The molecular epidemiology of norovirus outbreaks in Victoria, 2014 to 2015

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Abstract

Noroviruses are a leading cause of outbreaks of gastroenteritis. This study examined the incidence and molecular characteristics of norovirus outbreaks in healthcare and non-healthcare settings in Victoria, Australia, over 2 years (2014–2015). Norovirus was detected in 65.7% and 60.4% of gastroenteritis outbreaks investigated for the years 2014 and 2015 respectively. There was a significant decline in the number of norovirus outbreaks in the period 2014 to 2015 although in both years norovirus outbreaks peaked in the latter part of the year. Norovirus Open Reading Frame (ORF) 2 (capsid) genotypes identified included GI.2, GI.3, GI.4, GI.5, GI.6, GI.9, GII.2, GII.3, GII.4, GII.6, GII.7, GII.8, GII.13 and GII.17. GII.4 was the most common genotype detected. In addition, the following ORF 1/ORF 2 recombinant forms were confirmed: GII.P4_NewOrleans_2009/GII.4_Sydney_2012, GII.P12/GII.3, GII.Pb (GII.21)/GII.3, GII.Pe/GII.2 and GII.Pe/GII.4_Sydney_2012. A significant decline was noted in the chief norovirus strain GII.Pe/GII.4_Sydney_2012 between 2014 and 2015 but there was a re-emergence of a GII.P4_NewOrleans _2009 norovirus strain. Outbreaks involving the GII.P17/GII.17 genotype were also detected for the first time in Victoria. GI genotypes circulating in Victoria for the 2 years 2014 and 2015 underwent a dramatic change between the 2 years of the survey. Many genotypes could occur in both healthcare and non-healthcare settings although GI.3, GII.6, and GII.4 were significantly more common in healthcare settings. The study emphasises the complex way in which norovirus circulates throughout the community. *Commun Dis Intell* 2017;41(1):E21–E32.

Keywords: norovirus, outbreaks, genotypes, healthcare, non-healthcare, setting, RT-PCR, nucleotide sequencing

Introduction

Noroviruses are single-stranded positive sense RNA viruses, classified in the genus *Norovirus* within the Family Caliciviridae.[1] Noroviruses are currently classified into 6 genogroups of which genogroups I, II and IV (GI, GII, GIV) occur in human infections.[1] The incidence and clinical significance of GIV noroviruses in human infections are little understood.[2]

Noroviruses are now recognised as a major cause of morbidity and mortality and can cause gastroenteritis in individuals of all ages; it has been estimated that, annually, norovirus infection causes 70,000 to 200,000 deaths around the world.[3] Globally, norovirus is considered to be associated with approximately one-fifth of all diarrhoea cases[4] and in Australia, norovirus appears to be the major known gastroenteritis pathogen.[5] Although vaccine strategies against norovirus are under development,[3] the genetic diversity of the noroviruses has complicated this process.[3,6]

The human norovirus genome comprises 3 open reading frames (ORFs).[1] ORF 1 encodes the non-structural polyprotein, ORF 2 the major capsid protein and ORF 3 the minor capsid protein.[1] Norovirus genotype classification can be based on the ORF 1 region or the ORF 2 region[7] but recombination can occur at the ORF 1–ORF 2 intersect[8] so in some recombinant noroviruses the ORF 1 and ORF 2 genotypes are different.

At least 29 ORF 2 human norovirus genotypes have been identified[1] although the GII.4 genotype appears to be the most common norovirus genotype in human disease.[3,9] Furthermore, GII.4 noroviruses undergo mutation and recombination such that a major new GII.4 variant epidemic strain normally appears every 2 to 4 years.[3] It is therefore of interest that recent studies in China[10,11] and Japan[12] indicate that a new epidemic strain, GII.17, may have emerged. These observations prompted de Graaf et al.[13] to raise the question whether the emergence of a novel GII.17 norovirus is a sign that the GII.4 era was coming to an end.

Overview studies of norovirus molecular epidemiology remain an area of active interest in Australia.[14,15] The current report extends previous work by examining the characteristics of norovirus outbreaks in Victoria in 2014 to 2015 and their associated genotypes. In particular the study examined quantitative and qualitative aspects of 3 areas: what is the relationship between seasonality and norovirus incidence; what norovirus genotypes were detected and how did they change over time and what was the relationship between norovirus genotype and outbreak setting.

Materials and methods

Definition of gastroenteritis outbreak

For the purposes of this study an outbreak was defined as a gastroenteritis cluster that 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 2014 to 2015. VIDRL, which is National Association of Testing Authorities, Australia accredited, 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.

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 as previously described.[16] 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).[17]

Six 2-round RT-PCR procedures (protocols 1 to 6; Table 1) were then used for norovirus detection. For the first round of each of the 6 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, 2, 3 and 5 (Table 2). Protocol 4 was directed to the possibility of detecting a GI-GI recombinant and protocol 6 to the possibility of detecting a GII-GI recombinant but no PCR product was obtained with either protocol. Sequence analysis made use of the software MacVector (Oxford Molecular Limited, Madison, WI) and genotyping made use of the norovirus genotyping tool (http://www.rivm.nl/mpf/norovirus/typingtool).[24]

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 (ORF 2 GI and ORF 2 GII RT-PCRs). Nucleotide sequencing was then performed on all positive norovirus specimens from protocols 2 and 3. In addition ORF 1–ORF 2 RT-PCRs (protocols 4–6) were carried out to confirm the recombination status of specimens where the ORF 1 and ORF 2 RT-PCR protocols gave different genotypes.

Statistical analysis

Statistical analysis was carried out using the χ^2 test[25] and the partitioning of χ^2 test.[26] For the partitioning of χ^2 test 3 terms, 'period', 'grouping' and 'class' were defined as follows. A period was one of the 2-month intervals for which the outbreak frequency was used in the analysis. A grouping was a set of genotypes combined so that the total frequency was sufficient for the χ^2 test to be valid. A class was the set of 2-monthly periods or groupings such that the individual periods or groupings did not differ significantly from each other in the characteristic of interest.

To investigate the change in the number of norovirus outbreaks between 2014 and 2015, the null hypothesis was that the number of outbreaks was the same in each year and equal to half of the total number of outbreaks in 2014 to 2015. The χ^2 test (1 degree of freedom) was then applied. If the probability was less than 0.05 the difference was taken to be significant.

To investigate the distribution of norovirus outbreaks among different months of the year separately for 2014 and 2015, the partitioning of χ^2 test was used. First, the months were combined in pairs, to smooth out fluctuations, and the null hypothesis was that the number of outbreaks was

Table 1: Reverse transcription-polymerase chain reaction (RT-PCR). protocols used

Genogroup detected (Protocol number)	ORF	Primers (5' to 3')*	Comments	References	Fragment size for genotype analysis (position relative to reference strain)
GI and GII (protocol 1)	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.[18] Bruggink et al.[19]	440bp (4484–4923†)
GI (protocol 2)	ORF 2	**COG1F** CGY TGG ATG CGN TTY CAT GA **G1SKR** CCA ACC CAR CCA TTR TAC A	Two-round RT-PCR. The second round is a booster step, which uses the same primers as the first round.	Bruggink et al.[19] McIver et al.[20]	198bp (5415–5612‡)
GII (protocol 3)	ORF 2	**G2F3** TTG TGA ATG AAG ATG GCG TCG A **G2SKR** CCR CCN GCA TRH CCR TTR TAC AT	Two-round RT-PCR. The second round is a booster step, which uses the same primers as the first round.	McIver et al.[20] Dunbar et al.[21]	195bp (5133–5327†)
GI-GI (protocol 4)	ORF 1-ORF 2	**NV 4562** GAT GCD GAT TAC ACA GCH TGG G **G1SKR** CCA ACC CAR CCA TTR TAC A	Two-round RT-PCR. The second round is a booster step, which uses the same primers as the first round.	Bruggink et al.[22]	NA
GII-GII (protocol 5)	ORF 1-ORF 2	**NV 4692** GTG TGR TKG ATG TGG GTG ACT TC **G2SKR** CCR CCN GCA TRH CCR TTR TAC AT	Two-round RT-PCR. The second round is a booster step, which uses the same primers as the first round.	Bruggink et al.[22]	345–753bp (in range 4484–5273†)
GII-GI (protocol 6)	ORF 1-ORF 2	**NV 4692** GTG TGR TKG ATG TGG GTG ACT TC **G1SKR** CCA ACC CAR CCA TTR TAC A	Two-round RT-PCR. The second round is a booster step, which uses the same primers as the first round.	Bruggink et al.[23]	NA

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

† Reference strain Camberwell (accession number AF145896)

‡ Reference strain Norwalk (accession number M87661)

NA = not applicable

Table 2: Settings of norovirus positive outbreaks 2014 to 2015

Healthcare settings	Number of norovirus positive outbreaks	Percentage of healthcare outbreaks	Percentage of total norovirus positive outbreaks
Aged care facility	199	85.8	69.3
Disabled care facility	10	4.3	3.5
Early parenting centre	2	0.9	0.7
Hospital	16	6.9	5.6
Hospital – Maternity Ward	1	0.4	0.3
Hospital – Psychiatric Ward	3	1.3	1.0
Hospital – Rehabilitation Unit	1	0.4	0.3
Total	232	100.0	80.8
Non-healthcare settings	**Number of norovirus positive outbreaks**	**Percentage of non-healthcare outbreaks**	**Percentage of total norovirus positive outbreaks**
Camp	1	1.8	0.3
Camp – school	2	3.6	0.7
Childcare centre	27	49.1	9.4
Gathering	6	10.9	2.1
Restaurant	12	21.8	4.2
School	1	1.8	0.3
Special accommodation	2	3.6	0.7
Suspect food	4	7.3	1.4
Total	55	100.0	19.2

the same in each 2-monthly period. The χ^2 test (5 degrees of freedom) was then applied. In 2014 there was a difference in norovirus incidence for different 2-monthly periods ($\chi^2 = 30.53$, 5 degrees of freedom, $P = 0.00001$) so that it was valid to partition χ^2. In 2015 there was also a difference in norovirus incidence for different 2-monthly periods ($\chi^2 = 37.74$, 5 degrees of freedom, $P = 0.0000004$) so that it was again valid to partition χ^2. Secondly, to partition χ^2 for each year, 2-monthly periods were combined to give classes, with the null hypothesis that all the 2-monthly periods in each class had the same number of outbreaks. The 2-monthly periods for each class were chosen so that when a χ^2 test was applied with the null hypothesis used, the value of χ^2 was small and it could be taken that there was no significant difference between the number of outbreaks in the 2-monthly periods in the class. Thirdly, a new table was created with each entry being the total number of outbreaks in a class covering the total months corresponding to the class. A χ^2 test was applied to this new table with the null hypothesis being that the number of outbreaks per 2-monthly period was the same for all classes in the table. If the value of χ^2 was sufficiently high to correspond to a probability of less than 0.05 it was taken that there was a difference in the incidence per 2-monthly period of the different time intervals for the table.

To investigate the significance of the change in the proportion of norovirus outbreaks of the 2 major ORF 1/ORF 2 genotypes (GII.Pe/GII.4_Sydney_2012 and GII.Pe/GII.4) between 2014 and 2015 the ORF 1/ORF 2 genotypes were first divided into 3 groupings, GII.Pe/GII.4_Sydney_2012, GII.Pe/GII.4 and the other genotypes ('other genotypes'). The null hypothesis was that the proportion of outbreaks due to the 3 genotype groupings was the same in 2014 and in 2015. The χ^2 test (2 degrees of freedom) was then applied. A significant difference was found ($\chi^2 = 6.162$, 2 degrees of freedom, $P = 0.046$) so that partitioning of χ^2 could be applied. On this basis the genotype groupings could then be grouped into 2 classes, with GII.Pe/GII.4_Sydney_2012 and GII.Pe/GII.4 in the first class and 'other genotypes' in the second class. For the first class genotypes, the null hypothesis was that the proportion of the outbreaks associated with each of the 2 genotypes was the same in 2014 as in 2015. This null hypothesis was confirmed ($\chi^2 = 0.005$, 2 degrees of freedom, $P = 0.997$). Then a new table was set up, with each row giving the numbers of outbreaks in 2014 and 2015 for 1 of the 2 classes. The null hypothesis was that the proportion of outbreaks for each of the 2 classes was the same in 2014 and 2015. Application of the χ^2 test indicated the null hypothesis was not supported ($\chi^2 = 6.157$, 2 degrees of freedom, $P = 0.046$).

To investigate whether the proportion of outbreaks of a particular genotype was higher in a particular type of setting (healthcare vs non-healthcare) than for other genotypes, a table was set up giving the frequencies of outbreaks in the healthcare and non-healthcare settings for each genotype. The frequencies of the minor genotypes were then combined so that frequencies in the table were sufficiently high that the χ^2 test could be validly used. This gave a table with 5 groupings of genotypes, GI.3, GII.4, GII.6, GII.3 and 'other genotypes'. 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 each of the 5 groupings of genotypes. The χ^2 test was then applied. As there were significant differences in the fraction of outbreaks in healthcare settings among these 5 groupings of genotypes ($\chi^2 = 24.16$, 4 degrees of freedom, $P = 0.00007$), partitioning of χ^2 could be applied. On this basis, the genotype groupings could then be organised into classes, with the null hypothesis that the proportion of outbreaks in healthcare settings for each genotype grouping in the class was the same for all genotype groupings in the class. The first class comprised GI.3, GII.4 and GII.6 and a χ^2 test applied to the genotype groupings in the class gave $\chi^2 = 2.45$, 4 degrees of freedom, $P = 0.65$. The second class comprised GII.3 and 'other genotypes' and a χ^2 test applied to the genotype groupings in the class gave $\chi^2 = 0.83$, 4 degrees of freedom, $P = 0.935$. Thus the null hypothesis was confirmed for each class and the proportion of outbreaks in healthcare settings for each genotype grouping in the class could be taken to be the same.

A new table was set up, with each row giving the numbers of outbreaks in healthcare and non-healthcare settings for 1 of the 2 classes. The null hypothesis was that the proportion of outbreaks in healthcare settings (and in non-healthcare settings) for all the classes, was the same and the χ^2 test was applied to the new table. A high value of χ^2 corresponding to a probability of less than 0.05 was taken to indicate that the classes differed in the proportion of outbreaks of that genotype in healthcare settings (and consequently in non-healthcare settings) and this was found to be the case ($\chi^2 = 21.01$, 4 degrees of freedom, $P = 0.00003$). Each class could then be considered as a whole to determine the relative frequency in healthcare and non-healthcare settings. The first class was then tested to determine whether genotypes were more prevalent in healthcare than in non-healthcare settings, the null hypothesis being that the number of outbreaks in healthcare settings was the same as the number of outbreaks in non-healthcare settings. The null hypothesis was not supported ($\chi^2 = 102.76$, 1 degree of freedom, $P = 5 \times 10^{-24}$).

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.

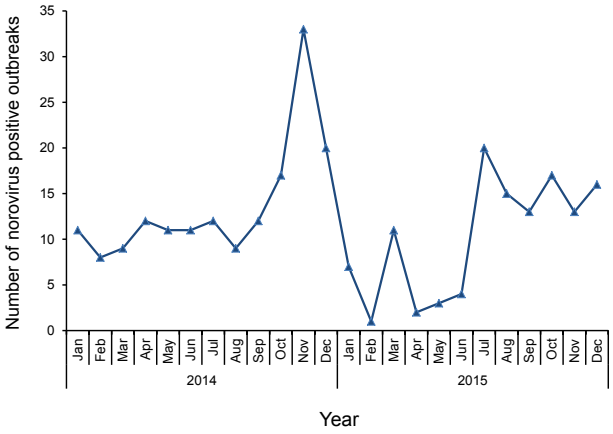
Results

Norovirus outbreak incidence, seasonal periodicity and setting

For the calendar year 2014, specimens from 251 gastroenteritis outbreaks were received for testing and of these 165 (65.7%) were positive for norovirus by the ORF 1 PCR (protocol 1) and/or an ORF 2 PCR (protocols 2 and 3). For the calendar year 2015, specimens from 202 gastroenteritis outbreaks were received for testing and of these 122 (60.4%) were positive for norovirus by the ORF 1 PCR (protocol 1) and/or an ORF 2 PCR (protocols 2 and 3). Thus norovirus was the chief viral agent associated with gastroenteritis outbreaks in Victoria for both 2014 and 2015. However, there was a significant decline in the number of norovirus outbreaks identified in 2015 compared with 2014 ($\chi^2 = 6.44$, 1 degree of freedom, $P = 0.011$).

The seasonal periodicity of all norovirus outbreaks for the period 2014 to 2015 is given in Figure 1. For 2014, partitioning of χ^2 was applied and it was found that outbreak incidence did not change significantly in the period January–October ($\chi^2 = 2.64$, 5 degrees of freedom, $P = 0.75$). However, there was a significant difference in incidence between January–October and November–December

Figure 1: Number of norovirus positive outbreaks (i.e. norovirus positive by ORF 1 and/or ORF 2 RT-PCR) per month for the years 2014 to 2015



($\chi^2 = 28.37$, 5 degrees of freedom, $P = 0.00003$), and the incidence rose significantly in the period November–December.

For 2015, partitioning of χ^2 was also applied. Outbreak incidence did not change significantly in the period January–June ($\chi^2 = 2.22$, 5 degrees of freedom, $P = 0.82$) and did not change significantly in the period July–December ($\chi^2 = 0.66$, 5 degrees of freedom, $P = 0.985$). However, there was a significant difference in incidence between January–June and July–December ($\chi^2 = 35.70$, 5 degrees of freedom, $P = 0.000001$), so that the incidence rose significantly in the period July–December compared with January–June.

Therefore in both years the number of outbreaks rose in the latter part of the year, but the time when the rise occurred was not the same in 2015 as in 2014.

Norovirus outbreak settings could be divided into 2 groups: healthcare and non-healthcare (Table 2). Although most outbreaks came from aged care facilities, a large number of settings were represented in the study. It was notable that the percentage of norovirus outbreaks from the healthcare and non-healthcare categories was similar in 2014 and 2015. In particular, in 2014, 135 (81.3%) of the norovirus outbreaks were from healthcare settings and 31 (18.7%) from non-healthcare settings. In 2015, 98 (80.3%) of the norovirus outbreaks were from healthcare settings and 24 (19.7%) from non-healthcare settings. Thus any alteration in frequency patterns of the various genotypes detected could not have resulted from altered sampling patterns.

Norovirus genotype analysis

A summary of all ORF 1 and ORF 2 norovirus genotypes identified in the study is given in Table 3. It can be seen that a broad range of norovirus genotypes were detected. In terms of ORF 2 (capsid) genotypes, the frequencies, in descending order, were: GII.4 (144/229), GI.3 (18/229), GII.6 (18/229), GII.3 (16/229), GI.2 (7/229), GII.2 (7/229), GII.17 (6/229), GI.4 (3/229), GI.9 (3/229), GII.7 (2/229), GI.5 (1/229), GI.6 (1/229), GII.8 (1/229) GII.13 (1/229) and the mixed outbreak GI.3 plus GII.3 (1/229).

The ORF 1 genotype sometimes differed from the ORF 2 genotype in a given outbreak (Table 3). To test whether these variable genotype combinations represented ORF 1/ORF 2 recombinant forms, nucleotide sequencing in the ORF 1-ORF 2 intersect region was carried out and the following recombinant forms were confirmed: GII.P4_NewOrleans_2009/GII.4_Sydney_2012, GII.P12/GII.3, GII.Pb(GII.P21)/GII.3, GII.Pe/GII.2 and GII.Pe/GII.4_Sydney_2012. A representative sequence of each of these 5 recombinant genotypes has been deposited in GenBank with the accession numbers KX064756 to KX064760 respectively.

There were significant changes in the incidence of some genotypes over the 2-year period 2014 to 2015, notably the decline in the incidence of GII.Pe/GII.4_Sydney_2012 and GII.Pe/GII.4, the re-emergence of GII.P4_NewOrleans_2009, the emergence of GII.17 norovirus and a dramatic alteration in the mix of GI norovirus genotypes (Table 3). These 4 areas are next considered.

Decline in GII.Pe/GII.4_Sydney_2012 and GII.Pe/GII.4 strains over 2014 to 2015

The chief norovirus strains over the period of the study, GII.Pe/GII.4_Sydney_2012 and GII.Pe/GII.4, declined in incidence from 2014 to 2015 (Table 3). In 2014, these strains were found in 60/117 (51.3%) and 27/117 (23.1%) respectively of outbreaks with both ORF 1 and ORF 2 sequence available, whereas in 2015 they were found in 32/80 (40.0%) and 14/80 (17.5%) respectively of outbreaks with both ORF 1 and ORF 2 sequence available. Application of the partitioning of χ^2 test then showed that the 2 chief genotypes did decline significantly from 2014 to 2015 ($\chi^2 = 6.157$, 2 degrees of freedom, $P = 0.046$).

Re-emergence of GII.P4_NewOrleans_2009

During the study period there was a re-emergence of the ORF 1 form GII.P4_NewOrleans_2009 norovirus. GII.P4_NewOrleans_2009 was not detected in 2014 and was first identified in July 2015; thereafter it was detected in a further 5 outbreaks. The 6 outbreaks occurred in a range of settings, 4 in healthcare settings and 2 in non-healthcare settings.

Three of the 6 ORF 1 GII.P4_NewOrleans_2009 norovirus strains were found to be linked to the ORF 2 genotype GII.4_Sydney_2012 (Table 3). Sequence analysis of the ORF 1 fragment showed 98.2% to 98.6% similarity with the reference strain GII.P4_NewOrleans_2009 (GU445325); sequence analysis of the ORF 2 fragment showed 97.4% to 97.9% similarity with the reference strain GII.4_Sydney_2012 (JX459908). Application of the bridging RT-PCR protocol 5 (Table 1) for 1 specimen confirmed GII.P4_NewOrleans_2009/GII.4_Sydney_2012 was a true recombinant form.

Emergence of GII.17 norovirus

Six outbreaks involving the GII.P17/GII.17 genotype were detected (Table 3). In 2014, there were 2 such outbreaks, both in aged care facilities. In

2015, 3 of 4 outbreaks occurred in aged care facilities and 1 in a boarding school. GII.P17/GII.17 could infect individuals over a broad range of ages; of 11 individuals from 6 outbreaks where ages were available and nucleotide sequences for both the ORF 1 and ORF 2 regions were obtained, the spread of ages was 17 to 95 years.

Sequence analysis indicated GII.17 norovirus underwent minor changes in 2014 to 2015. Sequence analysis (protocols 1 and 3; Table 1) indicated that both the GII.17 ORF 1 and ORF 2 regions respectively of the two 2014 GII.P17/GII.17 noroviruses were identical. When the 2014 ORF 1 region was compared with the ORF 1 region of the four 2015 GII.P17/GII.17 noroviruses, they were found to have 97.3% to 98.0% similarity; 1 of the nucleotide differences resulted in an amino acid change. When the 2014 GII.17 ORF 2 region was compared with the GII.17 ORF 2 region of the four 2015 GII.P17/GII.17 noroviruses, they were found to have 97.4% to 98.5% similarity; none of the nucleotide differences resulted in an amino acid change.

Table 3: Genotypes found in norovirus positive outbreaks 2014 to 2015

ORF 1	ORF 2	Number of norovirus positive outbreaks		
		2014	2015	Total
GI.P2	GI.2	7	0	7
GI.P3	GI.3	2	15	17
GI.P3	–	0	1	1
GI.P4	GI.4	3	0	3
GI.P5	GI.5	1	0	1
GI.P6	GI.6	1	0	1
GI.P9	GI.9	3	0	3
GI.Pa	GI.3	1	0	1
GII.P4_NewOrleans_2009	GII.4_Sydney_2012	0	3	3
GII.P4_NewOrleans_2009	GII.4*	0	1	1
GII.P4_NewOrleans_2009	–	0	2	2
GII.P12	GI.3 & GII.3	1	0	1
GII.P12	GII.3	3	6	9
GII.P16	GII.4*	0	1	1
GII.P16	GII.13	1	0	1
GII.P17	GII.17	2	4	6
GII.P17	–	0	1	1
GII.Pb (GII.P21)	GII.3	4	3	7
GII.Pb (GII.P21)	–	2	0	2
GII.Pe	GII.2	1	1	2
GII.Pe	GII.4_Sydney_2012	60	32	92
GII.Pe	GII.4*	27	14	41
GII.Pe	–	27	19	46
–	GII.2	0	5	5
–	GII.4_Sydney_2012	3	0	3
–	GII.4*	0	3	3
–	GII.6	11	7	18
–	GII.7	0	2	2
–	GII.8	1	0	1
–	–	4	2	6
Total		165	122	287

– No sequence available.

* GII.4 variant identity could not be determined by the norovirus genotyping tool.[24]

Nucleotide sequence analysis was also carried out to determine sequence similarity between the GII.P17/GII.17 strains found in this study and GII.P17/GII.17 strains recently identified in the United States of America (USA) and Japan. Analysis of a 753bp fragment corresponding to the ORF 1/ORF 2 intersect region showed a 2014 sequence from the current study was 98.4% similar to the USA strain KR083017[27] and 99.3% similar to the Japanese strain AB983218.[12] A similar analysis of 4 strains from the four 2015 GII.17 outbreaks from the current study showed a 99.3% to 99.7% similarity to KR083017[27] and a 98.1% to 98.5% similarity to AB983218.[12] Thus the Australian strains showed high similarity with the USA and Japanese strains.

One 753bp GII.P17/GII.17 sequence from an individual in the first GII.P17/GII.17 outbreak in 2015 has been lodged in GenBank as KT734635.

Alteration in genogroup I genotypes

Based on ORF 2 sequences it was noted that the proportion of genogroup I (GI) outbreaks in 2014 (14.4%) was similar to the proportion in 2015 (15.5%) (Table 3). However, examination of the GI genotypes circulating in Victoria for the 2 years 2014 and 2015 indicated a dramatic shift in the variety of genotypes detected from 2014 to 2015 (Table 3). In particular, in 2014, 7 GI ORF 1/ORF 2 genotype combinations were detected, whereas in 2015 only 1 GI ORF 1/ORF 2 genotype combination (GI.P3/GI.3) was detected.

An examination of representative GI.3 norovirus nucleotide sequences from both the ORF 1 and ORF 2 regions of the genome (Figure 2) indicates that there were substantial alterations in the genome in the period corresponding to the transition between 2014 and 2015. None of the 'definitive' nucleotide changes in either ORF 1 or ORF 2 resulted in an amino acid change (Figure 2).

It can be seen that for both ORF 1 sequences and ORF 2 sequences there was a major change early in 2015 and this is denoted by a horizontal line; the 3 sequences above the line correspond to the 2 GI.3 outbreaks in 2014 and the first GI.3 outbreak in 2015 and the 14 sequences below the line correspond to GI.3 outbreaks in the remainder of 2015. In Figure 2A (ORF 1) it can be seen that there were 25 (6%) definitive changes (i.e. a change that, once it had occurred, remained fixed for the rest of 2015) in a sequence 440 bp long. In Figure 2B (ORF 2) it can be seen there were 6 (3%) definitive changes in a sequence 198 bp long.

Relationship between ORF 2 genotype and outbreak setting

An examination of Table 4 indicates there was a relationship between some ORF 2 genotypes and outbreak setting and this was then examined by statistical methods. For the statistical analysis, 5 genotype groupings, based on genotype frequency, were chosen. These groupings were GI.3, GII.3, GII.4, GII.6 and all the other genotypes ('other genotypes'). Application of the partitioning of χ^2 test followed by the χ^2 test indicated that GI.3, GII.4 and GII.6 were much more common in healthcare settings than in non-healthcare settings ($\chi^2 = 102.76$, 1 degree of freedom, $P = 5 \times 10^{-24}$).

Discussion

The findings of the current study indicate that norovirus remained a common gastroenteritis virus that infected individuals in a broad range of settings. Norovirus outbreaks occurred throughout the year but a seasonal peak was noted in 2014 and 2015, although the timing was different.

A great diversity in norovirus genotypes was found to be circulating within Victoria in 2014 to 2015. This included 6 ORF 2 GI genotypes and 8 ORF 2 GII genotypes. The genotype diversity of circulating norovirus was further emphasised by the identification of 5 ORF 1/ORF 2 recombinant forms.

A key finding was that there was a progressive decline in norovirus outbreaks in Victoria in the period 2013 to 2015. Using the same sampling and testing protocols, 190 norovirus outbreaks were identified in 2013.[14] In the current study there were 165 norovirus outbreaks in 2014 and 122 in 2015. This decline was linked, to some degree, to changes in the prevalence of the predominant epidemic strain GII.Pe/GII.4_Sydney_2012. These findings indicate that this predominant GII.4 strain is losing its potency to infect, presumably as a result of increasing herd immunity,[28] and some speculation as to what may follow this strain is appropriate. Two potential candidates emerge, GII.P4_NewOrleans_2009 and GII.17.

The first potential candidate that may supplant the diminishing GII.Pe/GII.4_Sydney_2012 is a new strain of GII.P4_NewOrleans_2009 that appeared in 2015. Three of the new GII.P4_NewOrleans_2009 strains were identified by sequencing analysis as GII.4_Sydney_2012 in ORF 2. These observations indicate that a previously dominant ORF 1 form, GII.P4_NewOrleans_2009, had recombined with the currently dominant ORF 2 form GII.4_Sydney_2012 to produce a novel strain. At the end of 2015, GII.P4_NewOrleans_2009/GII.4_Sydney_2012 was still relatively rare.

Figure 2: Nucleotide sequence alignments of 1 specimen from each GI.3 outbreak where both ORF 1 (Figure 2A) and ORF 2 (Figure 2B) sequences were available for a given individual from a given outbreak

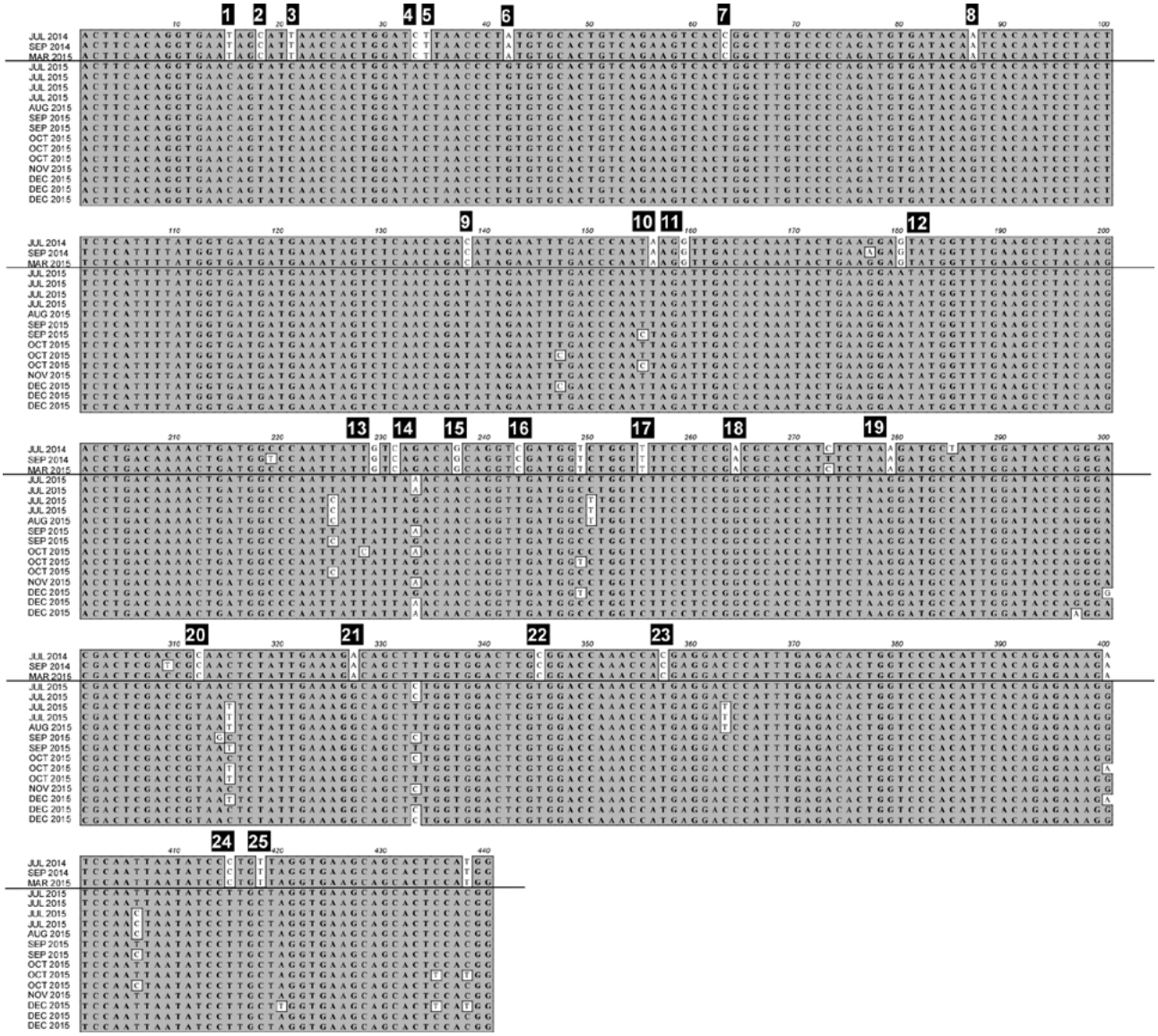


Figure 2B

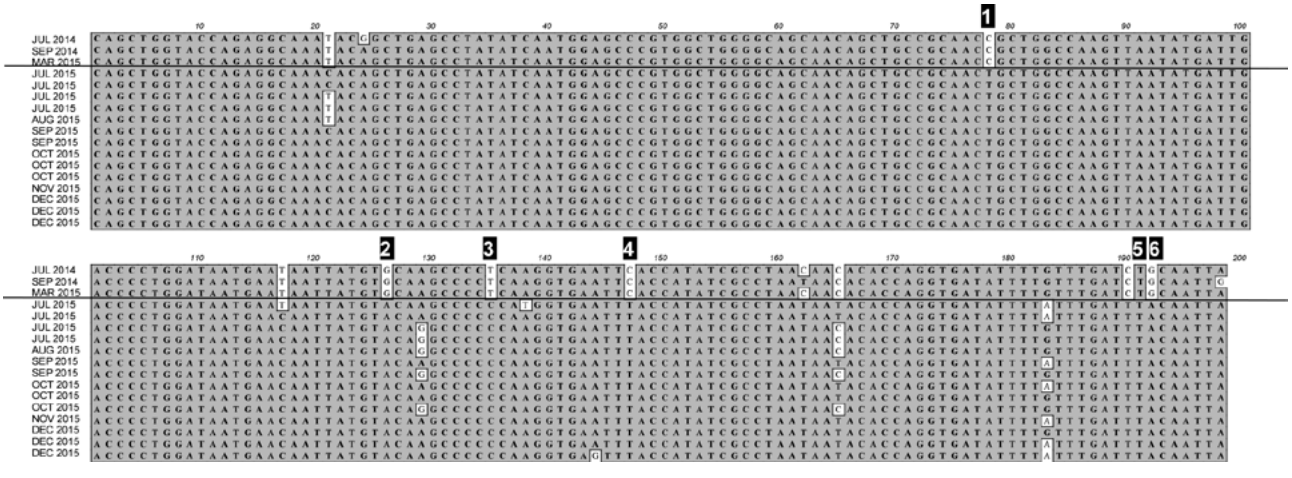


Table 4: Norovirus ORF 2 genotypes detected in healthcare and non-healthcare categories from 2014 and 2015

Norovirus ORF 2 genotypes	Norovirus positive outbreaks in healthcare settings				Norovirus positive outbreaks in non-healthcare settings			
	2014		2015		2014		2015	
	n	%	n	%	n	%	n	%
GI.2	3	2.2	0	0.0	4	12.9	0	0.0
GI.3	3	2.2	12	12.2	0	0.0	3	12.5
GI.3 and GII.3	0	0.0	0	0.0	1	3.2	0	0.0
GI.4	3	2.2	0	0.0	0	0.0	0	0.0
GI.5	1	0.7	0	0.0	0	0.0	0	0.0
GI.6	1	0.7	0	0.0	0	0.0	0	0.0
GI.9	3	2.2	0	0.0	0	0.0	0	0.0
GII.2	1	0.7	1	1.0	0	0.0	5	20.8
GII.3	3	2.2	5	5.1	4	12.9	4	16.7
GII.4	80	59.7	49	50.0	10	32.3	5	20.8
GII.6	9	6.7	5	5.1	2	6.5	2	8.3
GII.7	0	0.0	1	1.0	0	0.0	1	4.2
GII.8	1	0.7	0	0.0	0	0.0	0	0.0
GII.13	1	0.7	0	0.0	0	0.0	0	0.0
GII.17	2	1.5	3	3.1	0	0.0	1	4.2
No sequence available	23	17.2	22	22.4	10	32.3	3	12.5
Total	134	100.0	98	100.0	31	100.0	24	100.0

The second potential candidate that may supplant GII.Pe/GII.4_Sydney_2012, is GII.17 norovirus. Recent studies in China[10,11] and Japan[12] have identified a new GII.17 norovirus as an apparent new norovirus epidemic strain. Studies in the USA[27] and Taiwan[29] have confirmed the presence of GII.17 norovirus strains in these countries but reports from other areas are lacking. A comparison with published sequence data on GII.17 norovirus strains recently reported in the USA[27] and Japan[12] showed the Australian, USA and Japanese strains were similar. The current study indicates that GII.17 does not appear to be a major genotype in gastroenteritis outbreaks in Victoria and recent studies in New South Wales and Western Australia[15] indicate that GII.17 is not a major genotype in those states either.

Previous studies in Victoria (2002 to 2010)[22] have indicated that norovirus gastroenteritis outbreaks associated with GI norovirus are relatively rare compared with outbreaks associated with GII norovirus and the data for the current study confirms this finding. Nevertheless, a dramatic change in the diversity of GI noroviruses occurred in Victoria over the period 2013 to 2015. In 2013, ORF 2 sequence analysis identified 7 ORF 2 GI genotypes associated with gastroenteritis outbreaks in Victoria with GI.4 being the chief genotype detected.[14] In the current study, in 2014, 6 ORF 2 GI norovirus genotypes were detected, with GI.2 being the most common. In 2015, however, a dramatic change occurred with only 1 ORF 2 genotype detected, GI.3.

Genetic analysis of GI.3 noroviruses over 2014 to 2015 indicated there were substantial changes in the genome in the period corresponding to the transition between 2014 and 2015. Although the relative number of GI outbreaks, based on ORF 2 sequencing, was similar in 2014 (14.4%) and 2015 (15.5%), it remains to be seen whether the genetic changes that have occurred in GI norovirus in Victoria in 2015 will result in a greater incidence of GI norovirus in coming years.

A key area in the understanding of how norovirus circulates through the community involves an examination of the relationship between norovirus genotype and outbreak setting. Previous studies in this laboratory have established that such a relationship does exist [22,30,31] and the current study supports and extends these previous observations. In particular, it was shown that the ORF 2 genotypes GI.3, GII.6, and GII.4 were significantly more common in healthcare settings than in non-healthcare settings.

In summary, this study emphasises the complex way in which norovirus circulates throughout the community and the associated genetic changes

the virus undergoes as it does so. The ongoing monitoring of these variables may eventually lead to the development of a clear model of how human norovirus can continually re-invent itself.

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