The Comparative Molecular Epidemiology of GII.P7_GII.6 and GII.P7_GII.7 Norovirus Outbreaks in Victoria, Australia, 2012–2014

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Key Words

GII.6 norovirus · GII.7 norovirus · Gastroenteritis outbreaks · Age · Australia

Abstract

The comparative molecular epidemiology of the related GII.P7_GII.6 and GII.P7_GII.7 noroviruses has not been examined in detail. ORF 1, ORF 2 and ORF 1/ORF 2 RT-PCR as well as sequencing and phylogeny analysis were carried out on faecal specimens from 873 gastroenteritis outbreaks in Victoria, Australia (2012–2014). There were 575 (66%) detected as positive for norovirus by means of ORF 1 RT-PCR and/or ORF 2 RT-PCR. Of these, 24 (4.2%) were GII.6 (ORF 2) outbreaks, 7 (1.2%) were GII.7 (ORF 2) outbreaks, and 1 outbreak (0.2%) involved both GII.6 (ORF 2) and GII.7 (ORF 2) noroviruses. The median age of patients identified with GII.6 (ORF 2) (84 years) was significantly different from that of patients identified with GII.7 (ORF 2) (39 years). ORF 2 GII.6 and ORF 2 GII.7 sequences were always associated with a GII.P7 ORF 1 sequence, and GII.P7 sequences fell into two clusters, with one corresponding to the GII.6 ORF 2 genotype and the other to the GII.7 ORF 2 genotype, thereby indicating that the ORF 1 has been evolving separately for the two viruses. Thus, two closely related noroviruses can have a markedly different incidence and epidemiology.

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The noroviruses, which are now considered one of the major causes of gastroenteritis worldwide [1], are single-stranded positive-sense RNA viruses classified as the genus *Norovirus* within the family *Caliciviridae* [2]. Noroviruses have been classified into 6 genogroups, and 3 of these, genogroups I, II and IV (GI, GII and GIV), occur in human infections [2]. The noroviruses can be further subdivided into genotypes [2]; the GII.4 genotype appears to be the most common in humans [3].

The human norovirus genome comprises 3 open reading frames (ORFs) [2]. ORF 1 encodes the non-structural polyprotein, ORF 2 encodes the major capsid protein and ORF 3 encodes a minor capsid protein [2]. Norovirus genotype classification using nucleic acid sequencing can be based on the ORF 1 region or the ORF 2 region [4]. Recombination often occurs in the region of the junction of ORF 1 and ORF 2, so that recombination can result in differing genotype classifications for ORF 1 and ORF 2 in a given genome [5].

Although GII.6 (ORF 2) and GII.7 (ORF 2) noroviruses have been documented in various studies [6–8] and GII.6 (ORF 2) has been noted to be a relatively common norovirus in some settings [9–12], detailed molecular epidemiological analysis of these viruses is lacking. Furthermore, although a link between GII.6 and GII.7 noroviruses has been documented, in that both can be linked to

GII.P7 [13–16], the precise relationship between GII.P7_GII.6 and GII.P7_GII.7 does not appear to have been examined. This study reviews the molecular epidemiology of GII.P7_GII.6 and GII.P7_GII.7 noroviruses in gastroenteritis outbreaks in Victoria, Australia, and examines the relationship between the two.

For the purposes of the study, an outbreak of gastroenteritis was defined as an incident, apparently associated with a common event or location, in which ≥ 4 individuals had symptoms of gastroenteritis [17].

All outbreaks included in the study were those for which faecal specimens were sent to the Victorian Infectious Diseases Reference Laboratory (VIDRL) for the testing of outbreaks that occurred during the period 2012–2014. VIDRL 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 Department of Health. Outbreak specimens are also occasionally sent by other institutions such as hospitals. Outbreak documentation and management were carried out by staff linked to the Victorian Department of Health and/or staff at the affected setting. 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. Only one faecal specimen per person per outbreak was tested.

For the purposes of analysis, norovirus outbreak settings were classified as either ‘healthcare’ or ‘non-healthcare’. Healthcare outbreaks included the following settings: age care facility, disabled care facility and hospital. Non-healthcare outbreaks included the following settings: school camp, child-care centre, children’s activity centre, gathering, navy base, consumption of suspect food, consumption of suspect water and restaurant.

For the period 2012–2014, 3307 faecal specimens from 873 gastroenteritis outbreaks were tested for noroviruses. Faecal specimens were prepared as a 20% (vol/vol) suspension in Hanks’ complete balanced salt solution, and clarified by centrifugation [18]. RNA extraction was then carried out using the Corbett (now Qiagen GmbH, Hilden, Germany) automated nucleic acid extraction procedure [19].

Four norovirus RT-PCR protocols were utilised in the study. Protocol 1 was an ORF 1 RT-PCR which involved a 2-round, hemi-nested procedure, using the primers NV4562, NV4611, NV4692, NV5296, NV5298 and NV5366. It was carried out essentially as given previous-

ly [20, 21]. Protocol 2 involved the use of a GII ORF 2 region C RT-PCR procedure. It utilised primers G2F3 and G2SKR and was carried out essentially as given previously [22, 23]. Protocol 3 involved the use of a GII ORF 1 RT-PCR procedure. It utilised primers MON 431 and MON 433 and was carried out essentially as given previously [24, 25]. Protocol 4, a GII ORF 1/ORF 2 RT-PCR that crosses the junction of ORF 1 and ORF 2, was developed for this study and was carried out to confirm the presence of recombinant forms. The RT-PCR utilised the primers MON 431 and G2SKR in a two-round procedure. The first round comprised an RT step carried out at 50°C for 30 min, followed by PCR with initial denaturation at 94°C for 5 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 3 min. An additional extension step was then carried out at 72°C for 10 min. The second-round PCR was performed as for the first round but without the RT step.

Nucleotide sequencing was carried out for the products of the RT-PCR protocols 2, 3 and 4 essentially as given previously [26]. For protocol 2, a fragment of 195 bp corresponding to nucleotides 5,133–5,327 relative to the norovirus reference strain JX459908 was used. For protocol 3, a fragment of 105 bp corresponding to nucleotides 4,880–4,984 relative to the norovirus reference strain JX459908 was used. For protocol 4, a fragment of 368 bp corresponding to nucleotides 4,937–5,304 relative to the norovirus reference strain JX459908 was used.

Phylogenetic analysis of the nucleotide sequences was carried out using MacVector software v8.0 (Oxford Molecular Limited, Madison, Wis., USA). Genotype identification was carried out using the norovirus typing tool of Kroneman et al. [27]. Where the typing tool was unable to type the fragment, a BLAST search was performed and the closest match was put into the typing tool to identify the genotype. Phylogenetic trees were constructed using Phylip v3.68, essentially as described previously [26], to examine the clustering patterns of ORF 1 and ORF 2 fragments.

During the period 2012–2014, all faecal specimens from gastroenteritis outbreaks received at VIDRL were tested by RT-PCR protocol 1. In addition, 1 specimen chosen at random from each RT-PCR protocol 1 negative outbreak was then tested by the ORF 2 RT-PCR (protocol 2). Where a GII.6 or GII.7 ORF 2 genotype was detected, all specimens in that outbreak were tested by the protocol 2 RT-PCR and all positive specimens were then sequenced and phylogenetic analysis was carried out. As sequencing analysis indicated that RT-PCR protocol 1 did not normally detect GII.P6 or GII.P7, all specimens positive for

(For legend see next page.)

ORF 2 GII.6 or GII.7 were tested by ORF 1 RT-PCR protocol 3, an alternate ORF 1 procedure, which can detect ORF 1 GII.P7. All specimens positive for ORF 1 GII.P7 norovirus were then subjected to sequencing and phylogenetic analysis. In order to confirm the presence of the ORF 1/ORF 2 forms, GII.P7_GII.6 and GII.P7_GII.7, 1 specimen for each of these forms was tested by the ORF 1/ORF 2 RT-PCR protocol 4 and sequenced.

Faecal specimens were received from 873 gastroenteritis outbreaks and 575 (66%) were positive for norovirus by either the protocol 1 ORF 1 RT-PCR or the protocol 2 ORF 2 RT-PCR. Of these, 24 (4.2%) were GII.6 (ORF 2) outbreaks, 7 (1.2%) were GII.7 (ORF 2) outbreaks and 1 outbreak (0.2%) involved both the GII.6 (ORF 2) and GII.7 (ORF 2) noroviruses. Thus GII.6 norovirus outbreaks were about 3 times more common than GII.7 norovirus outbreaks. This difference was statistically significant ($p < 0.005$, χ^2 test; the calculation excluded the outbreak with both GII.6 and GII.7). Both GII.6 and GII.7 could occur in a variety of healthcare and non-healthcare settings and there was no statistical difference in the incidence of GII.6 and GII.7 outbreaks in healthcare compared to non-healthcare settings.

Application of the Kolmogorov-Smirnov method [28] indicates that the age distribution of all patients with GII.7 (ORF 2) was normal ($p > 0.20$) whereas the age distribution of all patients with GII.6 (ORF 2) was not normal ($p < 0.01$). The median age for the GII.6 patients was 84 years ($n = 56$; range 1–98 years; interquartile range 54.75 years) and the median age for GII.7 patients was 39 years ($n = 65$; range 1–89 years; interquartile range 37.0 years). Application of the median test [28] showed that the median age of those with GII.6 was significantly different to that of those with GII.7 ($p = 0.027$). Thus there

was a difference in the epidemiology of GII.6 (ORF 2) and GII.7 (ORF 2) outbreaks.

ORF 1 RT-PCR and sequencing were successfully carried out by protocol 3 in 28 of the 32 outbreaks; in the 4 outbreaks where an ORF 1 sequence could not be obtained, 3 had a GII.6 ORF 2 genotype and 1 had a GII.7 ORF 2 genotype. Application of the norovirus typing tool, in combination with BLAST analysis, indicated that all ORF 1 sequences linked to the ORF 2 GII.6 and GII.7 genotypes belonged to the ORF 1 genotype GII.P7. The phylogenetic tree corresponding to all unique ORF 1 sequences found in association with ORF 2 GII.6 and GII.7 noroviruses is shown in figure 1a. The phylogenetic tree corresponding to all unique ORF 2 GII.6 and GII.7 sequences is shown in figure 1b. A comparison shows that the upper cluster in the ORF 1 phylogenetic tree (fig. 1a) corresponded to GII.7 in ORF 2 (fig. 1b) and the lower cluster (fig. 1a) corresponded to GII.6 in ORF 2 (fig. 1b). These observations indicate that GII.P7_GII.6 and GII.P7_GII.7 have been evolving separately for some time.

The protocol 4 ORF 1/ORF 2 bridging RT-PCR procedure, in combination with sequence analysis, confirmed the identity of the GII.P7_GII.7 form and of the recombinant GII.P7_GII.6 form; 2 such representative sequences have been lodged in GenBank as KT970472 and KT970473, respectively.

This study is one of the first to examine the incidence, molecular epidemiology and relationship between GII.6 and GII.7 noroviruses linked to the GII.P7 ORF 1 genotype. In general, the findings indicate that these 2 viruses are relatively rare in gastroenteritis outbreaks in Victoria, Australia, and that they both occurred in a broad range of settings. However, although both genotypes affected individuals across a wide range of ages, GII.6 norovirus tended to occur more commonly in older people whereas GII.7 norovirus occurred more in younger people. This suggests that the viruses circulate in different ways in the community and, consequently, that their epidemiology may be different.

The results of our study indicate that GII.6 norovirus was more common than GII.7 norovirus, and this is in close agreement with the findings of Zhirakovskaia et al. [29], who examined sporadic gastroenteritis in children in Russia in the period 2003–2012. They found GII.6 in 20 faecal samples tested whereas GII.7 was only found in 3 faecal samples. In contrast, Tan et al. [8], in a study of norovirus in children in Nanning City, China, in 2010–2011, found that GII.7 (3/84) was only slightly more common than GII.6 (2/84), although the low number of cases here makes it impossible to draw any conclusions about

Fig. 1 a DNA (dist) Kimura phylogenetic tree showing the phylogenetic relationship of all unique GII.P7 ORF 1 sequences detected. The tree was based on a 105-bp ORF 1 sequence corresponding to nucleotides 4,880–4,984 relative to the norovirus reference strain JX459908. The reference strains included in the tree were: Baltimore/274/1993/US (AF414408) (GII.P7_GII.6), Gwynedd/373/1994/US (AF414409) (GII.P7_GII.7) and Sydney/2012/AU (JX459908) (GII.Pe_GII.4). **b** DNA (dist) Kimura phylogenetic tree showing the phylogenetic relationship of all unique GII.6 and GII.7 ORF 2 sequences detected. The tree was based on a 195-bp ORF 2 sequence corresponding to nucleotides 5,133–5,327 relative to the norovirus reference JX459908. The reference strains included in the tree were as for **a**. **a, b** The figures on the branches represent bootstrap values (%) after resampling 1,000 data sets. Only bootstrap values $\geq 70\%$ are shown. The scale marker represents substitutions per site.

the relative frequency of the 2 genotypes from this study. Therefore, the evidence indicates that, in general, GII.6 norovirus is more common than GII.7 norovirus.

Two GII.6 recombinant forms have been described previously. GII.P7_GII.6 has been identified in a number of studies around the world including China [15], South Africa [13] and Uruguay [14]. Truong et al. [30] suggested they had identified the recombinant GII.P4_GII.6 in South Korea, but this finding remains to be confirmed. Our study documents the first identification of the recombinant form GII.P7_GII.6 in Australia and no other ORF 1 recombinant forms linked with ORF 2 GII.6 have been found.

A key observation is that both capsid (ORF 2) genotypes, the GII.6 and GII.7 noroviruses, were linked to the

same ORF 1 genotype, GII.P7. An examination of the ORF 1 GII.P7 phylogenetic tree indicated that all GII.P7 sequences in this study fell into two clusters and that one cluster corresponded to the GII.6 ORF 2 genotype and the other cluster to the GII.7 ORF 2 genotype. This suggests that the recombination events occurred some time ago, and that since then the ORF 1 regions have evolved separately for these 2 viruses.

The findings of this study enable some speculation about the evolution of the noroviruses. Since the recombination of GII.P7 with GII.6, the 2 norovirus genotypes, GII.P7_GII.6 and GII.P7_GII.7, appear to have been evolving away from each other. This is possibly facilitated by differences in their epidemiology. It is notable that one of these viruses, GII.P7_GII.6, is the more successful.

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