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# The molecular epidemiology of norovirus outbreaks in Victoria, 2016

Leesa D. Bruggink, Megan J. Triantafilou and John A. Marshall

## Abstract

Noroviruses are a leading cause of outbreaks of gastroenteritis. This study examined the incidence and molecular characteristics of norovirus outbreaks in Victoria, Australia in 2016. Norovirus was detected in 52.4% of gastroenteritis outbreaks surveyed and the year was notable in that there was no significant temporal peak in norovirus outbreaks. Norovirus Open Reading Frame (ORF)2 (capsid) genotypes were successfully identified in 84 of 110 norovirus outbreaks and included GI.3, GI.6, GI.9, GII.2, GII.3, GII.4, GII.6, and GII.17. Norovirus GII.4 was the most common ORF2 genotype detected (55.9%). Other relatively common ORF2 genotypes included GII.2 (19.0%), GII.17 (9.5%), GI.3 (7.1%) and GII.3 (4.8%). The GII.P4\_NewOrleans\_2009/GII.4\_Sydney\_2012 recombinant emerged as an important new GII.4 form. The study also confirmed the identity of three ORF1/ORF2 recombinant forms as follows: GII.P16/GII.2, GII.P16/GII.3 and GII.P16/GII.4. Statistical analysis indicated GII.4 (ORF2) was much more common in healthcare settings than in non-healthcare settings. The study indicates 2016 was a transition year in Victoria, Australia, in that the previous norovirus epidemic strain had diminished to the point where it was no longer dominant but as yet no replacement epidemic strain had become obvious.

Keywords: Norovirus, outbreaks, genotypes, RT-PCR, nucleotide sequencing.

## Introduction

The noroviruses, a major cause of diarrhoea worldwide<sup>1</sup>, are single-stranded positive sense RNA viruses, classified in the genus *Norovirus* within the family *Caliciviridae*.<sup>2</sup> Noroviruses are currently classified into 6 genogroups of which genogroups I, II and IV (GI, GII, GIV) occur in human infections.<sup>2</sup> In decreasing order of frequency human norovirus infections are caused by GII, GI and, to a very limited extent, GIV.<sup>3</sup>

The norovirus genome is typically defined in terms of its open reading frames (ORFs) i.e. the strings of codons uninterrupted by a stop codon<sup>4</sup> and the human norovirus genome is known to comprise three ORFs.<sup>2</sup> ORF1 encodes the non-structural polyprotein, ORF2 the major capsid protein and ORF3 the minor capsid protein.<sup>2</sup> Norovirus genotype classification can be based on the ORF1 region or the ORF2 region<sup>5</sup> but

recombination can occur at the ORF1-ORF2 intersect so in some recombinant noroviruses the ORF1 and ORF2 genotypes are different.<sup>6</sup>

Twenty nine ORF2 human norovirus genotypes have been identified<sup>2</sup> although the GII.4 genotype appears to be the most commonly reported in human disease.<sup>3,7</sup> Furthermore, GII.4 noroviruses undergo frequent genetic change such that a major new GII.4 variant strain normally appears every 2 to 4 years.<sup>7</sup> Recent GII.4 variant forms have included Hunter\_2004 (2004), Yerseke\_2006a (2006), Den Haag\_2006b (2006), NewOrleans\_2009 (2009) and Sydney\_2012 (2012).<sup>5,8</sup>

The natural history of norovirus is complex in that norovirus prevalence can vary throughout the year and the prevalence patterns of the various norovirus genotypes appear to be dependent on a number of variables including the setting of



the outbreak<sup>9</sup> and the geographical region.<sup>10</sup> The current report extends previous work on norovirus prevalence in Victoria in the year 2013<sup>11</sup> and the years 2014-2015<sup>9</sup> by examining the characteristics of norovirus outbreaks in Victoria in 2016 and their associated genotypes. In particular, the study examined quantitative and qualitative aspects of 3 areas: what was the relationship between seasonality and norovirus incidence; what norovirus genotypes were detected and how did they relate to previous findings of norovirus prevalence; what was the relationship between genotype and outbreak setting.

## Materials and methods

### Definition of a gastroenteritis outbreak

For the purposes of this study an outbreak was defined as a gastroenteritis cluster which was apparently associated with a common event or location and in which 4 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 2016. VIDRL, which is National Association of Testing Authorities, Australia (NATA) accredited, is the main public health laboratory for viral identification in the state of Victoria, Australia. 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.

### Faecal processing, RNA extraction and Reverse Transcription-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 minute centrifugation (7000g) as previously described.<sup>12</sup> 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)<sup>13</sup> or the Qiagen (Qiagen Sciences, Germantown, MD, USA) QIAcube automated extraction procedure.

Four two-round RT-PCR procedures (protocols 1 to 4, Table 1) were then used for norovirus detection. For the first round of each of the 4 protocols, the Qiagen (GmbH, Hilden, Germany) One step RT-PCR kit that combined the RT step and the first round PCR was utilised. For the second round PCR the Qiagen *Taq* DNA polymerase kit was used. All PCR protocols utilised a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA).

### Nucleotide sequencing and genotype classification

Nucleotide sequencing was carried out for protocols 1 to 4 (Table 1). Sequence analysis made use of the software MacVector (Oxford Molecular Limited, Madison, WI, USA) and genotyping made use of the norovirus genotyping tool (<http://www.rivm.nl/mpf/norovirus/typingtool>).<sup>18</sup>

### Experimental plan

All faecal specimens in the study were initially tested by the protocol 1 RT-PCR. Nucleotide sequencing was carried out on 1 positive specimen, chosen at random, from each outbreak. One specimen from every outbreak was also tested by both protocols 2 and 3 (ORF2 GI and

Table 1: RT-PCR protocols used

Genogroup detected (Protocol No.)	ORF	Primers (5' to 3')*	Comments	References	Fragment size for genotype analysis (position relative to reference strain)
GI and GII (protocol 1)	ORF1	<b>NV 4562</b> GAT GCD GAT TAC ACA GCH TGG G	Two-round hemi-nested RT-PCR both detects and distinguishes between GI and GII noroviruses.	Yuen <i>et al.</i> <sup>14</sup> Bruggink <i>et al.</i> <sup>15</sup>	440bp (4,484-4,923 <sup>†</sup> )
		<b>NV 4611</b> CWG CAG CMC TDG AAA TCA TGG			
		<b>NV 4692</b> GTG TGR TKG ATG TGG GTG ACT TC			
		<b>NV 5296</b> CCA YCT GAA CAT TGR CTC TTG			
		<b>NV 5298</b> ATC CAG CGG AAC ATG GCC TGCC			
		<b>NV 5366</b> CAT CAT CAT TTA CRA ATT CGG			
		<b>COG1F</b> CGY TGG ATG CGN TTY CAT GA			
		<b>G15KR</b> CCA ACC CAR CCA TTR TACA			
		<b>G2F3</b> TTG TGA ATG AAG ATG GCG TCG A			
		<b>G25KR</b> CCR CCN GCA TRH CCR TTR TAC AT			
GI (protocol 2)	ORF2	<b>NV 4611</b> CWG CAG CMC TDG AAA TCA TGG	Two-round RT-PCR. The second round is a booster step which uses the same primers as the first round.	Bruggink <i>et al.</i> <sup>15</sup> McIver <i>et al.</i> <sup>16</sup>	198bp (5,415-5,612 <sup>†</sup> )
		<b>G25KR</b> CCR CCN GCA TRH CCR TTR TAC AT			
		<b>NV 4611</b> CWG CAG CMC TDG AAA TCA TGG			
		<b>G25KR</b> CCR CCN GCA TRH CCR TTR TAC AT			
GII (protocol 3)	ORF2	<b>NV 4611</b> CWG CAG CMC TDG AAA TCA TGG	Two-round RT-PCR. The second round is a booster step which uses the same primers as the first round.	McIver <i>et al.</i> <sup>16</sup> Dunbar <i>et al.</i> <sup>17</sup>	195bp (5,133-5,327 <sup>†</sup> )
		<b>G25KR</b> CCR CCN GCA TRH CCR TTR TAC AT			
GII-GII (protocol 4)	ORF1-ORF2	<b>NV 4611</b> CWG CAG CMC TDG AAA TCA TGG	Two-round RT-PCR. The second round is a booster step which uses the same primers as the first round.	This study.	753bp (4,484-5,236 <sup>†</sup> )
		<b>G25KR</b> CCR CCN GCA TRH CCR TTR TAC AT			

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

<sup>†</sup>Reference strain Sydney (accession number JX459908)

<sup>‡</sup>Reference strain Norwalk (accession number M87661)

ORF2 GII RT-PCRs). Nucleotide sequencing was then performed on all positive norovirus specimens from protocols 2 and 3. In addition an ORF1-ORF2 RT-PCR (protocol 4) was carried out to confirm the recombination status of specimens where the ORF1 and ORF2 RT-PCR protocols gave different genotypes.

## Statistical analysis

To investigate the distribution of norovirus outbreaks among different months of the year for 2016 the  $\chi^2$  test was used.<sup>19</sup> The null hypothesis was that the number of outbreaks in the first 6 months of the year was the same as in the second 6 months of the year. The year was also divided into 3 and/or 4 month periods and the null hypothesis was taken that the number of outbreaks was the same in all periods.

Frequency tables were set up to investigate whether the genotypes involved in outbreaks varied by setting (healthcare vs non-healthcare). In this table, the GII.4 variants were combined and compared to all other genotypes combined to ensure that frequencies were sufficiently high for the  $\chi^2$  test to be valid. The null hypothesis was

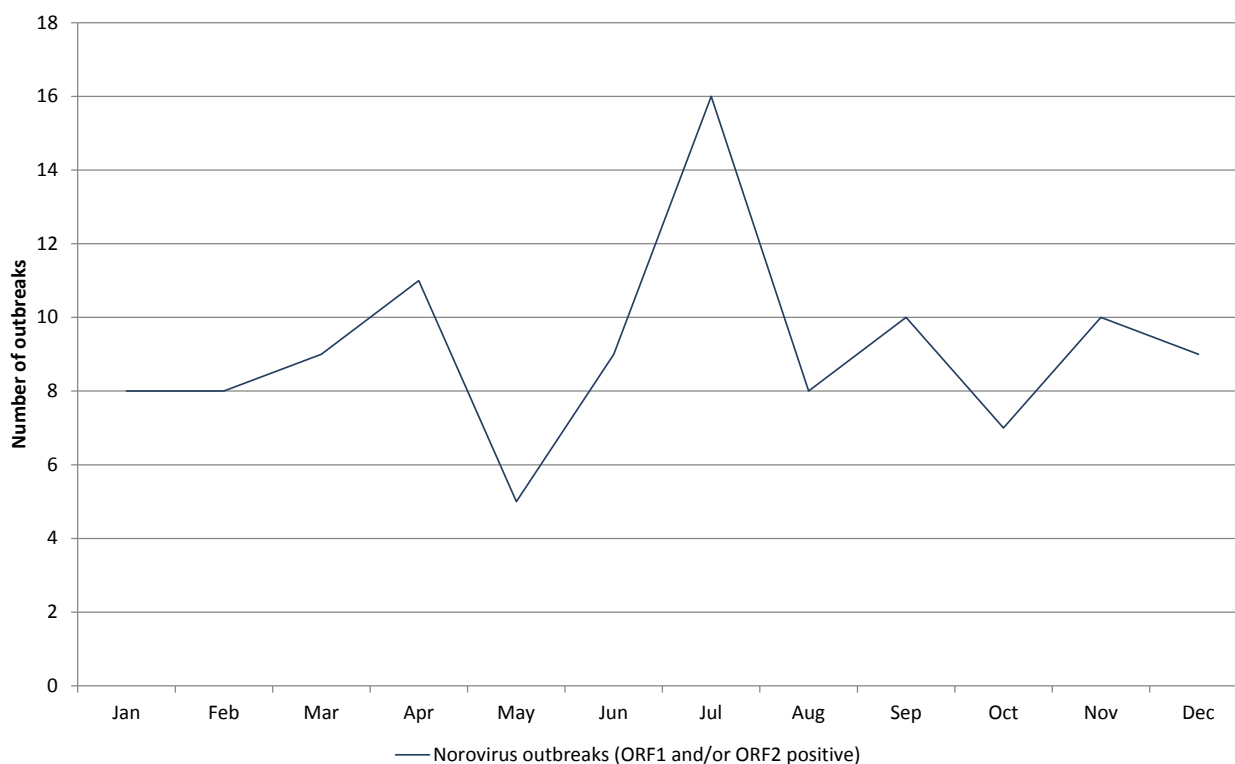
that the proportion of outbreaks in healthcare settings (and consequently the proportion of outbreaks in non-healthcare settings) was the same for GII.4 and for the other genotypes. A high value of  $\chi^2$  corresponding to a probability of less than 0.05 was taken to indicate that GII.4 differed significantly from the other genotypes in the proportion of outbreaks in healthcare settings (and consequently in non-healthcare settings). This was found to be the case ( $\chi^2 = 16.07$ , 1 degree of freedom,  $p = 0.000061$ ).

GII.4 and the other genotypes were then considered separately to determine the relative frequency in healthcare and non-healthcare settings. The null hypothesis for GII.4 was that the number of outbreaks in healthcare settings was the same as in non-healthcare settings. The same null hypothesis was used for the other genotypes.

## Ethics

Data collection for the current study is covered by public health legislation and specific ethics approval was not required. No information is given that would allow the identification of any individuals in the study.

**Figure 1: Number of norovirus positive outbreaks per month for the year 2016**



**Table 2: Norovirus outbreaks by setting in 2016**

Healthcare	Number of outbreaks	% of total (n=110)
Aged Care Facility	70	63.6
Disabled Care Facility	4	3.6
Hospital	7	6.4
Hospital - Rehabilitation Unit	4	3.6
<b>Total Healthcare</b>	<b>85</b>	<b>77.3</b>
Non-Healthcare	Number of outbreaks	% of total (n=110)
Camp - school	1	0.9
Child Care Centre	14	12.7
Gathering	4	3.6
Restaurant	6	5.5
<b>Total Non-Healthcare</b>	<b>25</b>	<b>22.7</b>

## Results

### Norovirus outbreak incidence, seasonal periodicity and settings

For the 2016 calendar year, 666 specimens from 210 gastroenteritis outbreaks were received for testing and of these, 110 outbreaks (52.4%) were positive for norovirus by the ORF1 (Protocol 1) and/or ORF2 (Protocols 2 and 3) RT-PCRs.

The seasonal periodicity of all norovirus outbreaks for 2016 is given in Figure 1. It can be seen that outbreaks occurred throughout the year with an apparent peak in the middle of the year. The null hypothesis, that the number of outbreaks in the first 6 months of the year was the same as in the second 6 months of the year, was confirmed ( $\chi^2 = 0.91$ , 1 degree of freedom,  $p = 0.34$ ). When the year was divided into 3 and four month periods, with the null hypothesis being that the number of outbreaks was the same in each period, the null hypothesis was again confirmed with  $p > 0.34$ . Thus statistical analysis indicated there was no seasonal peak for the year 2016.

Norovirus outbreak settings could be divided into 2 groups: healthcare and non-healthcare (Table 2). Within these groups a variety of settings were noted although most outbreaks received for testing were from the elderly (aged care facilities) or children (child care centres).

### Norovirus genotype analysis

A summary of all ORF1 and ORF2 norovirus genotypes identified in the study is given in Table 3. It can be seen that a broad range of norovirus genotypes were detected.

ORF2 (capsid) genotypes were successfully identified in 84 of 110 norovirus outbreaks and included, in descending order, GII.4 (47/84), GII.2 (16/84), GII.17 (8/84), GI.3 (6/84), GII.3 (4/84), GI.6 (1/84) GI.9 (1/84) and GII.6 (1/84).

The ORF1 genotype sometimes differed from the ORF2 genotype in a given outbreak (Table 3). Previous studies<sup>9</sup> for the period 2014-15 confirmed the ORF1/ORF2 recombinant forms GII.P4\_NewOrleans\_2009/GII.4\_Sydney\_2012, GII.P12/GII.3 and GII.Pe/GII.4\_Sydney\_2012. In the current study 3 new ORF1/ORF2 recombinant forms were confirmed: GII.P16/GII.4, GII.P16/GII.3 and GII.P16/GII.2. A representative sequence of each of these 3 recombinant genotypes has been deposited in GenBank with the accession numbers MF574092-4 respectively.

### Relationship between ORF2 genotype and setting

The relationship between ORF2 genotype and outbreak setting is given in Table 4. Statistical analysis indicated GII.4 was much more common in healthcare settings than in non-healthcare settings ( $\chi^2 = 35.77$ , 1 degree of freedom,  $p$

**Table 3: Norovirus genotypes detected in 2016**

ORF1	ORF2	Number of outbreaks
GI.P3	GI.3	6
GI.P6	GI.6	1
GI.P9	GI.9	1
GII.P4_NewOrleans_2009	GII.4_Sydney_2012	17
GII.P4_NewOrleans_2009	GII.4*	6
GII.P4_NewOrleans_2009	-	4
GII.P12	GII.3	2
GII.P12	-	8
GII.P16	GII.2	1
GII.P16	GII.3	1
GII.P16	GII.4*	4
GII.P16	-	3
GII.P17	GII.17	8
GII.Pe	GII.4_Sydney_2012	1
GII.Pe	GII.4*	6
GII.Pe	-	11
-	GII.2	15
-	GII.3	1
-	GII.4*	13
-	GII.6	1
<b>TOTAL</b>		<b>110</b>

\*GII.4 variant identity could not be determined by the norovirus genotyping tool.<sup>18</sup>

**Table 4: Norovirus ORF2 genotypes seen in healthcare and non-healthcare settings**

ORF2 genotype	Norovirus positive outbreaks in healthcare settings		Norovirus positive outbreaks in non-healthcare settings		Total number of outbreaks
	Number	%	Number	%	
GI.3	3	3.5	3	12.0	6
GI.6	0	0.0	1	4.0	1
GI.9	1	1.2	0	0.0	1
GII.2	8	9.4	8	32.0	16
GII.3	0	0.0	4	16.0	4
GII.4_Sydney_2012	18	21.2	0	0.0	18
GII.4*	26	30.6	3	12.0	29
GII.6	1	1.2	0	0.0	1
GII.17	8	9.4	0	0.0	8
No sequence available	20	23.5	6	24.0	26
<b>TOTAL</b>	<b>85</b>	<b>100.0</b>	<b>25</b>	<b>100.0</b>	<b>110</b>

\*GII.4 variant identity could not be determined by the norovirus genotyping tool.<sup>18</sup>



= 0.0000000011) whereas other genotypes were found with approximately equal frequency in healthcare settings and non-healthcare settings ( $\chi^2 = 0.68$ , 1 degree of freedom,  $p = 0.41$ ).

## Discussion

It is now established that norovirus outbreaks in Victoria occur throughout the year at a baseline level with one or more epidemics occurring every calendar year.<sup>20,21</sup> The findings of the current study, however, indicate 2016 was an exceptional year in that there was no significant epidemic peak.

Yearly norovirus epidemics appear to be controlled by two sets of factors, environmental and genetic.<sup>22</sup> Rainfall appears to be one important environmental factor in Victoria<sup>23</sup> and changes in the nature of GII.4 noroviruses appear to be an important genetic factor.<sup>24</sup> Previous studies in this laboratory<sup>9,11</sup> and the findings of the current study, indicate there was a progressive decline in the number of norovirus outbreaks in Victoria over the period 2013-2016, and this corresponded to a decline in the initially predominant GII.4 variant during 2014-2016.<sup>9</sup> This progressive decline, in turn, appears finally to have resulted in norovirus outbreaks remaining at baseline level throughout 2016. The detailed data for this are as follows.

Surveillance studies for the years 2013<sup>11</sup>, 2014<sup>9</sup>, 2015<sup>9</sup> and 2016 (the current study) indicate the number of norovirus outbreaks identified in Victoria over this four-year period, using similar norovirus testing and surveillance methods, was 190, 165, 122 and 110 respectively. In 2012, a new GII.4 epidemic strain, GII.Pe/GII.4\_Sydney\_2012 emerged.<sup>25,26</sup> ORF1 data was not available for 2013 but in 2014 and 2015, 60 and 32 GII.Pe/GII.4\_Sydney\_2012 outbreaks, respectively, were identified<sup>9</sup> and in the current study only one such outbreak was identified. As a percentage of all norovirus outbreaks for the years 2014, 2015 and 2016, where both an ORF1 and ORF2 genotype were obtained, fully identified GII.Pe/GII.4\_Sydney\_2012 norovirus made up 51.3%,

40.0% and 1.9% of all norovirus outbreaks. Thus, GII.Pe/GII.4\_Sydney\_2012 had become relatively unimportant by 2016.

The question then arises whether an alternate GII.4 norovirus variant form, or some other norovirus genotype, is emerging as a potential predominant norovirus. Of the various ORF1/ORF2 forms identified in 2016, two stand out: GII.P4\_NewOrleans\_2009/GII.4\_Sydney\_2012 (17 outbreaks) and GII.P17/GII.17 (8 outbreaks).

GII.P4\_NewOrleans\_2009/GII.4\_Sydney\_2012 will be considered first.

Bruggink et al.<sup>20</sup> recently examined 14 years of norovirus outbreak surveillance data and noted there was typically a delay of 2 to 7 months between the first detection of a new GII.4 variant and the subsequent epidemic linked to that variant. Bruggink et al.<sup>20</sup> noted that the recombinant form GII.P4\_NewOrleans\_2009/GII.4\_Sydney\_2012 was first detected in Victoria in August 2015 at low frequency and then re-emerged in June 2016, having undergone marked genetic change in the capsid region. Studies in 2017 should determine whether it will become the next major norovirus pandemic strain or whether it will be eclipsed by another GII.4 variant or some other genotype.

The possibility of GII.17 becoming predominant in the future also requires consideration. Recent studies in China<sup>27,28</sup> and Japan<sup>29</sup> have identified GII.17 as an important new epidemic strain in these countries. This in turn has encouraged some authors to suggest that GII.17 might replace GII.4 as the predominant norovirus strain around the world at some point.<sup>10</sup> GII.17 was first detected in Victorian norovirus outbreaks in 2014 and was also detected in 2015.<sup>9</sup> In both years it was relatively rare. GII.17 was detected in the current study, but only made up about 15% (8/54) of all fully characterized genotypes associated with Victorian norovirus outbreaks, thereby indicating it was not an especially important virus in Victorian norovirus outbreaks in 2016.

Surveillance studies of GI noroviruses in Victorian norovirus outbreaks in 2014 and 2015 indicated that a dramatic change in the mix of GI genotypes had occurred in those 2 years,<sup>9</sup> in that in 2014 seven GI ORF1/ORF2 genotype combinations were detected, whereas in 2015 only one GI ORF1/ORF2 combination (GI.P3/GI.3) was detected. In the current study the GI ORF1/ORF2 combination GI.P3/GI.3 remained predominant, but 2 other GI forms (GI.P6/GI.6 and GI.P9/GI.9) were also detected. Despite the greater variety of GI noroviruses detected in 2016 than in 2015, GI norovirus associated gastroenteritis outbreaks still only made up about 15% (8/54) of Victorian norovirus outbreaks of fully characterized genotypes.

It is now well established that there can be a relationship between norovirus genotype and outbreak setting. More specifically, recent studies in Victoria have indicated that the ORF2 genotypes GI.3, GII.6 and GII.4 were significantly more common in healthcare settings than in non-healthcare settings.<sup>9</sup> The data in the current study provided further confirmation that the ORF2 genotype GII.4 was significantly more common in healthcare than in non-healthcare settings.

A critical question in the development of vaccine strategies is whether norovirus genotypes in 1 region are similar to those in another region. The growing evidence is that this may not be the case. For example, it was notable that in Germany in 2016 there was a steep rise in the recombinant form GII.P16/GII.2<sup>30</sup> but this recombinant, whilst recognized in the current study, was not an important virus. Further, United States surveillance reports<sup>31</sup> indicate the recombinant form GII.P16/GII.4 became prominent but this was also not the case in Victoria.

The current study indicates 2016 was a transition year in Victoria, Australia, in that the previous norovirus epidemic strain had diminished to the point where it was no longer dominant but as yet no replacement epidemic strain had become obvious. There was some evidence that the recombinant form GII.P4\_NewOrleans\_2009/GII.4\_Sydney\_2012 may take the role of predominant norovirus strain in Victoria but fur-

ther studies in 2017 should answer this question. Ongoing studies are also important to determine how, and if, Australia is different from the rest of the world.

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