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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 GII.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 genoclusters 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].

Abbreviations: PHC, Primary Health Care; RSA, Republic of South Africa; UK, United Kingdom

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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 out-patient children under 5 years of age with and without diarrhoea, 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, GIISKF/GIISKR for 344 bp of a GII capsid fragment as previously described [22] or designed WGS 9F/WGS 9R primer pair to amplify a 751 bp product in those samples that did not yield products with the GIISK primers. Briefly a 10% diluted 5 µl of RNA extract was transcribed into cDNA for 10 min at 50 °C followed by heat inactivation of reverse transcriptase at 95 °C for 5 min, and then amplified using 0.5 µM of each oligonucleotide primer in a final reaction volume of 25 µl. PCR was carried out for 40 cycles using the following thermocycling conditions: denaturation for 10 s at 95 °C, annealing for 10 s at 56 °C (GII SK primers) or 50 °C (GISK primers or WGS 9 primers) and extension for 10 s at 72 °C followed by a final extension for 10 min at 72 °C.

Primers set JV12/JV13 were used to amplify 326 bp of the RdRp fragment as previously described [23]. PCR conditions for polymerase gene fragment amplification consisted of denaturation for 10 s at 95 °C, annealing for 10 s at 50 °C and extension for 10 S at 72 °C and a final extension for 10 min at 72 °C.

The amplicons were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and specific sized bands visualised using a UV light transilluminator. SimplyLoad 100 bp DNA ladder (Lonza) was used for every electrophoretic gel.

3.3. Genotyping and phylogenetic analysis of NoV strains

The PCR products of the amplified fragments were directly purified with a master mix of ExoSAP (Nucleics, Australia). Using the same specific primers, the Sanger sequencing was performed on the ABI 3500XL Genetic Analyzer POP7™ (Thermo-Scientific). The raw sequence reads were edited with Finch TV v1.4 (Geospiza, Seattle, USA).

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 Noronet typing tools [24] available at http://www.rivm.nlm/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 neighbor-joining 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
Primers used for Norovirus genotyping in this study.

Primers	Sequence(5′-3′)	Polarity	Genotype	Target	Size(bp)	Nucleotide position	Reference
G1SKF	CTGCCCGAATTYGTAAATGA	F	GI NoV	Capsid	330 bp	5342	Kojima et al. [22]
G1SKR	CCAACCCARCCATTRTACA	R	GI NoV	Capsid		5671	Kojima et al. [22]
G2SKF	CNTGGGAGGGCGATCGCAA	F	GII NoV	Capsid	344 bp	5058	Kojima et al. [22]
G2SKR	CCRCCNGCATRHCCRTTRTACAT	R	GII NoV	Capsid		5401	Kojima et al. [22]
JV12	ATACCACTATGATGCAGATTA	F	GI and GII NoV	RdRp	326 bp	4552	Vinje et al. [23]
JV13	TCATCATCACCATAGAAAGAG	R	GI and GII NoV	RdRp		4878	Vinje et al. [23]
WGS 9F	CACCCACAGTTGAGTCAAGAAC	F	GII NoV	Capsid	751 bp	5734[a]	
WGS 9R	GGAGCTGCCTCTTGGTAGA	R	GII NoV	Capsid		6484[a]	

[a] 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.

Genogroup	Genotype		Genotype		Genotype	
	RdRp	n (%)	Capsid	n (%)	RdRp/Capsid	n (%)
GI			GI.4	4 (22.2)		
			GI.5	2 (11.1)		
GII	GII.Pe	9 (81.8)	GII.14	2 (11.1)	GII.Pe/GII.4	8(100)
	GII.P15	1 (9.1)	GII.4	9 (50)[a]		
	GII.P21	1 (9.1)	GII.6	1 (5.6)		
Total		11		18		8

[a] Including GII.4 Sydney 2012(n = 7, 41.2%) variant and GII.4 not assigned variant (n = 2, 11.7%).

(Table 2) comprising:

1) GI.4, GI.5, GII.14, GII.6, GII.4 Sydney 2012 variant and GII.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/GII.4 Sydney 2012 variant for the sequences with both RdRp/Capsid fragments gene available.

As shown on Table 2, GII.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/GII.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 GI capsid fragment).

The phylogenetic analyses in Fig. 1 revealed sequences KY012282 and KY012284, genotyped as GII.4 not assigned variants (Table 2), were closely related to GII.4 Sydney 2012 variant reference strains. Clustal analyses showed that sequence KY012282 was 97–99% similar to the reference strains KR904507.1 (South Africa), KF060126.1 (New Zealand), KF008241.1 (Paris), HQ003277.1 (Russia) and KY012284 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 GIIP15, 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 GIIP15 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/GII.4 Sydney 2012 variant (n = 6) and GII.Pe/GII.4 not assigned

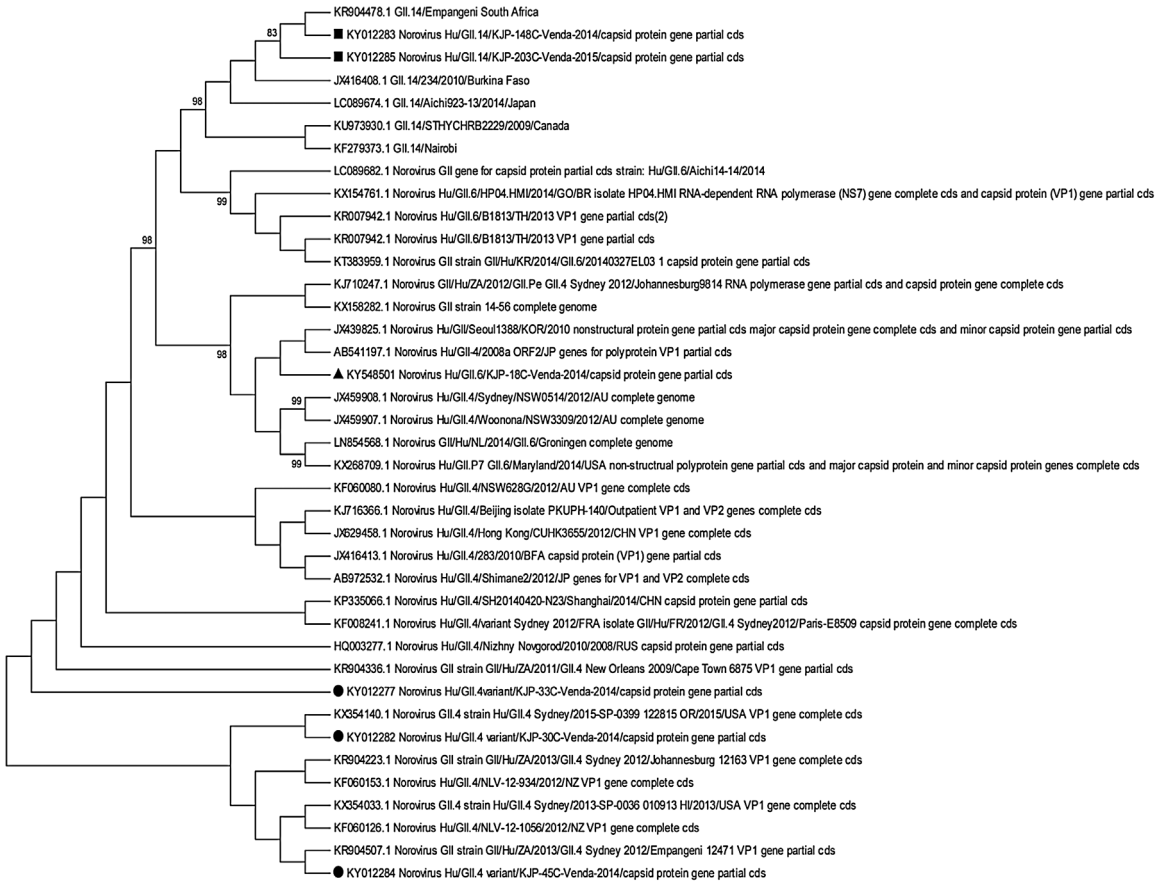

Fig. 1. Phylogenetic tree based on 344-nucleotide sequence of the NoV GII capsid gene fragment. The Neighbor-Joining tree of the GII capsid strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GII.4 capsid genotypes, squared dots for GII.14 capsid genotypes and triangle dots indicated GII.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.

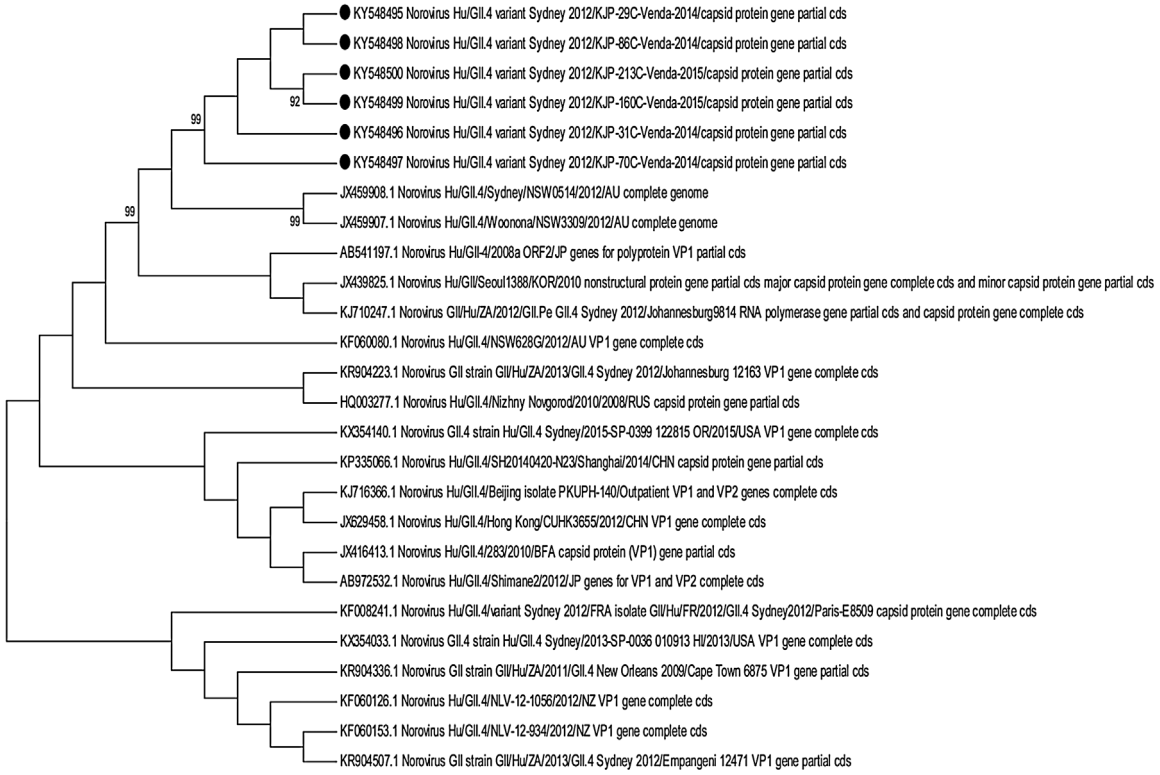

Fig. 2. Phylogenetic tree based on 751-nucleotide sequence of the NoV GII capsid gene fragment. The Neighbor-Joining tree of the GII.4 capsid strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GII.4 capsid genotypes from this study. Only bootstrap values greater than 70% are shown. Twenty representative references strains of NoV were selected from Genbank and used to build this phylogenetic tree.

(n = 2). 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 genetically 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 variant recombinants 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];

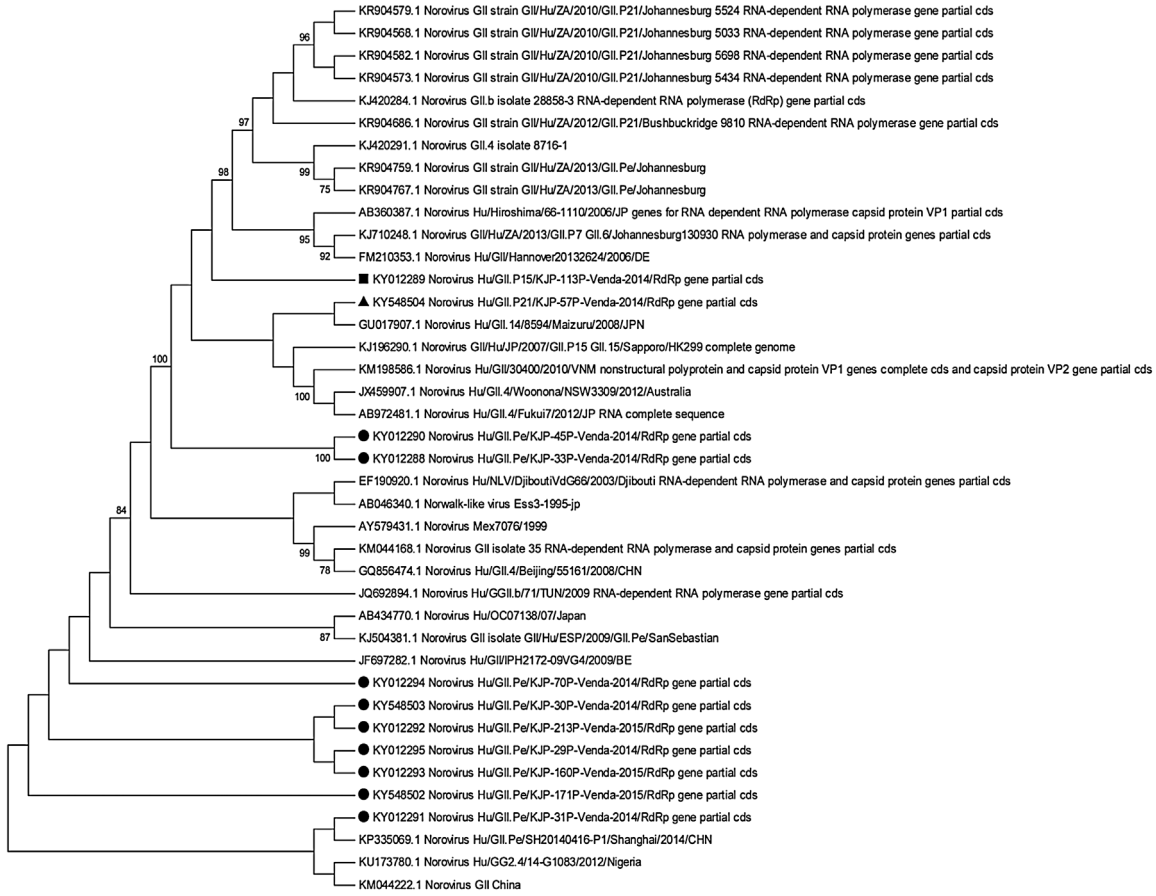

Fig. 3. Phylogenetic tree based on 326-nucleotide sequence of NoV GII polymerase gene fragment. The Neighbor-Joining tree of the GII polymerase strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GII.Pe polymerase 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].

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 GII.P15 strains in outpatients with sporadic gastroenteritis in China and classified them simply as GII genogroup since a genotype could not be assigned based on the partial polymerase sequence. Recently Fumian et al. [65] reported the presence of GIIP15/GII15 genotyped as a non-recombinant form in a NoV outbreak in Southern Brazil. The existence of numerous GII.4 recombinant variants circulating with the uncommon genotypes ensures enormous potential for novel viral recombination [68]. Continuous monitoring of the strains distribution in the population will allow the identification of epidemic-causing strains in real-time.

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 on-going 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 gastroenteritis in African continent.

Competing interest

None declared.

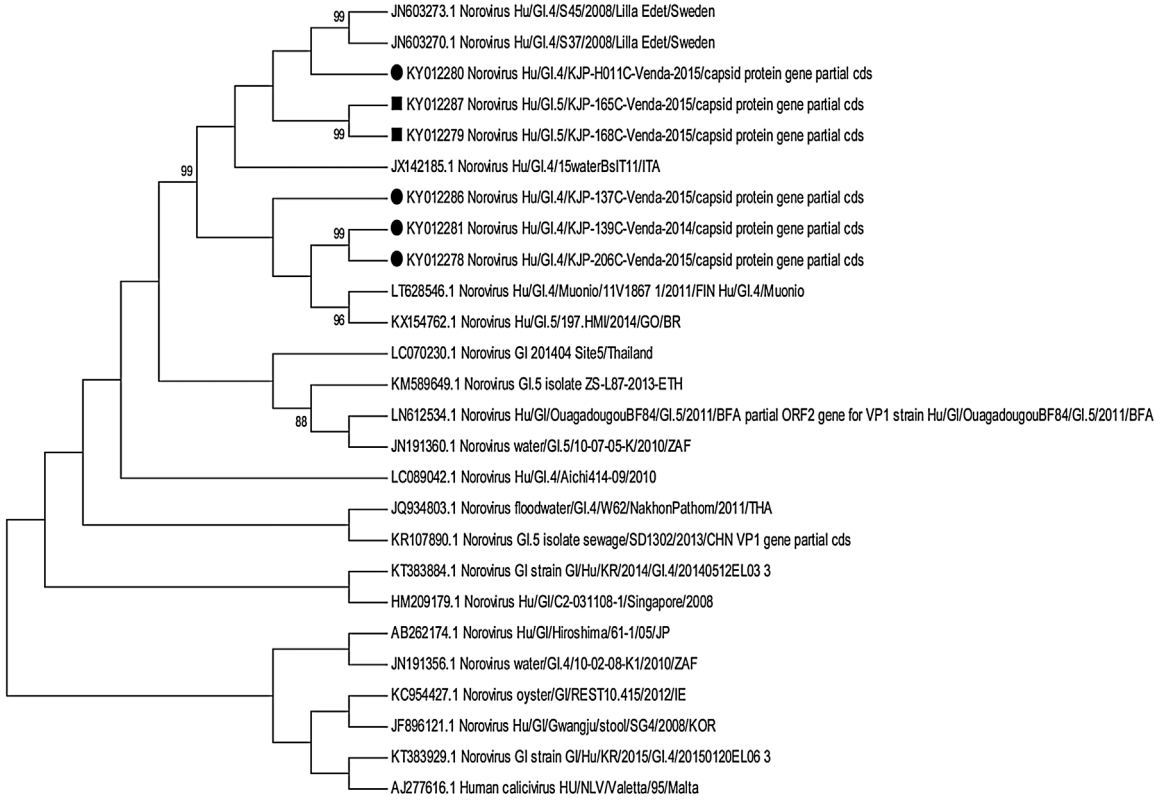

Fig. 4. Phylogenetic tree based on 330-nucleotide sequence of NoV GI capsid gene fragment. The Neighbor-Joining tree of the GI capsid strains circulating between July 2014 and April 2015 in the rural communities of Vhembe district/South Africa. Round Black dots indicated the GI.4 capsid genotypes and 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.

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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.

References

[1] L. Liu, S. Oza, D. Hogan, et al., Global, regional, and national causes of child mortality in 2000–13, with projections to inform post-2015 priorities: an updated systematic analysis, Lancet 385 (2015) 430–440.
[2] UNICEF/WHO, Diarrhoea: Why Children Are Still Dying and What can Be Done, (2009) Available at: http://www.who.int/child_adolescent_health/documents/9789241598415/en/index.html.
[3] UNICEF/WHO, Progress on Sanitation and Drinking Water-2015 Update and MDG Assessment, UNICEF, New York, 2015https://sanitationupdates.wordpress.com/2015/07/01/unicefwho-progress-on-sanitation-and-drinking-water-2015-update-and-mdg-assessment/.
[4] UNICEF, Progress for Children. A Report Card on Water and Sanitation, (2006) Available at. http://www.unicef.org/publications/files/Progress_for_Children_No._5_-_English.pdf.
[5] X. Jiang, M. Wang, K. Wang, M.K. Estes, Sequence and genomic organization of Norwalk virus, Virology 195 (1993) 51–61.
[6] X. Jiang, D.Y. Graham, K. Wang, M.K. Estes, Norwalk virus genome cloning and characterization, Science 250 (1990) 1580–1583.
[7] S. Ramani, R.L. Atmar, M.K. Estes, Epidemiology of Human noroviruses and updates on vaccine development, Curr. Opin. Gastroenterol. 30 (2014) 25–33.
[8] M.M. Patel, A.J. Hall, J. Vinje, U.D. Parashar, Noroviruses: a comprehensive review, J. Clin. Virol. 44 (2009) 1–8.
[9] L.C. Donaldson Ef Lindesmith, A.D. Lobue, R.S. Baric, Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations, Immunol. Rev. 225 (2008) 190–211.
[10] D.P. Zheng, T. Ando, R.L. Fankhauser, R.S. Beard, R.I. Glass, S.S. Monroe, Norovirus classification and proposed strain nomenclature, Virology 346 (2006) 312–323.
[11] K.E. Dingle, Norovirus infection control in Oxfordshine communities hospitals: mutation in a Lordsdale Norovirus epidemic strain as a potential indicator of transmission routes, J. Clin. Microbiol. 42 (9) (2004) 3950–3957.
[12] J. Rohayem, J. Munch, A. Rethwilm, Evidence of recombination in the Norovirus capsid gene, J. Virol. 79 (8) (2005) 4977–4990.
[13] Vinjé, Advances in laboratory methods for detection and typing of norovirus, J. Clin. Microbiol. 53 (2) (2015) 373–381.
[14] CDC-Division of viral diseases, national center for immunization and respiratory diseases, centers for disease control and prevention, Updated Norovirus outbreak management and disease prevention guidelines, MMWR. Morb. Mortal. Wkly. Rep. 60 (2011) 3.
[15] K.Y. Green, Caliciviridae: the norovirus, 5th edn, in: K. DM, H. PM (Eds.), Fields Virology, Vol. 1 Walters Klumer/Lippincott Williams & Wilkins, Philaldelphia, PA, 2007, pp. 949–979 (chapter 28).
[16] A. Kroneman, E. Vega, H. Vennema, et al., Proposal for a unified norovirus nomenclature and genotyping, Arch. Virol. 158 (10) (2013) 2059–2068, http://dx.doi.org/10.1007/s00705-013-1708-5 (Epub 2013 Apr 25).
[17] S.M. Karst, R.S. Baric, What is the reservoir of emergent human Norovirus strains? J. Virol. 89 (11) (2015) 5756–5759, http://dx.doi.org/10.1128/JVI.03063-14 (Epub 2015 Mar 18).
[18] J.S. Eden, M.M. Tanaka, M.F. Boni, W.D. Rawlinson, A. Peter, P.A. White, Recombination within the pandemic norovirus GII.4 lineage, J. Virol. 87 (11) (2013) 6270–6282.
[19] J.P. Kabue, E. Meader, P.R. Hunter, N. Potgieter, Human Norovirus prevalence in Africa: a review of studiesfrom 1990 to 2013, Trop. Med. Int. Health 21 (1) (2016) 2–17, http://dx.doi.org/10.1111/tmi.12617 (Review).
[20] World Health Organization (WHO), Treatment of Diarrhea: A Manual for Physicians and Senior Health Workers, (2005) http://whqlibdoc.who.int/publications .

(Accessed 24 June, 2015).
[21] J.P. Kabue, E. Meader, P.R. Hunter, N. Potgieter, Norovirus prevalence and estimated viral load in symptomatic and asymptomatic children from rural communities of Vhembe district, South Africa, J. Clin. Virol. 84 (2016) 12–18, http://dx.doi.org/10.1016/j.jcv.2016.09.005.
[22] S. Kojima, T. Kageyama, S. Fukushi, et al., Genogroup-specific PCR primers for detection of Norwalk-like viruses, J. Virol. Methods 100 (1–2) (2002) 107–114.
[23] J. Vinje, H. Vennema, L. Maunula, et al., International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses, J. Clin. Microbiol. 41 (4) (2003) 1423–1433.
[24] A. Kroneman, H. Vennema, K. Deforche, et al., An automated genotyping tool for enteroviruses and noroviruses, J. Clin. Virol. 51 (2) (2011) 121–125.
[25] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874, http://dx.doi.org/10.1093/molbev/msw054 (Advance Access publication March 22, 2016).
[26] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[27] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution 39 (1985) 783–791.
[28] M. Nei, S. Kumar, Molecular Evolution and Phylogenetics, Oxford University Press, New York, 2000.
[29] S. Fukuda, Y. Sasaki, S. Takao, M. Seno, Recombinant norovirus implicated in gastroenteritis outbreaks in Hiroshima prefecture, Japan, J. Med. Virol. 80 (2008) 921–928.
[30] J.S. Eden, J. Hewitt, K.L. Lim, et al., The emergence and evolution of the novel epidemic norovirus GII.4 variant Sydney 2012, Virology 450–451 (2014) 106–113.
[31] J. van Beek, K. Ambert-Balay, N. Botteldoorn, et al., Indications for worldwide increased norovirus activity associated with emergence of a new variant of genotype II.4, late 2012, Euro Surveill. 18 (2013) 8–9 (PMID: 23305715).
[32] M.C. Chan, P.K. Chan, Complete genome sequence of a novel recombinant human norovirus genogroup ii genotype 4 strain associated with an epidemic during summer of 2012 in Hong Kong, Genome Announc. 1 (1) (2013) 00140–212.
[33] CDC Prevention, Emergence of new norovirus strain GII.4 Sydney-United States, 2012, MMWR. Morb. Mortal. Wkly. Rep. 62 (2013) 55.
[34] J. Mans, T.Y. Murray, S. Nadan, R. Netshikweta, N.A. Page, M.B. Taylor, Norovirus diversity in children with gastroenteritis in South Africa from 2009 to 2013: GII.4 variants and recombinant strains predominate, Epidemiol. Infect. 144 (5) (2016) 907–916, http://dx.doi.org/10.1017/s0950268815002150 (Epub 2015 Sep 16).
[35] S.G. Morillo, A. Luchs, A. Cilli, et al., Norovirus GII. pe genotype: tracking a foodborne outbreak on a cruise ship through molecular epidemiology, Brazil, 2014, Food Environ. Virol. (2016), http://dx.doi.org/10.1007/s12560-016-9272-2.
[36] J.S. Kim, H. Kim, J. Hyun, H.S. Kim, W. Song, Molecular epidemiology of human Norovirus in Koreain 2013, BioMed. Res. Int. (2015) 468304.
[37] X. Wu, J. Han, L. Chen, et al., Prevalence and genetic diversity of norovirus in adults with acute gastroenteritis in Houzhou, China, 2013–2014, Arch. Virol. 160 (2015) 1705–1713.
[38] L.D. Bruggink, N.L. Dunbar, J.A. Marshall, Emergence of GII.e as a major ORF 1 norovirus genotype and its associated ORF 2 GII.4 variant forms, Infect. Genet. Evol. 22 (2014) 157–163.
[39] A. Thongprachum, W. Chan-it, P. Khamrin, et al., Molecular epidemiology of norovirus associated with gastroenteritis and emergence of norovirus GII.4 variant 2012 in Japanese pediatric patients, Infect. Genet. Evol. 23 (2014) 65–73, http://dx.doi.org/10.1016/j.meegid.2014.01.030 (Epub 2014, Feb 4).
[40] V. Martella, M.C. Medici, De Grazia, et al., Evidence for recombination between pandemic GII.4 norovirus strains New Orleans 2009 and Sydney 2012, J. Clin. Microbiol. 51 (2013) 3855–3857.
[41] E. Mathijs, S. Denayer, L. Palmeira, et al., Novel norovirus recombinants and of GII. 4 sublineages associated with outbreaks between 2006 and 2010 in Belgium, Virol. J. 8 (2011) 310, http://dx.doi.org/10.1186/1743-422X-8-310 (PubMed: 21682917).
[42] L. Puustinen, V. Blazevic, M. Salminen, M. Hamalainen, S. Rasanen, T. Vesikari, Noroviruses as a major cause of acute gastroenteritis in children in Finland, 2009–2010, Scand. J. Infect. Dis. 43 (2011) 804–808.
[43] J.S. Eden, R.A. Bull, E. Tu, et al., Norovirus GII. 4 variant 2006 b caused epidemics of acute gastroenteritis in Australia during 2007 and 2008, J. Clin. Virol. 49 (2010) 265–271.
[44] I. Di Bartolo, E. Pavoni, S. Tofani, et al., Waterborne norovirus outbreak during a summer excursion in Northern Italy, New Microbiol. 38 (2015) 109–112.
[45] K. Inoue, K. Motomura, M. Boonchan, et al., Molecular detection and characterization of noroviruses in river water in Thailand, Lett. Appl. Microbiol. 62 (3) (2016) 243–249, http://dx.doi.org/10.1111/lam.12529.
[46] G.C. Lee, M. Kim, J.I. Kim, C.H. Lee, Occurrence and molecular characterization of noroviruses in Korean surface water between 2007 and 2010, J. Microbiol. Biotechnol. 24 (4) (2014) 556–562, http://dx.doi.org/10.4014/jmb.1311.11089.
[47] J. Mans, R. Netshikweta, M. Magwalivha, W.B. Van Zyl, M.B. Taylor, Diverse norovirus genotypes identified in sewage-polluted river water in South Africa, Epidemiol. Infect. 141 (2013) 303–313.
[48] T.Y. Murray, J. Mans, M.B. Taylor, Human calicivirus diversity in wastewater in South Africa, J. Appl. Microbiol. 114 (2013) 1843–1853.
[49] N.P. Nenonen, C. Hannoun, C.U. Larsson, T. Bergstr€om, Marked genomic diversity of norovirus genogroup I strains in a waterborne outbreak, Appl. Environ. Microbiol. 78 (2012) 1846–1852.
[50] N. Potgieter, M. de Beer, M.B. Taylor, A.D. Steele, Prevalence and Diversity of Rotavirus strains in Children with acute diarrhea from rural communities in the Limpopo Province, South Africa, from 1998 to 2000, J. Infect. Dis. 202 (Suppl. (1)) (2010) 148–155.
[51] M. Jin, H.P. Xie, Z.J. Duan, et al., Emergence of the GII4/2006 b variant and recombinant noroviruses in China, J. Med. Virol. 80 (2008) 1997–2004.
[52] C.F. Manso, J.L. Romalde, Molecular epidemiology of norovirus from patients with acute gastroenteritis in northwestern Spain, Epidemiol. Infect. 143 (2) (2015) 316–324.
[53] W. Chan-It, A. Thongprachum, S. Okitsu, et al., Detection and genetic characterization of norovirus infections in children with acute gastroenteritis in Japan, 2007–2009, Clin. Lab. 57 (3–4) (2011) 213–220.
[54] J. de Andradea, J.S. Rochaa, F.A. Carvalho-Costa, et al., Noroviruses associated with outbreaks of acute gastroenteritis in theState of Rio Grande do Sul, Brazil, 2004–2011, J. Clin. Virol. 61 (2014) 345–352.
[55] D. Tan, L. Deng, M. Wang, X. Li, Y. Ma, W. Liu, High prevalence and genetic diversity of noroviruses among children with sporadic acute gastroenteritis in Nanning City, China, 2010–2011, J. Med. Virol. 87 (2015) 498–503.
[56] J. Mans, G.E. Armah, A.D. Steele, M.B. Taylor, Norovirus epidemiology in Africa: a review, PLoS One 11 (4) (2016) e0146280, http://dx.doi.org/10.1371/journal.pone.0146280.
[57] W. Chan-It, A. Thongprachum, P. Khamrin, et al., Emergence of a new norovirus GII.6 variant in Japan, 2008–2009, J. Med. Virol. 84 (2012) 1089–1096.
[58] L.F. Luo, K. Qiao, X.W. Wang, K.Y. Ding, H.L. Su, C.Z. Li, H.J. Yan, Acute gastroenteritis outbreak caused by a GII.6 norovirus, World J. Gastroenterol. 21 (17) (2015) 5295–5302.
[59] Z. Yang, J. Vinjé, C.A. Elkins, M. Kulka, Complete genome sequence of human norovirus strain GII. P7-GII. 6 detected in a patient in the United States in 2014, Genome Announc. 4 (5) (2016) e01211–e01216, http://dx.doi.org/10.1128/genomeA.01211-16.
[60] M.S.R. Ferreira, M. Victoria, F.A. Carvalho-Costa, et al., Surveillance of norovirus infections in the state of Rio De Janeiro, Brazil 2005–2008, J. Med. Virol. 82 (2010) 1442–1448.
[61] K.T. Franck, J. Fonager, A.K. Ersbøll, B. Böttiger, Norovirus epidemiology in community and health care settings and association with patient age, Denmark, Emerg. Infect. Dis. 20 (7) (2014) 1123–1131.
[62] Y.C. Lin, E. Hipfl, I. Lederer, F. Allerberger, D. Schmid, A norovirus GII.P21 outbreak in a boarding school, Austria 2014, Int. J. Infect. Dis. 37 (2015) 25–29.
[63] T. Hoa-Tran, T. Toyoko Nakagomi, D. Sano, et al., Molecular epidemiology of noroviruses detected in Nepalese children with acute diarrhea between 2005 and 2011: increase and predominance of minor genotype GII.13, Infect. Gen. Evol. 30 (2015) 27–36.
[64] A. Steyer, T.K. Godič, A.I. Gutierrez, M. Poljšak, High prevalence of enteric viruses in untreated individual drinking water sources and surface water in Slovenia, Int. J. Hyg. Environ. Health 214 (2011) 392–398.
[65] T.M. Fumian, J. da Silva Ribeiro de Andrade, J.P. Leite, M.P. Miagostovich, Norovirus recombinant strains isolated from gastroenteritis outbreaks in Southern Brazil, 2004–2011, PLoS One (2016) e0145391, http://dx.doi.org/10.1371/journal.pone.0145391 (eCollection 2016).
[66] T.G. Phan, K. Kaneshi, Y. Ueda, et al., Genetic heterogeneity, evolution, and recombination in noroviruses, J. Med. Virol. 79 (2007) 1388–1400.
[67] M. Jin, J. Chen, X.M. Zhang Jin, J. Chen, X.H. Zhang, M. Zhang, H.Y. Li, W.X. Cheng, N. Liu, M. Tan, T. Jiang, Z.J. Duan, et al., Genetic diversity of noroviruses in Chinese adults: potential recombination hotspots and GII-4/Den Haag-specific mutations at a putative epitope, Infect. Gen. Evol. 11 (2011) 1716–1726.
[68] R.A. Bull, P.A. White, Mechanisms of GII.4 norovirus evolution, Trends Microbiol. 19 (2011) 233–240, http://dx.doi.org/10.1016/j.tim.2011.01.002 (PMID: 21310617).