Norovirus genotype diversity associated with gastroenteritis outbreaks in Victoria in 2013

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Abstract

The noroviruses are now considered a leading cause of outbreaks of non-bacterial gastroenteritis worldwide. Vaccine strategies against norovirus are currently under consideration but depend on a detailed knowledge of the capsid genotypes. This study examined the incidence of norovirus outbreaks in Victoria over 1 year (2013) and documented the genotypes occurring in the different outbreak settings (healthcare and non-healthcare) and age groups. It was found that 63.1% of gastroenteritis outbreaks were associated with norovirus, thereby showing norovirus to be the major viral cause of illness in gastroenteritis outbreaks. Sixteen capsid genotypes were identified and included GI.2, GI.3, GI.4, GI.6, GI.7, GI.8, GI.9, GII.1, GII.2, GII.3, GII.4, GII.5, GII.6, GII.7, GII.13 and an as yet unclassified GII genotype. All genotypes found in the study, with the exception of GI.9, were detected in the elderly, indicating prior exposure to a norovirus genotype did not appear to confer long term immunity in many cases. The incidence of genotypes GII.1, GII.4 and GII.7 was linked with setting and age. As setting and age were correlated it was not possible to determine which variable was critical with the exception of GII.7, which appeared to be linked to age. The findings indicate that norovirus vaccine strategies should encompass a broad range of genotypes and, as setting or age may be important in determining genotype incidence, this should be taken into account as well. *Commun Dis Intell* 2015;39(1):E34–E41.

Keywords: norovirus, outbreaks, genotypes, vaccine strategies, healthcare, non-healthcare, setting, age

Introduction

The noroviruses, which are single-stranded positive sense RNA viruses classified in the genus *Norovirus* within the family Caliciviridae,[1] are now considered a leading cause of outbreaks of non-bacterial gastroenteritis worldwide.[1–3] Noroviruses are currently classified into 6 genogroups[1] and three of these, genogroups I, II and IV (GI, GII and GIV), occur in human infections,[1] although little is known concerning the incidence and clinical significance of GIV noroviruses in human infections.[4]

The norovirus genome comprises three open reading frames (ORFs).[1] ORF 1 encodes the non-structural polyprotein, ORF 2 encodes the major capsid protein and ORF 3 encodes the minor capsid protein.[1] Norovirus genotype classification using nucleic acid sequencing can be based on the ORF 1 region or the ORF 2 region.[5] Currently, 31 ORF 2 genotypes have been identified in norovirus genogroups I and II.[1]

The GII.4 genotype appears to be the most common in humans[6] and has been further subdivided into 'variants' or 'strains'[7,8] and successive variants are typically given a specific name.[7] In Australia, some recent GII.4 variants have been named 'Hunter' (2004–2005), 2006a (2006–2007) and 2006b (2006–2007).[7] In the period 2009–2012 the GII.4 'New Orleans 2009' variant appeared to be predominant and in late 2012 the GII.4 variant 'Sydney 2012' emerged as a major GII.4 variant in Australia.[9,10]

An understanding of norovirus genotypes is important in the ultimate management and eradication of norovirus infections. There are 2 main reasons for this. Firstly, genotype analysis enables an understanding of how noroviruses circulate throughout the community. Secondly, the development of vaccine strategies against norovirus depends on a knowledge of ORF 2 (capsid) genotypes in the community,[11,12] so documentation here is critical.

The current study documents the ORF 2 norovirus genotypes associated with norovirus gastroenteritis outbreaks in Victoria over 1 calendar year (2013) and represents the first detailed overview of ORF 2 norovirus genotypes associated with gastroenteritis outbreaks in Australia. The findings are discussed in terms of the great diversity of norovirus genotypes found and their relationship with the setting of the outbreak (healthcare vs non-healthcare) and the age of the patient. The significance of these findings for developing vaccine strategies against norovirus is discussed.

Materials and methods

Definition of gastroenteritis outbreak

For the purposes of this study an outbreak was defined as a gastroenteritis cluster, apparently associated with a common event or location, in which four or more individuals had symptoms of gastroenteritis. For an outbreak in a particular setting to be so defined at least 2 individuals had to develop gastroenteritis within 4 days of each other and for an outbreak linked to a suspect food source at least 2 individuals had to develop gastroenteritis within 4 days of consuming the suspect food.

Specimens

The faecal specimens included in this study were those sent to the Victorian Infectious Diseases Reference Laboratory (VIDRL) for norovirus testing from outbreaks that occurred during 2013. VIDRL is the main public health laboratory for viral identification in the State of Victoria. As such, it receives faecal material from gastroenteritis outbreaks reported to the Victorian health department. Outbreak specimens are also occasionally sent by other institutions such as hospitals. Only outbreaks that occurred in Victoria, were included in the study.

The date of an outbreak was taken as the onset date. If this was not available, the date the outbreak was first notified or the earliest date of collection of a specimen from the outbreak was taken as the date of the outbreak.

Outbreak setting

For data analysis, norovirus outbreaks were divided into the 2 groups of healthcare and non-healthcare. Healthcare settings included aged care facilities, disabled care facilities, hospitals, hospital geriatric ward, hospital palliative care and hospital rehabilitation unit. Non-healthcare settings included child care centres, children's activity centres, gatherings, a navy base, restaurants, schools and suspect food.

Faecal processing and RNA extraction prior to polymerase chain reaction testing

Faecal specimens were prepared as a 20% (vol/vol) suspension in Hanks' complete balanced salt solution (Sigma-Aldrich Company, Irvine, UK) and the suspension clarified with a single 10 min centrifugation spin as previously described.[13] This clarified extract was then used for RNA extraction followed by reverse-transcription polymerase chain reaction (RT-PCR). RNA extraction was carried out using the Corbett automated extraction procedure (now Qiagen Sciences, Germantown, MD, USA) essentially as described previously.[14]

Reverse-transcription polymerase chain reaction, nucleotide sequencing and phylogenetic analysis

Three 2 round RT-PCR protocols were used in the study (Table 1). For the first round of each of the 3 protocols the Qiagen (Qiagen GmbH, Hilden, Germany) OneStep RT-PCR kit that combined the RT step and the first round of the PCR was utilised. For the second round PCR the Qiagen *Taq* DNA polymerase kit was used. ORF 1 RT-PCR for GI and GII norovirus was carried out using a 2 round RT-PCR (Table 1). For studies on region C of ORF 2, GI and GII 2-round RT-PCR protocols were used (Table 1).

Nucleotide sequencing and phylogenetic analysis were carried out essentially as described previously.[18] The regions used for sequencing analysis are given in Table 1. Genotype analysis also made use of the norovirus automated genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtool).[19]

ORF 2 GII.4 variant status was determined in a 3 step process. Firstly, norovirus ORF 2 sequences were tested by the norovirus automated genotyping tool.[19] If the genotyping tool assigned a variant form, it was accepted. Secondly, if the typing tool classified a variant as 'unknown' it was classified as a 'like' variant if the ORF 2 sequence of 195 bases used was no more than 2 bases different from the accepted variant reference strain; this approach yielded the 'GII.4 New Orleans_2009-like' and 'GII.4 Sydney_2012-like' GII.4 forms found in the study. Thirdly, if an ORF 2 sequence had more than 2 base changes from the known variant reference strain it was classified as 'GII.4 (unknown)'. The reference strains used for this analysis comprised GII.4 New Orleans_2009 (GU445325) and GII.4 Sydney_2012 (JX459908).

Statistical analysis

Statistical significance of differences in genotype incidence between different groups was determined by the chi-square test or by Fisher's exact two-tailed test[20] as appropriate. Only sequenceable outbreaks were used to calculate the proportions. The significance of norovirus outbreak seasonality trends was evaluated by the method of partitioning of chi-square as given by Agresti.[21] The significance of differences in average age for different settings was determined by Student's t-test with the Welch approximation.[22] The correlation between setting and age was determined using Cohen's w index from Cramer's phi coefficient.[23]

Table 1: RT-PCR protocols used

Genogroup detected	ORF	Primers (5' to 3')*	Comments	References	Fragment size for phylogenetic analysis (position relative to reference strain)
GI and GII	ORF 1	**NV 4562** GAT GCD GAT TAC ACA GCH TGG G **NV 4611** CWG CAG CMC TDG AAA TCA TGG **NV 4692** GTG TGR TKG ATG TGG GTG ACT TC **NV 5296** CCA YCT GAA CAT TGR CTC TTG **NV 5298** ATC CAG CGG AAC ATG GCC TGC C **NV 5366** CAT CAT CAT TTA CRA ATT CGG	Two-round hemi-nested RT-PCR both detects and distinguishes between GI and GII noroviruses.	Yuen et al.[15] Bruggink et al.[16]	NA
GI	ORF 2	**COG1F** CGY TGG ATG CGN TTY CAT GA **G1SKR** CCA ACC CAR CCA TTR TAC A	Two-round RT-PCR.	McIver et al[17] Bruggink et al[16]	198bp (5415–5612†)
GII	ORF 2	**G2F3** TTG TGA ATG AAG ATG GCG TCG A **G2SKR** CCR CCN GCA TRH CCR TTR TAC AT	Two-round RT-PCR.	McIver et al. [17] Bruggink et al. [9]	195bp (5133–5327‡)

* D=AGT, H=ACT, W=AT, M=AC, R=AG, K=GT, Y=CT, N=AGCT.

† Reference strain Norwalk (accession number M87661).

‡ Reference strain Camberwell (accession number AF145896).

NA Not applicable.

RT-PCR Reverse-transcription polymerase chain reaction.

Experimental plan

All faecal specimens received at VIDRL for norovirus testing were initially screened by the ORF 1 RT-PCR, which detects and distinguishes between GI and GII noroviruses. If more than 1 specimen from a particular individual in a given outbreak was received, only the 1st specimen from that individual was tested. In addition, 1 specimen from every outbreak, chosen without bias, was also tested by both the ORF 2 GI and ORF 2 GII RT-PCRs and nucleotide sequencing performed if a positive result was obtained. The data were then analysed as follows: an outbreak was classified as norovirus positive if at least 1 specimen from the outbreak was positive by the ORF 1 and/or the ORF 2 assays. For the genotype analysis performed in the current study only sequences generated by the ORF 2 assays were used.

Results

Norovirus outbreak incidence, setting and temporal variation

For the calendar year 2013 faecal specimens from 301 gastroenteritis outbreaks were received for testing, and of these, 170 outbreaks were found to be positive for norovirus by the ORF 1 PCR and a further 20 were negative by the ORF 1 PCR but positive by an ORF 2 PCR. Thus a total of 190 outbreaks (63.1% of all outbreaks) were positive for norovirus, indicating it was the major cause of gastroenteritis.

Of these 190 outbreaks, 165 (86.8%) could be classified as healthcare and 25 (13.2%) as non-healthcare. A breakdown of the individual outbreak settings within these 2 categories is given in Table 2.

The monthly incidence of norovirus outbreaks and all outbreaks tested for 2013 is given in the Figure. It was noted that the 2-monthly incidence of norovirus outbreaks in January–February (i.e. late summer) was significantly higher than in any of the other 2-monthly periods in the year ($P < 0.005$, partitioning of chi-square).

Figure: The monthly incidence of norovirus outbreaks and all outbreaks tested, Victoria, 2013

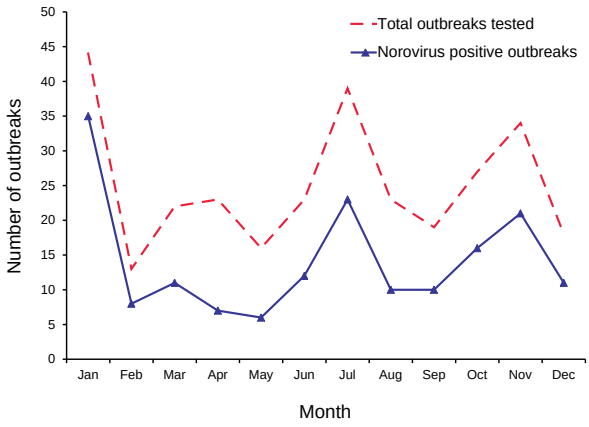

Table 2: Settings of norovirus outbreaks, Victoria, 2013

Healthcare	Number of norovirus outbreaks	Percentage of healthcare	Percentage of all norovirus outbreaks
Aged care facility	135	81.8	71.1
Disabled care facility	4	2.4	2.1
Hospital	21	12.7	11.1
Hospital – geriatric ward	2	1.2	1.1
Hospital – palliative care	1	0.6	0.5
Hospital – rehabilitation unit	2	1.2	1.1
Total	165	100.0	86.8
Non-healthcare	**Number of norovirus outbreaks**	**Percentage of non-healthcare**	**Percentage of all norovirus outbreaks**
Child care centre	8	32.0	4.2
Children's activity centre	2	8.0	1.1
Gathering	5	20.0	2.6
Navy base	1	4.0	0.5
Restaurant	5	20.0	2.6
School	1	4.0	0.5
Suspect food	3	12.0	1.6
Total	25	100.0	13.2

ORF 2 genotypes and settings

Sixteen ORF 2 genotypes were identified in the study (Table 3). Of these, the GII.4 genotype was the most common in both healthcare settings (63%) and non-healthcare settings (32%). However, GII.4 was significantly more common in outbreaks in healthcare settings than in non-healthcare settings ($P < 0.001$, chi-square test). A number of GII.4 variants were identified including GII.4 New Orleans_2009-like, GII.4 Sydney_2012 and GII.4 Sydney_2012-like. It was also noted that the genotypes GII.1 and GII.7 were significantly more common in non-healthcare settings than in healthcare settings ($P < 0.0012$ and $P < 0.032$, respectively, Fisher's exact two-tailed test).

Using the norovirus automated genotyping tool, an 'untypeable' GII ORF 2 norovirus sequence was detected in a 90-year-old individual associated with a gastroenteritis outbreak in November 2013. A BLAST search indicated the sequence had 99% nucleotide identity with the 2013 Korean strain KF774001. The strain identified in the current study has been lodged in GenBank as KM025343.

Table 3: ORF 2 norovirus genotypes and settings

Norovirus ORF 2 genotypes seen in healthcare settings	Number of norovirus outbreaks	Percentage of healthcare	Percentage of all norovirus outbreaks
GI.2	1	0.6	0.5
GI.3	2	1.2	1.1
GI.4	4	2.4	2.1
GI.6	2	1.2	1.1
GI.7	1	0.6	0.5
GI.8	1	0.6	0.5
GII.1	1	0.6	0.5
GII.2	1	0.6	0.5
GII.3	1	0.6	0.5
GII.4 New Orleans_2009-like	1	0.6	0.5
GII.4 Sydney_2012	67	40.6	35.3
GII.4 Sydney_2012-like	25	15.2	13.2
GII.4 unknown	11	6.7	5.8
GII.5	4	2.4	2.1
GII.6	2	1.2	1.1
GII.7	3	1.8	1.6
GII.13	8	4.8	4.2
GII.unknown	1	0.6	0.5
No sequence available	29	17.6	15.3
Total	165	100.0	86.8
Norovirus ORF 2 genotypes seen in non-healthcare settings	**Number of norovirus outbreaks**	**Percentage of non-healthcare**	**Percentage of all norovirus outbreaks**
GI.2	1	4.0	0.5
GI.9	1	4.0	0.5
GII.1	4	16.0	2.1
GII.3	1	4.0	0.5
GII.4 Sydney_2012	4	16.0	2.1
GII.4 Sydney_2012-like	4	16.0	2.1
GII.6	2	8.0	1.1
GII.7	3	12.0	1.6
GII.13	1	4.0	0.5
No sequence available	4	16.0	2.1
Total	25	100.0	13.2

ORF 2 genotypes and age

The relationship between age and norovirus ORF 2 genotype is presented in Table 4. It can be seen that healthcare settings tended to represent an older age demographic whereas non-healthcare settings tended to represent a younger age demographic. The average age of individuals (of known age) in healthcare settings was 82.6 years (standard deviation = 12.6 years, range = 23–98 years, n = 163) whereas the average age of individuals (of known age) in non-healthcare settings was 26.0 years (standard deviation = 25.2 years, range = 0–77 years, n = 24). This difference in average ages was statistically significant ($P < 0.001$, Student's t test with the Welch approximation).

All genotypes found in the study occurred in the older age group (66 years of age or over) with the exception of GI.9. Many of the genotypes found in the older age group were also detected in younger individuals (Table 4).

GII.4 norovirus was significantly more common in individuals 66 years of age or over than in those 65 years of age or under ($P < 0.001$, chi-square test). In contrast, GII.1 and GII.7 were significantly more common in those 65 years of age or under than in those 66 years of age and over ($P < 0.031$ and $P < 0.0006$ respectively, Fisher's exact two-tailed test).

Table 4: Age ranges and ORF 2 genotypes found in one representative individual from each outbreak

ORF 2 genotypes	Age ranges				
	0–15 years	16–45 years	46–65 years	>65 years	Unknown
Healthcare settings					
GI.2				1	
GI.3				2	
GI.4				4	
GI.6		1		1	
GI.7				1	
GI.8				1	
GII.1				1	
GII.2				1	
GII.3				1	
GII.4		2	7	94	1
GII.5				4	
GII.6			1	1	
GII.7		1	1	1	
GII.13			1	7	
GII.unknown				1	
No sequence available			2	26	1
Total	0	4	12	147	2
Non-healthcare settings					
GI.2	1				
GI.9			1		
GII.1	2		1		1
GII.3	1				
GII.4	3	3	2		
GII.6	1			1	
GII.7	1	2			
GII.13	1				
No sequence available	1	2		1	
Total	11	7	4	2	1

ORF 2 genotypes, setting and age

The incidence of GII.1 and GII.4 was linked to both setting and age. However, setting and age were closely correlated; chi-square analysis of setting vs age gave a value for Cohen's w index of 0.8, which indicates a strong correlation between setting and age.[19] Thus the data does not permit discrimination as to whether the critical variable was setting or age for these genotypes.

The data also indicates that there was a significant relationship between the incidence of the genotype GII.7 and setting and age. However, in this case it is possible to discriminate which variable is the critical one. In healthcare settings, GII.7 was significantly more common in those 65 years of age or under ($P < 0.029$, Fisher's exact two-tailed test). In non-healthcare settings, GII.7 was only found in individuals 65 years of age or under. These results suggest that for this genotype age was the critical variable.

Discussion

The current study indicates that in 2013 norovirus outbreaks occurred throughout the year although they were found to peak in warmer months of the year. Previous studies on the periodicity of norovirus outbreaks in Victoria have shown a similar trend,[24] indicating that 2013 was a typical year for norovirus incidence. The outbreaks occurred in a broad range of settings and individuals of all ages were affected.

This report is the first to systematically examine ORF 2 (capsid) genotypes associated with gastroenteritis outbreaks in Australia and a marked diversity of genotypes was found. Sixteen capsid genotypes were identified and included GI.2, GI.3, GI.4, GI.6, GI.7, GI.8, GI.9, GII.1, GII.2, GII.3, GII.4, GII.5, GII.6, GII.7, GII.13 and an unknown GII genotype. This unknown strain probably represents a new, as yet unclassified, norovirus genotype.

The current study indicates that the incidence of some genotypes, notably GII.1, GII.4 and GII.7, was linked to both setting and age. Since setting and age were linked to each other it is difficult to determine which was the more important. However, for one genotype, GII.7, age appeared to be the critical variable, with significantly greater incidence in younger age groups, possibly indicating long term immunity with this genotype. The relationship between the incidence of GII.7 and the age of infected individuals does not appear to have been examined in detail in the mainstream literature and warrants further study.

Of the genotypes detected, GII.4 was by far the most common in both healthcare settings and non-healthcare settings but was significantly more common in healthcare settings. This finding reinforces the findings of an earlier study in this laboratory, which examined the incidence of GII.4 norovirus, classified on the basis of ORF 1 sequencing,[25] in Victoria. In contrast to GII.4, genotypes GII.1 and GII.7 were found to be more common in non-healthcare settings than in healthcare settings. The precise relationship between the frequency of detection of these latter 2 genotypes and outbreak setting does not appear to have been examined in the mainstream literature and also warrants further study.

Although GII.4 (ORF 2) norovirus was the most common genotype in this and related studies[1], the findings of the current report indicate that norovirus vaccine strategies that target only GII.4 could exclude numerous other norovirus genotypes in both healthcare and non-healthcare settings. Norovirus vaccine strategies should encompass a broad range of genotypes and, as setting/age may be important in determining genotype incidence, this should be taken into account as well.

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