genotype the strains.

Results: The sequence analyses demonstrated multiple NoV genotypes including GI.4 (13.8%), GI.5 (6.9%), GII.14 (6.9%), GII.4 (31%), GII.6 (3.4%), GII.P15 (3.4%), GII.P21 (3.4%) and GII.Pe (31%). The most prevalent NoV genotypes were GII.4 Sydney 2012 variants (n = 7) among the capsid genotypes, GII.Pe (n = 9) among the polymerase genotypes and GII.Pe/GII.4 Sydney 2012 (n = 8) putative recombinants among the RdRp/Capsid genotypes. Two unassigned GI.4 variants were found.

Conclusions: The findings highlighted NoV genetic diversity and revealed continuous pandemic spread and predominance of GII.Pe/GII.4 Sydney 2012, indicative of increased NoV activity. An unusual RdRp genotype GII.P15 and two unassigned GII.4 variants were also identified from rural settings of the Vhembe district/South Africa. NoV surveillance is warranted to help to inform investigations into NoV evolution and disease burden, and to support on-going vaccine development programmes.

1. Background

Diarrhea is more prevalent in the developing world [1,2], due mainly to the lack of safe drinking water and poor or non-existent sanitation infrastructure and hygiene practices. There are a billion more people without improved sanitation in rural areas than in urban areas [3]. Subsequently, a large proportion of rural communities are exposed to bacterial, viral and parasitic pathogens [2,4].

NoVs are single stranded, non-enveloped, positive-sensed RNA viruses [5,6] and the genus includes more than 35 different strains which are divided into seven genogroups. Each genogroup is further subdivided into genocenters based on sequence similarity and phylogenetic analysis [7–10]. The genetic diversity of the NoV genomes is a result of point mutations [11] and recombination between strains [12].

Based on amino acid variations in the major structural protein (VP1) [7,10], the seven genogroups of NoV are subdivided into 41 genotypes with 9 genotypes in GI, 22 in GII, 3 in GIII, 2 each in GIV–VI and one in GVII [7,10,13–15].

Based on the RNA-dependent RNA polymerase (RdRp) and capsid gene sequences, 31 polymerase genotypes and 22 capsid genotypes of GII NoV have been determined to date [16]. Of these, GII.4 is predominant, causing at least six epidemics of gastroenteritis worldwide over the past 20 years (1995–1996, 2002, 2004, 2006, 2009, and 2012) with the emergence and rapid global spread of viral variants [17]. The emergence of novel NoV GII.4 variants is likely to be driven by genetic recombination [18].
Currently there is scarcity of data on the NoV genotypes circulating in rural communities of Africa [19] and the impact of inadequate sanitation on the genetic diversity, disease burden and transmission of circulating NoV strains.

2. Objectives

This study aimed to provide genetic characteristics and distribution of NoV genotypes circulating in outpatient children with and without diarrhea from rural communities of Vhembe district/South Africa.

3. Study design

3.1. Sample collection, RNA extraction and NoV detection

Between July 2014 and April 2015, a cross-sectional study was performed involving outpatient children under 5 years of age with and without diarrhea, attending Primary Health Care (PHC) centres. Diarrhea was defined as three or more episodes of watery stool in the previous 24 h [20]. The study protocol and consent procedures were approved by the ethics committees of the Department of Health in the Limpopo Province (Ref. 4/2/2) and University of Venda (Ref. SMNS/13/MBY/0212). Written, informed consent was given by the parent or guardian of the child before stool sample collection. Stool samples were randomly collected at different clinics located in the rural communities of Vhembe district in Limpopo Province, South Africa and analysed at the molecular laboratory of the department of Microbiology, University of Venda. A total of 40 clinics were selected as sampling sites for this study. Demographic and clinical data from this study have been previously reported [21].

NoV RNA extraction using the Boom method and NoV detection using the one-step RIDA® GENE NOROVIRUS I & II real-time RT-PCR assay performed in this study were previously published [21].

3.2. NoV RT-PCR

Fifty RNA extracts that tested NoV positive by One-step Real-time PCR [21] were randomly selected and subjected to RT-PCR amplification for the purpose of nucleotide sequencing. The primers used for RT-PCR are listed in Table 1.

One step Ahead RT-PCR (QIAGEN) was performed using oligonucleotide primer sets GISKF/GISKR to amplify 330 bp of GI capsid fragment, GISKF/GISKR for 344 bp of a GII capsid fragment as previously described [22] or designed WGS 9F/WGS 9R primer pair to fragment, GISKF/GISKR for 344 bp of a GII capsid fragment as previously described [22] or designed WGS 9F/WGS 9R primer pair to amplify 330 bp of GI capsid for the purpose of nucleotide sequencing. The primers used for RT-PCR are listed in Table 1. The nucleotide sequences of NoV obtained from this study were compared with those of the reference strains available in the NCBI GenBank using BLAST tool available at http://www.ncbi.nlm.nih.gov/blast and were then analysed for their genotypes using Norona typing tools [24] available at http://www.rivm.nl/norovirus/typingtool before subjecting to phylogenetic tree construction using MEGA 7 software [25]. The reference strains from GenBank were randomly selected among the Blast hits with > 80% similarities on the query sequence of the NoV strains identified from this study. The neighboring method [26] was used to build the phylogenetic tree and the reliability of the different phylogenetic groupings was evaluated by bootstrap analysis (1000 replicates) [27]. The evolutionary distances were computed using the p-distance method [28].

Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to generate percent identity matrix of aligned sequences that were GII.4 not assigned.

The nucleotide sequences of NoV strains obtained from this investigation were submitted to NCBI GenBank under the following accession numbers: KY012277-KY012287 and KY548495-KY548501 for the capsid genotypes, KY012288-KY012295 and KY548502-KY548504 for the RdRp genotypes.

4. Results

4.1. Sequence analysis of capsid and polymerase region fragments

Out of 50 NoV positive samples subjected to RT-PCR amplification, Twenty-one specimens (42%, 21/50) were successfully amplified and sequenced for capsid or polymerase fragment region. Failure to obtain amplicons or good quality sequence could be due to the low viral load or RNA degradation in the samples. At least nine genotypes were identified among twenty-nine nucleotide sequences of GI (n = 6) and GII (n = 23) NoV strains obtained

Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence(5'-3')</th>
<th>Polarity</th>
<th>Genotype</th>
<th>Target</th>
<th>Size(bp)</th>
<th>Nucleotide position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GISKF</td>
<td>CTGGGCGAATGTTGAATAGA</td>
<td>F</td>
<td>GI NoV</td>
<td>Capsid</td>
<td>330 bp</td>
<td>5342</td>
<td>Kojima et al. [22]</td>
</tr>
<tr>
<td>GISKR</td>
<td>CCACACACACCTTCTTACTAC</td>
<td>R</td>
<td>GI NoV</td>
<td>Capsid</td>
<td>356</td>
<td>5671</td>
<td>Kojima et al. [22]</td>
</tr>
<tr>
<td>G2SKF</td>
<td>CNTGGGAGGGGCAATGCCGAA</td>
<td>F</td>
<td>GI NoV</td>
<td>Capsid</td>
<td>344 bp</td>
<td>5058</td>
<td>Kojima et al. [22]</td>
</tr>
<tr>
<td>G2SKR</td>
<td>CCGCNGCTACRCRTRRTRRAC</td>
<td>R</td>
<td>GI NoV</td>
<td>Capsid</td>
<td>546</td>
<td>5461</td>
<td>Kojima et al. [22]</td>
</tr>
<tr>
<td>JV12</td>
<td>ATACCACTAGTAYGGCAGATTA</td>
<td>F</td>
<td>GII and GI NoV</td>
<td>RdRp</td>
<td>326 bp</td>
<td>4552</td>
<td>Vinje et al. [23]</td>
</tr>
<tr>
<td>JV13</td>
<td>TCAATACATCCACGAGGAGGAG</td>
<td>R</td>
<td>GI and GII NoV</td>
<td>RdRp</td>
<td>487</td>
<td>4878</td>
<td>Vinje et al. [23]</td>
</tr>
<tr>
<td>WGS 9F</td>
<td>CACCCACAGTTGAGTCAAGAAC</td>
<td>F</td>
<td>GI NoV</td>
<td>Capsid</td>
<td>751 bp</td>
<td>5734</td>
<td></td>
</tr>
<tr>
<td>WGS 9R</td>
<td>GAGACTGCGCTCTTGATGAA</td>
<td>R</td>
<td>GI NoV</td>
<td>Capsid</td>
<td>6484</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Accession number of reference strain from Genbank used to design the primer WGS9F/9R: JN595867.1.
Table 2: Genotype distribution of identified NoV strains in stool specimens between July 2014 and April 2015 in Rural communities of Vhembe district/South. Africa.

<table>
<thead>
<tr>
<th>Genogroup</th>
<th>Genotype</th>
<th>RdRp n (%)</th>
<th>Capsid n (%)</th>
<th>RdRp/Capsid n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI</td>
<td>GI.4</td>
<td>4 (22.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GI.5</td>
<td>2 (11.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GII</td>
<td>GII.Pe</td>
<td>9 (81.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GII.14</td>
<td>2 (11.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GII.4</td>
<td>9 (50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GII.P15</td>
<td>1 (9.1)</td>
<td>1 (5.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GII.P21</td>
<td>1 (9.1)</td>
<td>1 (5.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>18</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*including GI.4 Sydney 2012 variant and GI.4 not assigned variant (n = 2, 11.7%).

Table 2 comprising:

1) GI.4, GI.5, GI.14, GI.6, GI.4 Sydney 2012 variant and GI.4 not assigned variant for the sequences with only the capsid fragment available.
2) GII.Pe, GII.P15 and GII.P21 for the sequences with only the RdRp fragment gene available.
3) GII.Pe/GI.4 Sydney 2012 variant for the sequences with both RdRp/Capsid fragments gene available.

As shown on Table 2, GI.4 Sydney 2012 variants (7/17, 41.2%) were predominant followed by GI.4 (4/17, 23.5%) among capsid genotypes. GII.Pe (9/11, 81.8%) was the predominant polymerase genotype and GII.Pe/GI.4 Sydney 2012 variants (8/8, 100%) were the predominant RdRp/Capsid genotypes.

4.2. Phylogenetic analyses of NoV GI, GII capsid genotypes and GII polymerase genotypes

Representative phylogenetic trees based on partial nucleotide sequences of RdRp and capsid genes were generated by the neighbor-joining method (Fig. 1 for the 344 bp of a GII capsid fragment; Fig. 2 for the 751 bp of a GII capsid fragment; Fig. 3 for 326 bp of the RdRp fragment and Fig. 4 for the 330 bp of GII capsid fragment).

The phylogenetic analyses in Fig. 1 revealed sequences KY102282 and KY102284, genotyped as GII.4 not assigned variants (Table 2), were closely related to GI.4 Sydney 2012 variant reference strains. Cluster analyses showed that sequence KY102282 was 97–99% similar to the reference strains KR904507.1 (South Africa), KF060126.1 (New Zealand), KF008241.1 (Paris), HQ003277.1 (Russia) and KY102284 was 95–98% similar to KX354140.1 (USA), KP335066.1 (China), KF060080.1 (Australia) and HQ003277.1 (Russia) reference strains (Supplement A).

With note, the phylogenetic analyses in Fig. 3 revealed that the unusual genotype GIP.15, identified in this study, has a common ancestor with Hiroshima/66/1110/06/JP accession number AB360387.1 clustering with other GII strains (KJ710248.1 and FM210353.1). This GIP.15 strain from Japan was previously reported as GII/untypeable recombinant strain implicated in a gastroenteritis outbreak [29].

Differing RdRp and Capsid genotypes, suggesting putative recombinant strains, were found in 8 NoV positive specimens including GII.Pe/GI.4 Sydney 2012 variant (n = 6) and GII.Pe/GI.4 not assigned.

Fig. 1. Phylogenetic tree based on 344-nucleotide sequence of the NoV GI capsid gene fragment. The Neighbor-Joining tree of the GI capsid strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GI.4 capsid genotypes, squared dots for GI.14 capsid genotypes and triangle dots indicated GI.6 genotype from this study. Only bootstrap values greater than 70% are shown. Thirty-three references strains of NoV were selected from Genbank and used to build this phylogenetic tree.
Further confirmatory analysis is currently pending.

5. Discussion

This study aimed to investigate the genetic characteristics of NoV strains circulating in rural communities of Vhembe district, South Africa, between July 2014 and April 2015, and represents one of the few studies on NoV-associated gastroenteritis from outpatients in rural settings of the African continent. A combined analysis of partial ORF1 and ORF2 regions that allow differentiation of RdRp polymerase genotypes, capsid genotypes and RdRp/Capsid genotypes of NoV was performed in this study. Multiple NoV genotypes (Table 2) found in the current study highlighted the diverse range of NoV genotypes circulating in rural communities of Vhembe district, South Africa.

The study findings showed the predominance of the GII.4 Sydney 2012 variant, which has been widely associated with an increased NoV activity and outbreaks across the globe [30–33]. Mans et al., 2016 reported GII.4 Sydney 2012 variants from hospitalised children in some regions of South Africa between 2009 and 2013 [34]. Our findings indicate the continuous pandemic spread of GII.4 Sydney 2012 variants between 2014 and 2015 in rural South Africa.

GII.Pe, which was the predominant polymerase genotype in the current study, has been involved in NoV outbreaks in several countries including Brazil [35], South Africa [34], Korea [36], China [37], Australia [38] and Japan [39]. Interestingly, the GII.Pe strains (Fig. 3) identified during this study period are generally far less related to the GII.Pe genotypes previously reported in South Africa (KR904759.1 and KR904767.1) between 2009 and 2013. These findings indicate the continuous evolution of NoVs in these rural communities of South Africa, possibly via accumulation of mutations and/or recombination [40] and support the need for surveillance of NoV genotypes. GII.Pe has been frequently reported in combination with capsid genotypes including GII.4 Sydney 2012 variant, GII.3 and GII.12 [38,41–43]. Bruggink et al., 2014 speculated that GII.Pe is considered as an obligatory recombinant in that no single GII.Pe genotype ever been reported [38]. This corroborates with our findings, where eight out of nine GII.Pe genotypes identified were in combination with GII.4 Sydney 2012 variant (Table 2).

GII.Pe/GII.4 Sydney variants emerged for the first time in Australia and became a pandemic NoV GII.4 variant [31]. GII.Pe/GII.4 Sydney variants are recombinants of GII.Pe polymerase and GII.4 capsid gene variant that have the common ancestor with Apeldoorn 2008 and New Orleans 2009 GII.4 variants [31]. The GII.Pe/GII.4 Sydney 2012 variant (n = 8) predominantly reported in this study have been considered as putative recombinants, although further confirmation needs to be done.

GI.4 (n = 4) and GI.5 (n = 2) capsid genotypes reported in this study have been previously found in waterborne NoV outbreaks and environmental sources in several countries including Italy, Thailand, Korea, South Africa and Sweden [44–49]. The presence of these GI capsid genotypes in clinical specimens from children in rural communities with poor hygiene practice and polluted environments [50] indicate water-borne NoV transmission despite the absence of direct molecular evidence of NoV contamination of water in this study. Environmental NoV studies are warranted to ascertain the environmental transmission route of NoV in the study area.

Other globally distributed genotypes identified in this study include:

- GII.14 genotypes first detected in 1999 in China [51] and later emerged as major genotype in Spain [52] and Japan [53]. GII.14 strains have been weakly associated with sporadic gastroenteritis in Brazil [54], in China [55] and several countries of Africa [56].
- GII.6, which has been associated with sporadic gastroenteritis and outbreaks in Japan [57], China [58], USA [59], Brazil [60], Finland [42] and numerous African countries [56];
GII.P21, which was previously reported in Denmark [61], Austria [62], India [63], Slovenia [64], Brazil [65] and some countries of Africa [56].

The rare GII.P15 polymerase genotype has also been found in this study and to our knowledge this is the first report of this unusual genotype in the African continent. GII.P15 has previously been identified as an intergenogroup (GII.P15/GVI) recombinant genotype (Mex 7076/99) in Japan [66] and was implicated in sporadic gastroenteritis cases. In studies of recombinant NoV implicated in gastroenteritis in Hiroshima Prefecture/Japan, Fukuda et al. [29] reported GII.P15 (GQ856474) as GII/untypeable strain (Hiroshima/66/1110/06/JP), which shares a common ancestor with the GII.P15 identified in this study (Fig. 3).

Jin et al. [67] found GILP15 strains in outpatients with sporadic gastroenteritis in Hiroshima Prefecture/Japan, Fukuda et al. [29] reported GILP15 (GQ856474) as GIL/untypeable strain (Hiroshima/66/1110/06/JP), which shares a common ancestor with the GILP15 identified in this study (Fig. 3).

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This study has some limitations. First, the NoV genotyping of isolates was only performed for selected number of NoV-positive samples because of the cost limit. However we have identified multiple genotypes within the selected sampling set. In addition, we were not able to complete the recombination analyses to confirm the recombinant genotypes, given that the sequences used for our NoV characterisation did not span the entire region of the ORF1-ORF2 junction where most of NoV recombinant events are detected. This report describes the NoV genetic diversity detected from children under five with diarrhea, attending PHC centres in rural communities. We previously reported a 41.1% prevalence of NoV in this study area [21] demonstrating frequent exposure of young children to enteric pathogens. Considering that all the children with gastroenteritis disease may not present to any healthcare facilities, what we have reported could be the tip of iceberg. The finding of high levels of NoV prevalence rate in this study area and the predominance of GII.4 Sydney 2012 genotype observed in the current report, which has been widely associated with increased NoV outbreaks [31], suggest that there was increased NoV activity in the study region. This has not been described before since there is no surveillance of NoV in South Africa, and this finding may also be the case throughout Africa. Detection and characterisation of NoV genotypes in Africa may be useful to confirm the role of the NoV in diarrheal disease across the continent and assist the ongoing NoV vaccine development. Further efforts should be deployed to promptly investigate epidemic cases of NoV. This requires the implementation of systematic surveillance of NoV associated gastro-enteritis in African continent.

Fig. 3. Phylogenetic tree based on 326-nucleotide sequence of NoV GII polymerase gene fragment. The Neighbor-Joining tree of the GII polymerase strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GII.Pe polymerase genotypes, squared dot for GII.P15 polymerase genotype and triangle dot for GII.P21 polymerase genotypes from this study. Only bootstrap values greater than 70% are shown. Seventeen reference strains of NoV were selected from Genbank and used to build this phylogenetic tree.

GII.P21, which was previously reported in Denmark [61], Austria [62], India [63], Slovenia [64], Brazil [65] and some countries of Africa [56].

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Competing interest

None declared.
Fig. 4. Phylogenetic tree based on 330 nucleotide sequence of NoV GI capsid gene fragment. The Neighbor-Joining tree of the GI capsid strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GI.4 capsid genotypes and squared dots for GI.5 capsid genotypes from this study. Only bootstrap values greater than 70% are shown. Twenty reference strains of NoV were selected from Genbank and used to build this phylogenetic tree.

Acknowledgements

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We are grateful to the provincial executive of Limpopo department of Health, the district executive and public health care managers as well as nurses who help to collect stool specimens and complete this research study in Vhembe district/South Africa. We thank Prof Sali Troare and Prof Amidou Samie (Department of Microbiology, University of Venda) for their assistance and advice during this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2017.07.005.

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