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Emerging norovirus GII.4 2008 variant detected in hospitalised paediatric patients in South Africa

Janet Mans[a,*], J. Corrie de Villiers[b], Nicolette M. du Plessis[c], Theunis Avenant[c], Maureen B. Taylor[a,b]

[a] Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, P.O. Box 2034, Pretoria 0001, Gauteng, South Africa
[b] National Health Laboratory Service, Tshwane Academic Division, Pretoria, South Africa
[c] Paediatric Infectious Diseases Unit, Faculty of Health Sciences, University of Pretoria, South Africa

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ABSTRACT

Background: Noroviruses (NoVs) are important enteric pathogens that cause gastroenteritis worldwide. The first documented NoV outbreaks in South Africa (SA) were described in 1993. The current NoV prevalence and circulating genotypes are unknown. SA lacks NoV outbreak reporting systems and therefore the number and impact of NoV infections is underestimated.
Objectives: This study aimed to determine the prevalence and genetic diversity of NoV infections in hospitalised paediatric patients with gastroenteritis in SA during 2008.
Study design: Stool specimens referred for virological analysis from hospitalised children ≤13 years, with gastroenteritis, were screened for rotavirus, human adenovirus and human astrovirus by enzyme immunoassay and for NoV genogroup I (GI), II (GII) and sapovirus by real-time RT-PCR. NoV strains were genotyped, and variants identified, based on sequence and phylogenetic analyses of the 5′ end or the full length of the capsid gene, respectively.
Results: Rotavirus was the most prevalent virus detected in 24.2% (61/252) of specimens, followed by NoV in 14.3% (35/245) and adenovirus, astrovirus and sapovirus in 9.6%, 6.7% and 4% of specimens, respectively. NoVs were only detected in children ≤2 years. The GII NoVs (89%) predominated and eight types were identified with GII.4 (43%) detected most frequently. The emerging 2008 GII.4 variant represented 80% of the GII.4 strains.
Conclusions: A diverse range of NoV genotypes were identified in hospitalised children with gastroenteritis. The 2008 GII.4 variant was the most frequently detected strain in the study. This is the first report of NoV GII.4 viruses in SA.

1. Background

Viral gastroenteritis can cause life threatening disease in infants and young children, especially in the developing world.[1] Viruses implicated most frequently are group A rotaviruses (RV), human caliciviruses (HuCVs), enteric adenoviruses (AdVs) types 40/41 and human astroviruses (HAstVs).[2] Noroviruses (NoVs), classified in the *Caliciviridae* family, are small, non-enveloped RNA viruses (single-stranded, positive-sense genome, ~7.7 kb). Based on RNA polymerase or capsid gene sequence analysis, NoVs are divided into five genogroups. The genogroups are further divided into genotypes, currently there are eight types in genogroup I (GI), at least 17 in GII and two in GIII.[3] GI, GII and GIV infect humans[4] with GII.4 strains currently the leading cause of NoV outbreaks worldwide.[5] GII.4 variants emerge almost annually, spread rapidly and some become dominant for several seasons.[6] NoVs infect persons of all ages and cause gastroenteritis 10–51 h after infection. Symptoms may include fever, nausea, abdominal cramps, vomiting and watery diarrhoea.[4]

Initially described after a gastroenteritis outbreak at a school in Norwalk, Ohio in 1968,[7] NoVs are now known to cause large gastroenteritis epidemics worldwide.[8–10] Outbreaks often occur due to contaminated food or water and are difficult to control because of the high environmental stability, low infectious dose and high rate of secondary spread characteristic of NoVs.[11] Over the last 3 years a number of studies have investigated the prevalence of NoV infection in hospitalised children.[5] A wide range of prevalences (6–48%) were reported with a median of 14%,[5] and in some studies NoV-associated gastroenteritis was almost as common as RV infections[12–14] indicating that NoVs may be clinically more relevant than previously thought.

Abbreviations: AdV, adenovirus; EIA, enzyme immunoassay; G, genogroup; HAstV, human astrovirus; HIV, human immunodeficiency virus; NoV, norovirus; RT-PCR, reverse transcriptase-polymerase chain reaction; RV, rotavirus; SaV, sapovirus; SA, South Africa.

* Corresponding author. Tel.: +27 12 319 2534; fax: +27 12 325 5550.
E-mail addresses: janet.mans@tuks.co.za, janetmans0001@yahoo.com (J. Mans).

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Table 1
(a) Prevalence of different enteric viruses in hospitalised paediatric patients with gastroenteritis in 2008 at the Steve Biko and Kalafong Academic hospitals, Gauteng, South Africa, (b) Norovirus (NoV) genogroup distribution, and (c) Viral co-infections in NoV positive specimens.

(a) Virus	Positive specimens/Total number tested	Prevalence (%)
Group A RV[a]	61/252	24.21
NoV GI + GII[b]	35/245	14.29
AdV 40/41[a]	23/240	9.58
HAstV[a]	16/239	6.69
SaV[b]	10/245	4.08
(b) NoV genogroup	NoV positive samples/Total	(%)
Genogroup I	3/245	1.22
Genogroup II	31/245	12.65
GI + GII	1/245	0.41
Total	35/245	14.29
(c) Distribution of mixed and single-virus infections in NoV positive specimens		(%)
Positive for NoV	35[c]	100
NoV only detected	27	77.1
Two viruses detected		
NoV, RV	2	5.7
NoV, AdV	1	2.9
NoV, SaV	1	2.9
Three viruses detected		
NoV, RV, AdV	3	8.5
NoV, RV, HAstV	1	2.9

[a] Tested by EIA.
[b] Tested by real-time RT-PCR. Adenovirus (AdV), human astrovirus (HAstV), norovirus genogroup I or II (NoV GI, GII), rotavirus (RV), sapovirus (SaV).
[c] 12/35 NoV positive specimens were screened for bacteria and parasites and all were negative.

NoV-associated gastroenteritis outbreaks were first reported in South Africa (SA) in 1993.[15] The Norwalk (GI.1) and Hawaii (GII.1) strains were identified as the causative agents in the respective outbreaks. In 1995, Mexico-like viruses (GII.3) were identified in paediatric stool specimens from sporadic gastroenteritis cases.[16] In 1997, the prevalence of HuCV infection in SA was estimated to be 3.3%, with NoVs representing 89% and sapoviruses (SaVs) 11%.[17] NoV GI and GII viruses were identified, but the GII strains (Mexico-like) predominated. A seroepidemiological study in the Pretoria area of SA indicated that 57% of children were exposed to NoV by 1–2 years of age and that 62% of the population was seropositive by the age of 40.[18] A later study reported NoV antibody prevalence levels of 94–96% in both urban and rural South African populations.[19] It is evident that NoV infections in SA occur early in life and affect all groups of the population. In the past 10 years no data on NoV prevalence or circulating genotypes have been reported for SA. There is also no mandatory gastroenteritis outbreak reporting system in the health care/old-age home setting. Therefore sporadic cases and outbreaks of NoV may be underreported and the prevalence of these infections underestimated.

2. Objectives

The aim of this study was to determine the prevalence and genetic diversity of NoV infections in South African hospitalised paediatric patients with gastroenteritis and to compare the circulating genotypes with NoV strains that are currently found worldwide.

3. Study design

3.1. Sample collection and processing

In a retrospective study, stool specimens, from hospitalised patients (≤13 years old) with gastroenteritis symptoms, submitted from January to December 2008 to a routine diagnostic laboratory for gastroenteritis virus testing were subsequently screened for NoV and SaV. Specimens were received from the Steve Biko and Kalafong Academic Hospitals, Gauteng, SA and stored at 4 °C until processing. Stool specimens were routinely tested by enzyme immunoassay (EIA) for RV (Premier Rotaclone, Meridian Biosciences Inc., Cincinatti, OH) and/or AdV 40/41 (Premier Adenoclone 40/41, Meridian Biosciences Inc.) and/or HAstV (Amplified IDEIA Astrovirus, Oxoid, Ely, UK) according to the manufacturer's instructions. Screening for NoV and SaV was done by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Stool suspensions (10–20%) were prepared in water and nucleic acids were extracted from 200 μl suspension with a total nucleic acid isolation kit (Roche Diagnostics, Mannheim, Germany) in a MagNA Pure LC robotic instrument (Roche Diagnostics). Total nucleic acid was eluted in 50 μl elution buffer, aliquoted and stored at −70 °C before use.

3.2. Statistical analyses

Descriptive statistics were used to summarise demographic data. Human immunodeficiency virus (HIV) positive and negative groups were compared in terms of acute or chronic infection using the Mantel-Haenszel Chi-square test (http://www.openepi.com/TwobyTwo/TwobyTwo.htm).

3.3. Detection and genotyping of NoVs

Published primer sets, Taqman probes and the QuantiTect Probe RT-PCR kit (Qiagen Inc., Valencia, CA) were used to detect NoV GI[20,21] or GII[22,23] and SaV.[24] For genotyping the 5′ end of the NoV capsid gene (Region C) was amplified and sequenced. Random primed cDNA was prepared from 10 μl total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Conventional PCR was performed with primers G1SKF, G1SKR, G2SKF and G2SKR[25] and HotStarTaq (Qiagen Inc.) according to the manufacturer's recommendations. M13(-21) and M13REV primer sequences[26] were added at the 5′ end of primers to facilitate sequencing using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3130 automated analyser (Applied Biosystems, Foster City, CA).

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Table 2
Distribution of (a) norovirus (NoV) and rotavirus (RV) infections among different age groups, (b) NoV and RV infections by month during 2008.

(a) Age distribution of NoV and RV infections

Age groups	NoV positive/Total	RV positive/Total
0–6 months	15/103	25/105
7–12 months	11/54	28/87
13–18 months	5/25	2/4
19–24 months	4/26	3/19
>2–4 years	0/14	0/8
>4–6 years	0/10	0/8
>6–8 years	0/9	2/10
>8–13 years	0/4	0/5
Unknown	–	1/6
Total	35/245	61/252

(b) Seasonal distribution of NoV and RV infections

Month	NoV positive/Total	RV positive/Total
January	2/19	5/20
February	4/27	5/26
March	2/25	4/26
April	1/18	10/21
May	4/29	10/31
June	1/17	6/17
July	2/22	8/22
August	1/16	7/16
September	1/7	0/6
October	3/14	2/15
November	6/19	1/18
December	8/32	3/34
Total	35/245	61/252

Nucleotide sequences were analysed using Sequencher™ 4.9 (Gene Codes Corporation, Ann Arbor, MI), BioEdit Sequence Alignment Editor (V.7.0.9.0)[27] and BLAST-N.[28] Sequences were aligned with reference sequences from genogroup I, II and IV using MAFFT Version 6 (https://align.bmr.kyushu-u.ac.jp/mafft/online/server/). After manual adjustment of the alignment, phylogenetic analysis was performed with MEGA4 using neighbour-joining methods.[29] GII.4 variants were identified by phylogenetic analysis of full length capsid sequences using GII.4 variant reference strains isolated from 1996 to 2008. Full capsid sequences were obtained by amplification of randomly primed cDNA with primers GR-JS1, GR19B, GR20A, GR21, GR22, GR23newR (TTATACAGCACGTCTACGCC), GR24A and GR25[30] and sequencing of four overlapping fragments that span the capsid gene. Only nucleotide sequences with less than 98% identity to NoV sequences in GenBank were submitted to the database. GenBank accession numbers are shown in Fig. 2.

4. Results

4.1. Prevalence of NoV and other gastroenteritis viruses in diarrhoeal specimens from hospitalised children

The median age of the study population was 8 months (range 1 day to 13 years) and the female to male ratio was 1:1.45. Of the stool specimens submitted for testing, 252 were tested for RV, 245 for NoV and SaV, 240 for AdV and 239 for HAstV. RV was identified in 24.2% (61/252) of specimens and was the most prevalent virus detected (Table 1a). NoVs, including GI and GII were detected in 14.3% (35/245) of the specimens whereas AdV 40/41, HAstV and SaV were detected in 9.6%, 6.7% and 4% of the specimens, respectively. NoVs were only detected in children up to 2 years old, as was 96.6% of RV (Table 2a), 95.7% of AdV 40/41 and 93.8% of HAstV (data not shown). NoVs were detected throughout 2008 with an increase in the positive specimens in November and December (Table 2b). RV infections peaked between April and August of 2008 (Table 2b). NoV GI (3/245) and GII (31/245) as well as one mixed GI + GII infection was detected (Table 1b).

Viral co-infections were identified in 23% of the NoV positive specimens (Table 1c). Co-infections with RV, AdV or SaV alone or a combination of RV/AdV and RV/HAstV were identified. Twelve of the 35 NoV positive specimens had been screened for bacteria and parasites but the results were negative. Approximately 80% of the NoV infections were community acquired infections whereas 20% of the patients possibly represented nosocomial infections. Community acquired infections predominated in children up to 18 months of age (Fig. 1a).

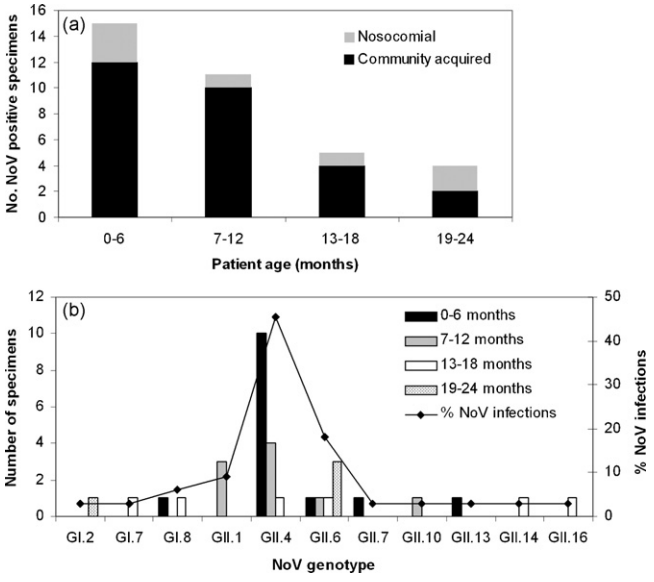

Fig. 1. (a) Distribution of community acquired and nosocomial norovirus (NoV) infections in hospitalised children of different ages. (b) NoV genotype distribution within four age groups (0–6 months, 7–12 months, 13–18 months and 19–24 months) of hospitalised paediatric patients.

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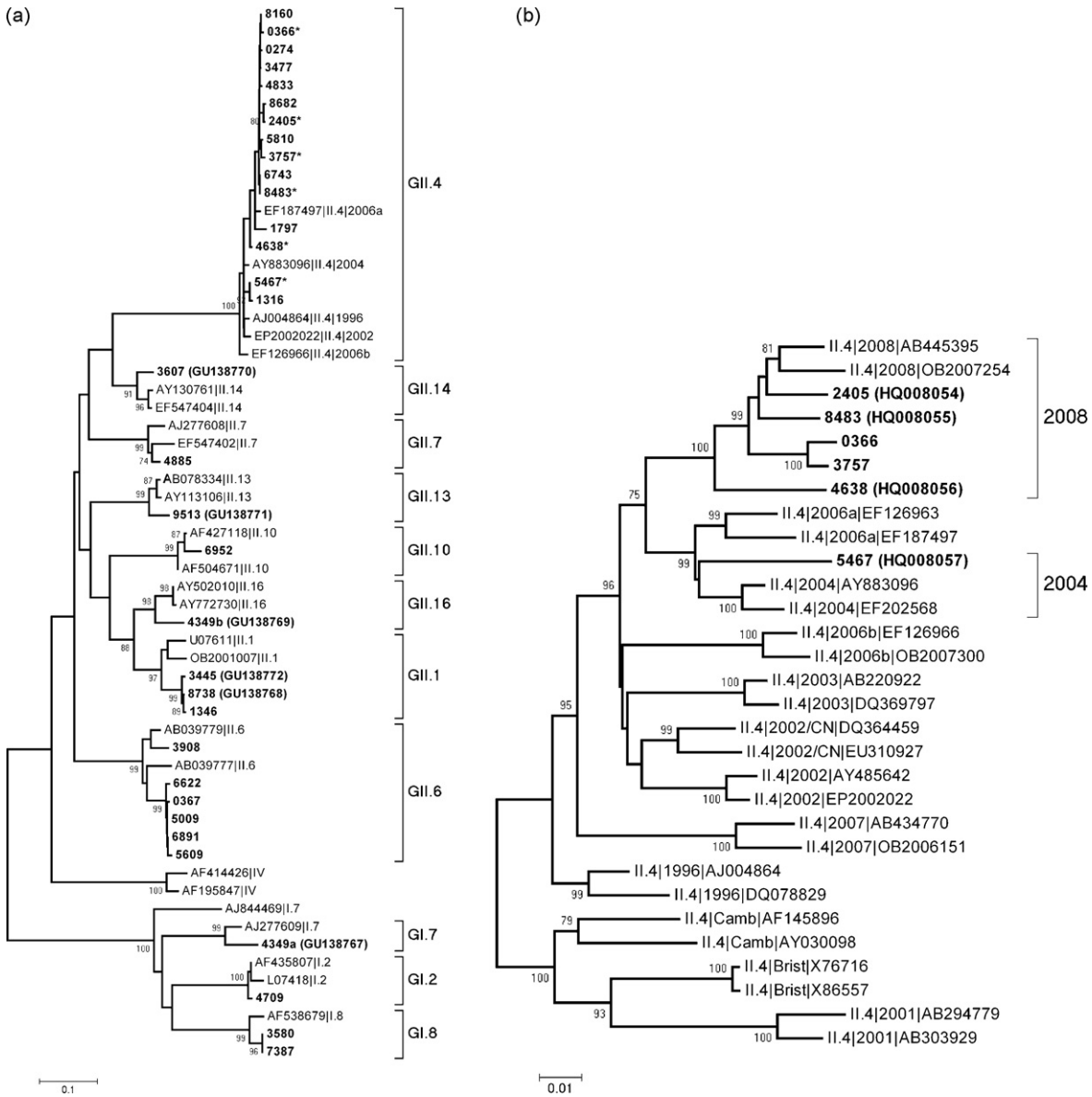

Fig. 2. Neigbour-joining analysis of (a) the 5′ end of the capsid gene (274 bp) of 33 norovirus strains and (b) the entire capsid gene of six GII.4 variant strains detected in hospitalised paediatric patients in Gauteng, South Africa during 2008. Bootstrap support (5000 replicates) above 70% is indicated. Specimen codes are highlighted in bold and reference strains for the respective genotypes or GII.4 variants are indicated by GenBank accession numbers. Only nucleotide sequences with <98% identity to sequences in GenBank were submitted to the database. *Indicates representative GII.4 variant strains selected for full capsid sequencing.

4.2. NoV genotypes identified in diarrhoeal specimens from hospitalised children

Phylogenetic analysis of the 5′ end of the NoV capsid gene revealed a variety of NoV genotypes circulating in the hospitalised paediatric population. NoV could not be amplified by conventional RT-PCR from three specimens, originally detected by GII-specific real-time RT-PCR using Taqman probes (Cp > 36), and these remain untyped. Three different GI strains, GI.2 (25%), GI.7 (25%) and GI.8 (50%), were characterised (Fig. 2a and Table 3). The GII NoVs (89%) predominated and eight GII types were identified (Fig. 2a and Table 3) with GII.4 (43%), GII.6 (17%) and GII.1 (9%) detected most frequently. Several other GII types were found in single specimens. Phylogenetic analyses of region C of the GII.4 strains indicated clustering of 73% of the strains with the GII.4 2008 variant, however without significant bootstrap support (data not shown). Therefore the full capsid sequences of six representative GII.4 strains were determined. Neighbour-joining analysis of these sequences with GII.4 variant reference sequences confirmed that the strains cluster with the 2008 variant (Fig. 2b and Table 3). In addition two 2004 variants were identified. The full capsid sequence of isolate 1797, which groups separately from the other GII.4 strains (Fig. 2a), could not be determined and therefore no variant could be assigned.

In Fig. 1b the distribution of NoV genotypes within the age groups 0–6, 7–12, 13–18 and 19–24 months is shown. GII.4 strains were detected in 66% of the patients in the 0–6 month group, whereas both GII.4 (44%) and GII.1 (33%) strains were predominant in the 7–12 month age group. The other genotypes were distributed randomly.

Of the 35 NoV positive specimens, 15 were collected from HIV positive patients, 14 from HIV negative patients and six from patients of unknown HIV status. No correlation was observed between NoV genotype and HIV status (Table 3). Diarrhoeal cases were classified as acute (60%) or chronic (37%). The median duration of hospitalisation for the acute cases was 2 days (range 1–155), and 24 days (range 4–117) for patients with chronic infections.

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Table 3
Norovirus genotypes and GII.4 variants detected in hospitalised paediatric patients in Gauteng, South Africa, during 2008.

Specimen code	Patient age (months)	HIV status	Community acquired/nosocomial	Acute/Chronic diarrhoea (duration of hospitalisation in days)	Genotype based on 5′ end of capsid gene	GenBank accession number[a]
	0–6 months					
0366[b]	2	Neg	CA	Acute (2)	GII.4 2008	GQ845367.1
8733	2	Neg	CA	Acute (1)	Not typed	
6622	3.5	Pos	CA	Acute (11)	GII.6	EU007781.1
1797	4	Neg	CA	Chronic (23)	GII.4	AB496912.1
3757[b]	4	Pos	NS	Acute (26)	GII.4 2008	GQ845367.1
7387	4	ND	CA	Chronic (10)	GI.8	FJ788383.1
8160	4	ND	CA	Acute (2)	GII.4 2008	GQ845367.1
8483[b]	4.5	Neg	NS	Chronic (117)	GII.4 2008	*HQ008055*
4885	5	Pos	NS	Chronic (25)	GII.7	AB453773.1
5467[b]	5.5	ND	CA	Acute (1)	GII.4 2004	*HQ008057*
3477	5.5	Pos	CA	Acute (7)	GII.4 2008	GQ845367.1
0274	5.75	ND	CA	Acute (3)	GII.4 2008	GQ845367.1
9513	6	Neg	CA	Acute (10)	GII.13	*GU138771*
1316	6	Pos	CA	Chronic (24)	GII.4 2004	EU876886.1
5810	6	ND	CA	Acute (1)	GII.4 2008	GQ845367.1
	7–12 months					
2405[b]	7.5	Pos	CA	Chronic (13)	GII.4 2008	*HQ008054*
6743	7.75	Pos	NS	Acute (93)	GII.4 2008	GQ845367.1
6952	8	Pos	CA	Chronic (110)	GII.10	*GU724780*
8738	8	Neg	CA	Chronic (7)	GII.1	*GU138768*
1346	8.5	Pos	CA	Chronic (66)	GII.1	GU138768
5609	9	Pos	CA	Acute (13)	GII.6	EU007781.1
2055	9.25	Neg	CA	ND (16)	Not typed	
9899	11	Neg	CA	Acute (2)	Not typed	
4833	11	Neg	CA	Acute (1)	GII.4 2008	GQ845367.1
3445	12	Neg	CA	Acute (2)	GII.1	*GU138772*
8682	12	Pos	CA	Acute (11)	GII.4 2008	AB491291.1
	13–18 months					
4638[b]	13.25	Pos	CA	Chronic (37)	GII.4 2008	*HQ008056*
4349	14.5	Pos	NS	Acute (155)	GI.7/GII.16	*GU138767/GU138769*
0367	15	Pos	CA	Chronic (10)	GII.6	EU007781.1
3607	17	ND	CA	Chronic (4)	GII.14	*GU138770*
3580	17.5	Neg	CA	Acute (2)	GI.8	FJ788383.1
	19–24 months					
3908	19	Pos	NS	Chronic (46)	GII.6	EU007768.1
4709	20	Neg	CA	Acute (1)	GI.2	FJ383881.1
5009	20	Neg	CA	Acute (1)	GII.6	EU007781.1
6891	24	Neg	NS	Acute (114)	GII.6	EU007781.1

[a] Accession number of most closely related sequence in GenBank (>98% identity). Accession numbers in italics represent sequences identified in this study that were submitted to GenBank. ND = not determined, CA = community acquired, NS = nosocomial. Specimens collected from patients within 48 h of admission were considered cases of community acquired gastroenteritis and specimens collected more than 48 h after admission, were classified as nosocomial infections. NoV infections were classified as acute (≤7 days) or chronic (≥7 days) based on the duration of symptoms.
[b] Six GII.4 variants were assigned based on analysis of full length capsid sequences, the other variants were assigned based on >98.5% nucleotide sequence identity with 0366, 3757, 8483, 5467, 2405 and 4638 in the 5′ end region.

In the HIV positive group chronic infections were observed frequently (8/15), whereas HIV negative patients were often acutely infected (10/14) (Table 3). However, this trend was not statistically significant.

5. Discussion

Between January and December 2008, NoVs were detected in 35/245 (14.3%) stool specimens from hospitalised children with gastroenteritis in the Pretoria region, SA. After RV, NoV was the most prevalent virus detected followed by AdV 40/41, HAstV and SaV. These results are consistent with recent studies that describe RV as the most prevalent and NoV as the second most important viral agent causing gastroenteritis in children (RV 16.6–71.4%; NoV 12–17.3%).[31–35] The NoV and SaV prevalence determined in the current study is higher than previously estimated in the same region,[17] which could be attributed to the more sensitive real-time RT-PCR detection method applied. NoVs were detected only in children up to 2 years old (median age 8 months), which is in agreement with studies in Brazil and Nicaragua where more frequent NoV infections in children ≤2 years old were reported.[36,37] This could indicate that short term immunity is generated to NoV, since NoV infections in older children and adults are common.[38] Alternatively, NoV infections in infants could cause more serious symptoms that require hospitalisation whereas older children may not be as severely affected.

A variety of NoV genotypes (3 GI; 8 GII) were identified in this study and up to five different strains circulated during the same time period. These results are similar to studies in Brazil, Nicaragua and Tunisia where the predominance of the GII strains in hospitalised children with gastroenteritis (88–96% of NoV positive specimens) was also noted.[13,36,37] However, other studies in Brazil[39] and Mexico[40] found GI and GII strains at comparable frequencies. These studies were conducted over different time intervals several years ago (1998 and 1998–2005) and used different study populations. Furthermore, in years in which a new GII.4 NoV variant emerges one might expect an increase in the frequency of detection of GII strains, as observed in this study. NoV outbreaks

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are mostly associated with peaks in winter,[41] whereas sporadic NoV gastroenteritis are detected continuously.[39,42] In this investigation NoVs were detected year round, with a peak in November and December. GII.4 strains were detected in 9 months of the year, comprising 43% of the NoV strains. In other recent studies GII.4 strains were detected even more frequently ranging from 62.7% to 68%.[13,36,37] This is the first report of GII.4 strains occurring in SA. Variant analysis indicated that the newly emerging GII.4 2008 variant (12/15) was most prevalent but the older 2004 variant (2/15) was also detected. The results imply that the 2008 variant is well established in the community under investigation. This study focused on a small region and more widespread NoV screening is needed to determine the true prevalence of the GII.4 2008 variant in SA.

The data suggests that 80% of the NoV infections in this study were community acquired. Nosocomial infections occurred at both hospitals, however, no outbreaks were reported and no correlation was found between community acquired cases that were admitted to hospital and the nosocomial infections. The HIV status of the patients did not significantly correlate with either acute or chronic infection or NoV genotype.

In conclusion, this study has shown that a diverse range of NoV genotypes are found in children hospitalised with gastroenteritis. The GII.4 strains dominated and the emerging GII.4 2008 variant was present in SA and probably responsible for a significant proportion of hospitalisation due to NoV infection. The high prevalence of NoVs recorded in this study highlights the necessity for a larger study and suggests that routine NoV testing should be initiated to elucidate the contribution of NoV to the burden of gastroenteritis.

Conflict of interest

None.

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