Applicability of Bio-wipes for the collection of human faecal specimens for detection and characterisation of enteric viruses

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Abstract

OBJECTIVE To determine whether gastroenteritis viruses and other enteric viruses could be detected in faecal specimens collected with Bio-wipes.
METHODS Faecal specimens, self-collected with Bio-wipes, from 190 individuals (94 diarrhoeal, 93 non-diarrhoeal, 3 unknown) were screened for eight human enteric viruses (enterovirus, hepatitis A virus, adenovirus, astrovirus, norovirus GI and GII, sapovirus and rotavirus) by real-time (reverse transcription)-polymerase chain reaction. Rotaviruses and noroviruses from positive specimens were genotyped. RESULTS At least one enteric virus could be detected in 82.6% (157/190) of faecal specimens. Mixed infections of up to four different viruses could be detected in both diarrhoeal and non-diarrhoeal specimens. Enteroviruses were detected most frequently (63.7%), followed by adenoviruses (48.4%) and noroviruses (32.2%). Genotyping was successful for 78.6% of rotaviruses and 44.8% of noroviruses.
CONCLUSIONS Bio-wipes provide a user friendly, easier method for stool collection that facilitates enteric virus detection and genetic characterisation.

keywords Bio-wipe, enteric virus, faecal specimen, virus detection

Introduction

Diarrhoeal disease contributes significantly to the overall burden of disease worldwide (WHO 2008). In 2010, it was estimated that 11% of deaths in children younger than 5 years were due to diarrhoea (Liu *et al.* 2012). The majority of acute gastroenteritis episodes are viral in origin (de Wit *et al.* 2001; Hall *et al.* 2011), and among the enteric viruses, rotaviruses (RVs) are the major cause of diarrhoeal disease in children (Parashar *et al.* 2006) followed by norovirus (NoV) (Glass *et al.* 2009). Human adenovirus (HAdV), human astrovirus (HAstV) and sapoviruses (SaV) also cause gastroenteritis, but at lower frequencies than RV and NoV (Hall *et al.* 2011). Noroviruses are now recognised as the leading cause of gastroenteritis outbreaks, causing >90% of viral gastroenteritis outbreaks and up to 50% of all gastroenteritis outbreaks (Patel *et al.* 2008). In the USA, it has been estimated that NoVs cause 58% of foodborne illnesses (Scallan *et al.* 2011). Although the hospitalisation rate of NoV infections is estimated at only 0.03% NoV represented 26% of all hospitalisations due to foodborne illness. Even though highly specific and sensitive diagnostic tests have been developed for most known enteric pathogens, an aetiologic agent is identified in only approximately 30–40% of gastroenteritis cases (Hall *et al.* 2011; Tam *et al.* 2012). Collection of whole stool samples is recommended for the identification, characterisation and study of enteric pathogens including viruses (Bresee *et al.* 2012), but the collection of faecal specimens is viewed as unpleasant and technically challenging. Therefore, very few people with acute gastroenteritis volunteer to provide specimens and most whole stool samples are collected from hospitalised children and adults or the elderly in old age homes. To enable epidemiological studies on enteric pathogens in the general population, including individuals with less severe symptoms and individuals living in remote or rural areas, different, more accessible and convenient methods of faecal specimen collection should be explored. Rectal swabs have been used as an alternative

to whole stool specimens, but besides being intrusive, a recent study showed that pathogens were detected less often from rectal swabs than from whole stool specimens (Bresee *et al.* 2012). The Bio-wipe kit is a novel, less invasive and convenient faecal collection technique. This method has successfully been used for the collection of faecal specimens for the detection of bacterial diarrhoeal pathogens (Mieta *et al.* 2010). The aim of the current study was to determine whether enteric viruses representing the major gastroenteritis viruses (RV, NoV, HAdV, HAstV, SaV) as well as other important enteric viruses such as hepatitis A (HAV) and enteroviruses (EV) could be detected in faecal specimens collected using Bio-wipes.

Materials and methods

Faecal specimen collection using the Bio-wipe kit and Bio-wipe processing

One hundred-ninety participants, enrolled in a larger study on the impact of a ceramic pot filter point-of-use water treatment device in rural households in the Limpopo Province of South Africa (July 2007–December 2008; Potgieter *et al.* 2011) collected their own stool specimens using the Bio-wipe kit. Ethical clearance for this study was obtained from the National Department of Health in Polokwane and the ethical committee of the University of Venda. 94 participants collected samples during diarrhoeal episodes (self-reported), and 93 participants collected samples at times without any overt diarrhoeal symptoms. Diarrhoea was defined as the passage of three or more loose or liquid stools in a 24 h period. The diarrhoeal status of three samples was unknown. The age of the population ranged from 1 month to 87 years with a median of 14 years.

The Bio-wipe kit was developed by Professor MD Sobsey, University of North Carolina, USA, in collaboration with Prof. Chris Ohl, Wake Forest Baptist Medical Center, USA. The materials, assembly and instructions for use of the Bio-wipe kit have been described (Mieta *et al.* 2010). In brief, the kit consists of a square piece of absorbent fibrous material with an orange plastic backing, provided in a sterile re-sealable plastic bag. In addition, a piece of polyester batting material, soaked in storage/transport medium is provided in a second plastic bag. The Bio-wipe is used in the same manner as toilet tissue paper. After use, it is placed onto the batting material, folded together and replaced in the original bag. Used Bio-wipes were stored at room temperature by each participant until collection by field workers on a weekly basis. The Bio-wipes were kept on ice during transportation and stored at 4 °C in the laboratory until processing. Faecal material was recovered from the Bio-wipes with 6–10 ml of PBS (Sigma-Aldrich, St. Louis, MO, pH = 7.2) supplemented with 0.1% Tween buffer (vol/vol) as described by Mieta and co-workers (Mieta *et al.* 2010).

Nucleic acid extraction

Total nucleic acid was extracted from 1 ml of buffer containing faecal matter recovered from the Bio-wipe using the MagNA Pure LC Total Nucleic Acid Isolation kit (large volume) (Roche Diagnostics, Mannheim, Germany) in a robotic MagNA Pure LC instrument (Roche Diagnostics) according to the manufacturer's instructions. Nucleic acid was eluted in 100 µl and stored at −70 °C in 6 µl aliquots until use.

Enteric virus detection

Each Bio-wipe sample was analysed for eight different human enteric viruses using single-plex real-time polymerase chain reaction (PCR) (HAdV) or real-time, reverse transcription (RT)-PCRs (HAst, HAV, EV, NoV GI, NoV GII, RV and SaV).

Human adenovirus

Five microlitres of extracted nucleic acid were analysed in a total volume of 25 µl with the TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, Warrington, UK) and primers AQ1, AQ2 and Taqman probe AP (Table 1) on the LightCycler v1.5 real-time platform (Roche Diagnostics). The cycling conditions were as follows: 10 min at 95 °C, 45 cycles of 95 °C for 3 s, 55 °C for 10 s, 65 °C for 1 min.

Enterovirus and hepatitis A virus

Two-step RT-PCRs were used to detect EV and HAV. Randomly primed cDNA was generated from 10 µl of RNA using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) in a total volume of 20 µl. Reverse transcription conditions were: 25 °C for 10 min, 50 °C for 60 min, 85 °C for 5 min. Five microlitres of cDNA were tested for each virus using the LightCycler® TaqMan® Master Kit (Roche Diagnostics) on the LightCycler v1.5 real-time platform. The primers used for EV and HAV are shown in Table 1. The cycling conditions for both tests were as follows: 95 °C for 15 min, 45 cycles of 95 °C for 15 s, 60 °C for 1 min, 65 °C for 1 min.

Table 1 Primer and probe sequences and final concentrations used for real-time PCR detection of human adenovirus and real-time RT-PCR detection of enterovirus, human astrovirus, hepatitis A virus, norovirus GI, GII, rotavirus and sapovirus

Virus Detection	Primer /Probe	Final Concentration (nM)	Sequence (5′-3′)[j]	Position	Reference
Human adenovirus	AQ1	500	GCCACGGTGGGGTTTCTAAACTT	18 989–18 967[a]	Heim *et al.* (2003)
	AQ2	500	GCCCCAGTGGTCTTACATGCACATC	18 858–18 882[a]	
	AP	400	FAM-TGCACCAGACCCGGGCTCAGGTACT CCGA-TAMRA	18 926–18 898[a]	
Human astrovirus	AV1	500	CCGAGTAGGATCGAGGGT	2395–2412[b]	Le Cann *et al.* (2004)
	AV2	500	GCTTCTGATTAAATCAATTTTAA	2426–2459[b]	
	Probe AVS	100	FAM-CTTTTCTGTCTCTGTTTAGATTATTTTA ATCACC-TAMRA	2462–2484[b]	
Hepatitis A virus	HAV 68	500	TCACCGCCGTTTGCCTAG	68–85[c]	Costafreda *et al.* (2006)
	HAV 240	900	GGAGAGCCCTGGAAGAAAG	223–241[c]	
	HAV 150	250	FAM-TTAATTCCTGCAGGTTCAGG-TAMRA	150–169[c]	
Enterovirus	EntV1	500	GATTGTCACCATAAGCAGC	579–597[d]	Fuhrman *et al.* (2005)
	EntV2	500	CCCCTGAATGCGGCTAATC	451–469[d]	
	Probe EV	100	FAM-CGGAACCGACTACTTTGGGTGTCCGT-BHQ	532–557[d]	
Norovirus GI	QNIF4	200	CGCTGGATGCGNTTCCAT	5291–5308[e]	da Silva *et al.* (2007)
	NV1LCR	200	CCTTAGACGCCATCATCATTTAC	5354–5376[e]	Svraka *et al.* (2007)
	NVGG1p	200	FAM-TGGACAGGAGAYCGCRATCT-TAMRA	5321–5340[e]	
Norovirus GII	QNIF2	200	ATGTTCAGRTGGATGAGRTTCTCWGA	5012–5037[f]	Loisy *et al.* (2005)
	COG2R	200	TCGACGCCATCTTCATTCACA	5080–5100[f]	Kageyama *et al.* (2003)
	QNIFSp	200	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	5042–5061[f]	Loisy *et al.* (2005)
Rotavirus Group A	ROTFOR	400	ACCATCTWCACRTRACCCTCTATGAG	963–988[g]	Zeng *et al.* (2008)
	ROTREV	400	GGTCACATAACGCCCCTATAGC	1028–1049[g]	
	ROTprobe	200	FAM-AGTTAAAAGCTAACACTGTCAAA-MGB	995–1017[g]	
Sapovirus	CU-SV-F1	900	GACCAGGCTCTCGCYACCTAC	5074–5094[h]	Chan *et al.* (2006)
	CU-SV-F2	900	TTGGCCCTCGCCACCTAC	786–803[i]	
	CU-SV-R	900	CCCTCCATYTCAAACACTAWTTTG	5177–5154[h]	
	CU-SVp	450	FAM-TGGTTYATAGGYGGTAC-MGB	5101–5117[h]	

GenBank accession numbers: [a]J01917, [b]L06802.1, [c]M14707, [d]NC_002058, [e]M87661, [f]X86557, [g]X81436, [h]NC_006269, [i]U95644, [j]IUPAC codes used to indicate degenerate positions.

Human astrovirus, norovirus GI and GII, rotavirus and sapovirus

Human astrovirus, NoV GI and GII, RV and SaV were detected with individual one-step real-time RT-PCRs using the Quantitect® Probe RT-PCR Kit (Qiagen, Hilden, Germany), 5 µl of RNA and published primers (Table 1) in a total volume of 20 µl. All reactions were performed on the LightCycler v1.5 real-time platform (Roche Diagnostics). The cycling conditions for NoV GI, GII and RV were identical: 50 °C for 45 min, 95 °C for 15 min, 45 cycles of 95 °C for 15 s, 60 °C for 1 min, 65 °C for 1 min. Identical conditions were used for HAstV (55 °C) and SaV (56 °C) except for the indicated annealing temperatures.

Genotyping of NoV and RV strains

The 5′- end of the NoV capsid gene (Region C) was amplified and sequenced for genotyping as described previously (Mans *et al.* 2010). G and P genotypes of RV strains were determined using multiplex nested PCR methods to amplify the VP7 and the VP4 genes, respectively, as described by Kiulia *et al.* (2010).

Statistical analysis

The proportions of virus-positive specimens in diarrhoeal and non-diarrhoeal groups were compared with a two-tailed chi square test in OpenEpi.com. P values < 0.05

were considered significant (http://www.openepi.com/v37/TwobyTwo/TwobyTwo.htm).

Results

Detection of enteric viruses in Bio-wipe faecal specimens

One or more enteric viruses could be detected in 82.6% (157/190) of faecal specimens collected by means of Bio-wipes. Enteroviruses were detected most frequently, followed by HAdV, NoV, SaV, RV, HAV and HAstV (Table 2). Up to four different viruses could be detected from a single Bio-wipe specimen, and one to two viruses were detected in 68.9% of the specimens (Table 2). The faecal specimens could be divided into diarrhoeal (94), non-diarrhoeal (93) and unknown status (3) specimens (Table 3). Enteroviruses were detected more frequently in non-diarrhoeal specimens (70/93 vs 49/94, $P = 0.001$), whereas HAdVs were detected at similar rates in diarrhoeal (41/94) and non-diarrhoeal (49/93) specimens. Human astrovirus, HAV and NoV GI were only detected in non-diarrhoeal specimens. Norovirus GII was detected more frequently in non-diarrhoeal specimens than in diarrhoeal specimens (20/93 vs 7/94, $P = 0.006$). Sapovirus was detected in more non-diarrhoeal specimens, however, the difference was not statistically significant. Of the RV-positive specimens, 93% were from diarrhoeal specimens. Enterovirus, HAdV and NoV were detected in a wide range of age groups, from a few months old to over 80 years of age. The median age of the infected individuals ranged from 11 to 14 years (Table 2). The age distribution of RV-infected individuals was different, with a median age of 8 years (range 3 months to 45 years) whereas the median age of the SaV positive group was 24 years (range 1 month to 62 years).

Table 2 Detection of eight enteric viruses in human stool specimens collected by means of a Bio-wipe

Virus	Number of virus positive Bio-wipe samples/Total tested	%	Median age (years) of infected individuals (Min–Max)
Enterovirus	121/190	63.7	12 (2 months –87 years)
Hepatitis A virus	4/190	2.1	5.5 (3 months –37 years)
Human adenovirus	92/190	48.4	14 (7 months –87 years)
Human astrovirus	1/190	0.53	31
Norovirus GI	1/190	0.53	26
Norovirus GII	28/190	14.7	11 (3 months –82 years)
Sapovirus	16/190	8.4	24 (1–62 years)
Rotavirus A	14/190	7.3	8 (3 months –45 years)
No virus detected	33/190	17.4	17(1 month –78 years)
Mixed infections			
Single virus	61/190	32.1	
2 viruses	70/190	36.8	
3 viruses	23/190	12.1	
4 viruses	3/190	1.6	
Total*	157/190	82.6	

*Total number of samples in which one or more viruses were detected.

Genotyping of RV and NoV

To assess whether viruses could also be genotyped from faecal specimens collected using Bio-wipes, the RV and NoV-positive specimens were selected for genotyping. The majority (11/14; 78.6%) of RV-positive specimens could be genotyped, yielding five types within this set of specimens (Table 4). The typing efficiency of the NoVs was much lower (13/29; 44.8%). The real-time RT-PCR cycle threshold (Ct) values of NoV specimens that could be typed ranged from 15 to 35 with a mean of 27.5, whereas the untypeable specimens had C_t values ranging

Table 3 Enteric viruses detected in diarrhoeal versus non-diarrhoeal faecal specimens collected with Bio-wipes

Virus	Diarrhoeal specimens ($n = 94$)	Non-diarrhoeal specimens ($n = 93$)	*P*-value	Unknown ($n = 3$)
Enterovirus	49 (52.1%)	70 (75.3%)	0.001	2 (66.7%)
Hepatitis A virus	0	4 (4.3%)	–	0
Human adenovirus	41 (43.6%)	49 (52.7%)	0.214	2 (66.7%)
Human astrovirus	0	1 (1.1%)	–	0
Norovirus GI	0	1 (1.1%)	–	0
Norovirus GII	7 (7.4%)	20 (21.5%)	0.006	2 (66.7%)
Sapovirus	5 (5.3%)	11 (11.8%)	0.111	0
Rotavirus A	13 (13.8%)	1 (1.1%)	0.001	0

Table 4 Genotyping of norovirus and rotavirus positive specimens

Virus	Typed samples/ Total (%)	Genotypes
Norovirus GI and GII	13/29 (44.8)	GI.3, GII.1, GII.4, GII.14
Rotavirus	11/14 (78.6)	G2P[4], G2P[4]/P[6], G12P[6], G12P[6]/P[8], G?P[4]/P[8]

from 32 to 38 with a mean of 35.4. Four NoV genotypes (Table 4; Figure 1) were identified within the 13 strains that could be amplified for nucleotide sequence analysis, with GII.1 being the most frequent genotype that could be characterised (9/13; 69.2%).

Discussion

Although no direct comparison was made, this study showed that the use of Bio-wipes to collect faecal specimens for studies on enteric viruses was a practical alternative to collecting whole stool specimens. More than 80% of the Bio-wipe specimens tested positive for at least one enteric virus. Furthermore, mixed infections of up to four different viruses were detected. Ideally, whole stool specimens and rectal swabs should be collected in parallel with the Bio-wipe specimens to facilitate direct comparison of virus detection rates between different types of faecal specimens. However, this was not feasible in the current study population and setting. The results obtained using Bio-wipes, however, favourably correspond to virus recoveries of several other reports on the prevalence of enteric viruses, with viruses being detected in specimens from both symptomatic and asymptomatic individuals. In a study on the prevalence of RV, NoV and HAdV in hospitalised children in China, the viruses were detected in 68.7%, 20.4% and 5% of symptomatic children, respectively, and in 13.2%, 35.9% and 9.4% of asymptomatic children, respectively (Zhang *et al.* 2011). Rotaviruses were less prevalent in the current study, which likely reflects the demographic and health differences between the Chinese study (hospitalised children <5 years) and the current study (sporadic gastroenteritis in non-hospitalised children with a wider age range and median age of 14) in terms of study population and disease severity. However, RVs were detected more frequently in symptomatic children with diarrhoea than asymptomatic children in both studies. Noroviruses were detected significantly more often in asymptomatic individuals in both the current study and in the Chinese study (Zhang *et al.* 2011). Several reports have described varying rates of asymptomatic NoV infections. The following prevalences were found in children: 9.6% in Cameroon (Ayukekbong *et al.* 2011), 13% in Brazil (Barreira *et al.* 2010), 3.5–5.5% in Korea (Cheon *et al.* 2010), 49% in Mexico (Garcia *et al.* 2006), 5.2% in a community study in the Netherlands (de Wit *et al.* 2001). Clearly, NoV infections and other enteric virus infections are often asymptomatic and using Bio-wipes to collect faecal specimens allows detection of both symptomatic and asymptomatic infections.

After detection of an enteric virus, genotyping is the next important step to study the epidemiology of these pathogens. The genotyping success rate differed greatly between RVs and NoVs in this study. Rotaviruses are generally shed at higher levels (10^{12} *vs.* 10^{10}/g of faeces) than NoVs (Bishop 1996; Atmar *et al.* 2008) which could explain why 78% of the RV strains from positive samples could be genotyped, whereas only 45% of the strains in NoV-positive specimens could be amplified for genotyping. Average real-time RT-PCR Ct values of the NoV-positive specimens indicated that samples which could be typed had at least a 100-fold higher virus concentration than specimens that could not be amplified in the genotyping PCR. The low NoV genotyping success rate in this study probably reflects the low viral loads in the specimens, rather than inadequate specimen collection with Bio-wipes.

Diverse RV genotypes were identified in the 11 specimens typed in this study. The G2P[4] strain, which was shown to represent 5% of the RV strains detected in African children with diarrhoea (Todd *et al.* 2010), was detected in 5/14 specimens, including stool from one adolescent and two adults. Furthermore, G2 strains with mixed VP4 genotypes P[4] and P[6] were also detected. The G2P[6] strain was previously reported at a prevalence of 2.7% in children with acute diarrhoea in the same rural region in SA (Potgieter *et al.* 2010). In addition, the globally emergent G12 strain was detected in association with P[6] and P[8] VP4 types. One adult participant had a mixed G12P[6]/P[8] infection. The RV G type of one specimen remained undetermined but P[4] and P[8] types were found in this specimen.

Of the NoV strains identified in this study, GII.1, GII.4 and GII.14 have been detected in 2008 in children hospitalised with gastroenteritis in SA (Mans *et al.* 2010), whereas GI.3, GII.1 and GII.4 have been detected in surface water in the Gauteng province of SA (Mans *et al.* 2013). The majority of typed NoV strains in this study belonged to GII.1 which was detected in both symptomatic and asymptomatic individuals. The GenBank NoV sequence most closely related to the GII.1 strains characterised in this study was detected in Belgium in 2010 and was characterised as a polymerase GII.g/capsid GII.1 recombinant virus (Mathijs *et al.* 2011). Whether the

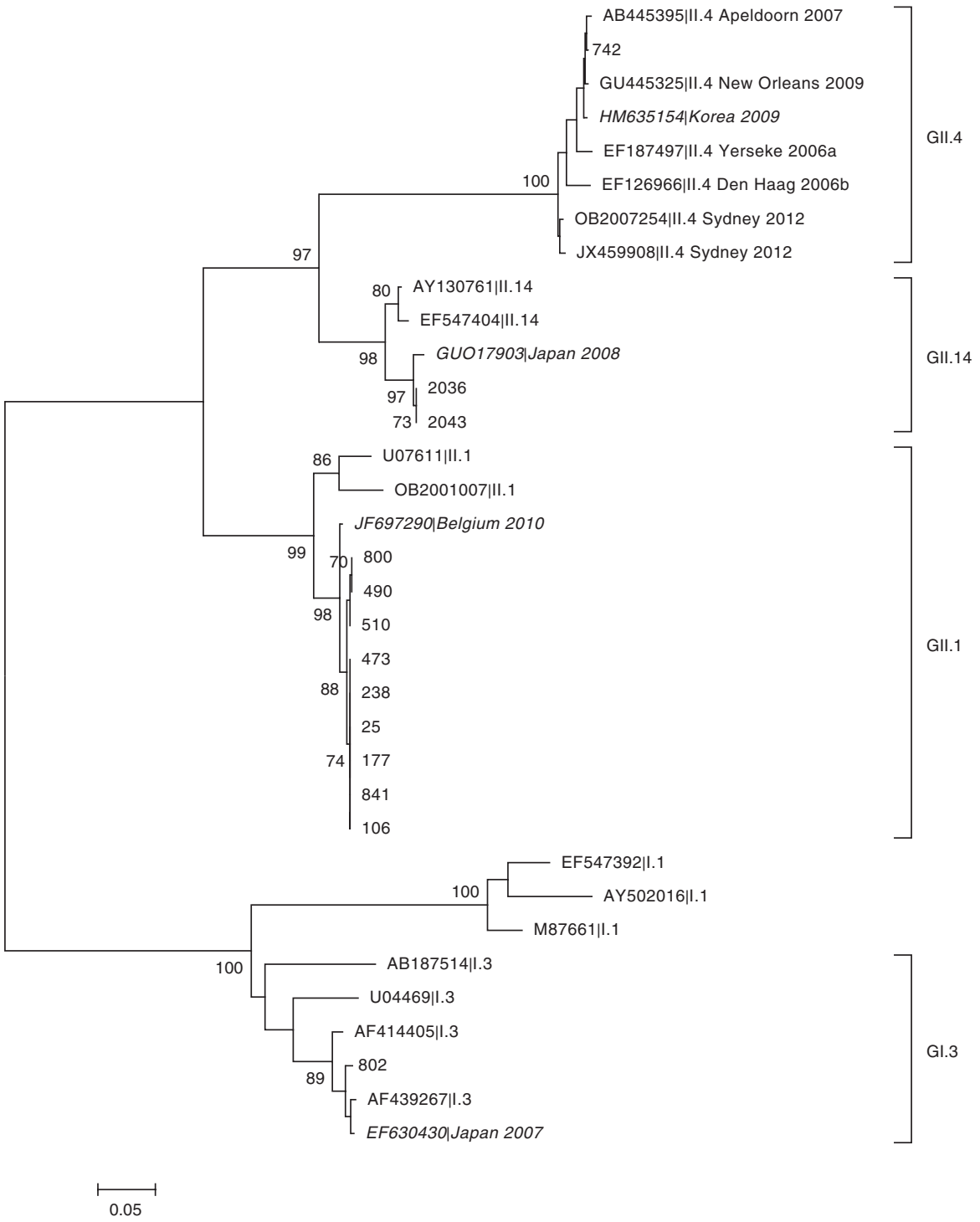

Figure 1 Neighbour-joining phylogenetic analysis of NoV reference strains and partial capsid gene (280 bp at 5′-end) sequences of 13 NoV strains (shown in boldface) detected in diarrhoeal and non-diarrhoeal specimens collected with Bio-wipes from individuals living in a rural setting in the Limpopo province of South Africa. Most closely related sequences on GenBank are shown in italics. Bootstrap support of >70% is indicated.

GII.1 strains from the Limpopo province also are recombinants requires further investigation.

The advantages of stool collection with Bio-wipes over whole stool collection in universal sterile containers are the higher acceptability, less intrusiveness and more convenient storage and transport of the sample. This method was shown to be effective for stool specimen collection from all age groups. An important factor for successful use of Bio-wipes for stool collection is thorough training of study participants to use Bio-wipes, as incorrect use could lead to collection of insufficient amounts of faeces or inappropriate Bio-wipe storage. This study showed that eight enteric viruses could be detected as single or co-infections from Bio-wipe faecal specimens and that RVs and NoVs could be genotyped successfully. Whether the faecal material is adequate for further characterisation of viruses, such as full genome sequencing, remains to be determined. Overall, the results from this study correspond to enteric virus prevalence patterns reported worldwide, suggesting that faecal specimens collected using Bio-wipes would be useful and convenient for epidemiological studies on enteric pathogens.

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