

Molecular epidemiology of norovirus in Edinburgh healthcare facilities, Scotland 2007–2011

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SUMMARY

Norovirus (NoV) is a leading cause of outbreaks of gastroenteritis worldwide, and a major burden for healthcare facilities. This study investigated the NoV genotypes responsible for outbreaks in Edinburgh healthcare facilities between June 2008 and July 2011, and studied their temporal distribution to enable a better understanding of the epidemiology of the outbreaks. A total of 287 samples positive for NoV genogroup II (GII) RNA by reverse transcription–polymerase chain reaction (RT–PCR) during routine diagnostic testing were investigated. Nested RT–PCR (nRT–PCR) and sequencing was used to genotype the NoV strains. Overall, a total of 69 NoV strains belonging to six different genoclusters (GII.1, GII.2, GII.3, GII.4, GII.6, GII.13) were detected. The predominant genotype was GII.4 that included four variants, GII.4 2006a, GII.4 2006b, GII.4 2007 and GII.4 2010. Importantly, increases in NoV activity coincided with the emergence of new GII.4 strains, highlighting the need for an active surveillance system to allow the rapid identification of new strains.

Key words: Gastrointestinal infections, infectious disease control, infectious disease epidemiology, molecular epidemiology, Norwalk agent and related viruses.

INTRODUCTION

Noroviruses (NoV) belong to the Caliciviridae family, within which the NoV genus consists of five main genogroups, each of which can be further subdivided into genoclusters. Three NoV genogroups (GI, GII, GIV) are known to infect humans; however, most of

contemporary NoV outbreaks, and sporadic cases, of gastroenteritis worldwide are caused by GII genotypes, in particular genocluster 4 (GII.4) [1]. In the past 15 years, several distinct variants of GII.4 have emerged every few years causing five pandemics of gastroenteritis [2–4]. Most notable recently was the simultaneous emergence of NoV variants GII.4 2006a and 2006b, both of which were first reported in Europe and have subsequently spread to many countries [5]. Initially NoV variant GII.4 2006a was the predominant strain in most areas with the

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exclusion of Asia, where this variant has been rarely detected [1, 5–8]. Following which, NoV variant GII.4 2006b quickly replaced 2006a as the dominant strain with a global distribution [1, 8]. Over time, epidemic waves of GII.4 viruses have been getting closer together, with pandemic strains being replaced by a new dominant strain every 1–2 years [3].

The emergence of new strains is associated with increased incidence of infection and an increase in out-of-season (April–September) outbreaks [9]. Such outbreaks of NoV gastroenteritis are a major problem in healthcare facilities as they most often result in ward closures [10]. In Scotland, due to the lack of a national NoV genotyping programme, there is limited data on the molecular epidemiology of NoV. An increase in NoV infections due to NoV variant GII.4 2006a was reported in Scotland in 2006 [11, 12]. Recent years have seen an increase in the number of laboratory-confirmed NoV outbreaks in Edinburgh healthcare institutions. To determine the NoV variants associated with these outbreaks, sequence analysis of the capsid gene and the adjacent region of *orf1* was performed for a representative number of NoV outbreaks in samples collected from Edinburgh healthcare facilities between 1 June 2008 and 31 July 2011. In addition to determining the genotypes responsible for NoV outbreaks, we also investigated the temporal occurrence of the genotypes to enable a better understanding of the epidemiology of the outbreaks.

METHODS

Samples

A total of 1871 samples that had previously been confirmed as positive for NoV GII RNA by reverse transcription–polymerase chain reaction (RT–PCR) during routine diagnostic testing were used as the pool to select the representative samples for sequence analysis. The samples were selected so as to be representative of the temporal patterns in observed clusters of outbreaks and from sporadic cases in healthcare institutions. An outbreak of gastroenteritis was defined as two or more cases in a ward with dates of onset within 7 days of each other. An outbreak was considered to be caused by NoV if two or more cases with gastrointestinal illness met the Kaplan criteria [13] after assessment of the situation by the infection control team. In some outbreaks only one sample was submitted for NoV detection by real-time PCR.

Between one and four samples, previously confirmed as being NoV GII positive, from each outbreak were sequenced.

Nested RT–PCR (nPCR)

Reverse transcription and first-round RT–PCR was performed using the Qiagen One-Step RT–PCR kit (Qiagen, UK). Briefly, 25 μ l reactions contained 1 \times One Step RT–PCR buffer, 0.4 μ M dNTPs; 0.6 μ M each outer primer [14] (Eurogentec, Belgium); 1 μ l enzyme mix; and 10 μ l RNA extract. RT was performed at 50 °C for 30 min, followed by PCR amplification: 95 °C for 15 min followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. Second-round PCR was performed in 50 μ l reactions with 1x HotStart PCR Mastermix (Qiagen); 0.6 μ M each inner primer [14] (Eurogentec); and 5 μ l of either neat or 1/10 diluted first-round product. PCR was performed at 95 °C for 15 min followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. A final extension step of 72 °C for 10 min was performed on the GeneAmp[®] PCR System 7500 (Applied Biosystems, UK).

Region amplified

The primers NV2oF2 and NV2oR amplified a 378-bp fragment encompassing 55 bp at the 3' end of the RNA-dependent RNA polymerase gene and 344 bp at the 5' end of the major capsid gene. Primers G2F3 and G2SKR amplified a 311-bp fragment within this region [14].

DNA Sequencing and phylogenetic analysis

PCR products were purified using Exosap-IT (GE Healthcare UK Ltd, UK). Cleaned products were sequenced using the BigDye Terminator v.3.1 Cycle Sequencing kit on an ABI 3130xl Genetic Analyser (Applied Biosystems, UK). Sequences were aligned and analysed using Bionumerics software version 5.1 (Applied Maths, Belgium), and database searches for related sequences were conducted using BLAST. The sequenced isolates were designated as being a new strain based on analysis of point mutations across a 270-bp region at the 5' end of the capsid gene when compared to the predominant circulating NoV variant group. The new strains were designated v1, v2, v3, etc. based on the time sequence of their emergence.

Table 1. *Laboratory-confirmed norovirus outbreaks between July 2008 and June 2011 including the responsible genotype where sequencing available*

| Observation period | July 2008 to June 2009 | July 2009 to June 2010 | July 2010 to June 2011 | Total |
|-------------------------------|---------------------------|---------------------------|---------------------------|-------|
| Number of norovirus outbreaks | 68 | 177 | 110 | 355 |
| Sequenced (%) | 40 (59%) | 59 (33%) | 35 (32%) | 134 |
| GII.1 | 1 | 2 | 2 | 5 |
| GII.2 | 3 | — | — | 3 |
| GII.3 | 1 | 2 | — | 3 |
| GII.4 2006a | 1 | 4 | 1 | 6 |
| GII.4 2010 v1 | — | 21 | — | 21 |
| GII.4 2010 | — | 24 | 12 | 36 |
| GII.4 2006b | 9 | 1 | 7 | 17 |
| GII.4 2006b v3 | 19 | — | — | 19 |
| GII.4 2006b v8 | — | 1 | 12 | 13 |
| GII.4 2007 | 6 | — | — | 6 |
| GII.6 | — | — | 1 | 1 |
| GII.13 | — | 4 | — | 4 |

A phylogenetic dendrogram was generated by using the neighbour-joining method with unweighted pair-group method using arithmetic averages (UPGMA). Reference strains included: AB434770 OC07138, DQ676865 Lincoln House 2006b, DQ665819 Rhyl440/2005/UK 2006a, X86557 Lordsdale Bristol variant, AJ004864 Grimsby USA95/96 and HQ230937 New Orleans 2010. To determine how well the phylogenetic trees were supported by the data bootstrap analysis (100 iterations of the alignments) were performed using Bionumerics.

RESULTS

In order to investigate the molecular epidemiology behind the NoV outbreaks, 287 of the 1871 (15.3%) NoV-positive specimens submitted to the laboratory during this period were sequenced. NoV was successfully amplified and sequenced from a representative number of cases in outbreaks ($n=268$) and from sporadic cases ($n=19$). This represents a minimum of one NoV successfully sequenced from 134 (38%) of the 355 confirmed NoV-positive outbreaks between July 2008 and June 2011 (Table 1).

Phylogenetic analysis of the nRT-PCR products identified 69 strains belonging to six different GII genoclusters (GII.1, GII.2, GII.3, GII.4, GII.6, GII.13). Overall, the predominant genotype was GII.4, which included 51 strains separated into four variants, designated 2006a, 2006b, 2007 and 2010. Of the six non-GII.4 genoclusters, GII.1, GII.6,

GII.13, GII.2 and GII.3 were identified (Table 1, Fig. 1).

Between June 2008 and July 2009 a total of 21 NoV strains belonging to five different genoclusters (GII.1, GII.2, GII.3, GII.4, GII.6) were detected. The predominant genocluster was GII.4, which included 15 strains separated into three variants, 2006a, 2006b and 2007. Analysis of the point mutations in the 270-bp sequenced region between the NoV GII.4 2006a variants found isolates which had 100% homology to a previously sequenced strain (Rhyl440/2005/UK) and revealed two further 2006a strains that differed from this NoV variant by four and five nucleotides, respectively (i.e. 98–100% nucleotide identity in the sequenced region). These strains were given the designation of 2006a v1 and v2, respectively. The NoV variant 2006b was the largest containing eight strains; NoV variant 2006b (Lincoln House/2006/UK) and two isolates which varied at a single locus (2006b v1 and v7) in the sequenced region; three double locus differences (2006b v2, v5, v6); one triple locus nucleotide change (2006b v4) and a strain which differed at four nucleotide locuses (2006b v3). The NoV GII.4 2007 variant consisted of four strains that differed from each other by one, two or three nucleotides (2007 v1–2007v3). Of the four non-GII.4 clusters, a single GII.1 strain and two closely related GII.2 and GII.3 strains were identified. One GII.6 strain was isolated from a sporadic case of gastroenteritis in a hospital patient. The majority (70%) of sequenced outbreaks in this time period were due to NoV variant

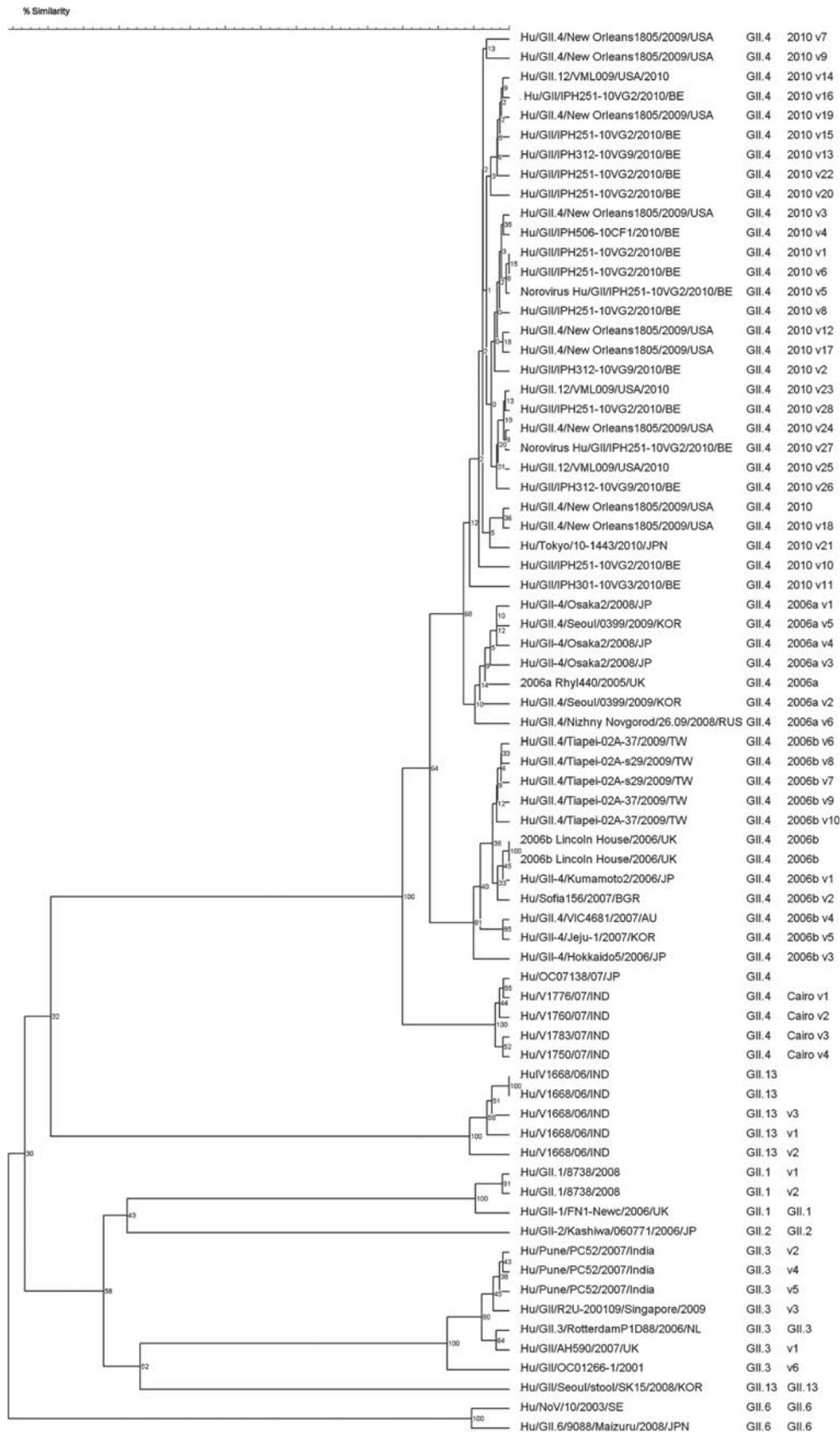


Fig. 1. A phylogenetic dendrogram based on sequences from the 5' end (270 bp) of the capsid gene of norovirus GII strains. Representative norovirus strains from selected outbreaks and sporadic cases were used to produce the dendrogram.

GII.4 2006b, most of which were caused by one strain, 2006b v3 (Hu/GII.4/VIC4681/2007/AU) that predominated from January 2009 until May 2009, while 15% of sequenced outbreaks were caused by a new NoV variant, GII.4 2007.

The following season, 2009/2010, four different genocusters (GII.1, GII.3, GII.4, GII.13) were found to be circulating in Edinburgh. Between November 2009 and June 2010 NoV variant GII.4 2006a emerged along with a large number of NoV GII.4 2010 variants. In the former there were four strains and 23 in the latter. The 2006a cluster contained one double locus nucleotide substitution (2006a v5); one triple locus change (2006a v4); one quintuple locus change (2006a v3) and one quadruple locus change (2006a v6) from the original NoV variant 2006a strain (Rhy1440/2005/UK). Overall there was 98–100% nucleotide identity between the NoV GII.4 2006a variants. The NoV GII.4 2010 variant first emerged in November 2009 and contained two isolates that had 100% homology over the sequenced region to NoV variant GII.4 2010 (Hu/GII.4/New Orleans1805/2009/USA) plus 22 isolates that differed from each other by either one (v2, v12, v18, v21), two (v1, v3, v4, v5, v6, v8, v9, v13, v19), three (v7, v11, v16, v17, v22) or four nucleotides (v10, v14, v15, v20) compared to the reference strain. Sequencing of isolates showed that the majority of outbreaks were due to one strain of the GII.4 2010 variant (v1), which was isolated from 36% of the typed outbreaks that season, predominantly between November 2009 and January 2010. Of the non-GII.4 genotypes, the GII.3 cluster consisted of five strains that differed from each other by either one, three, four or five nucleotides. Together they were responsible for 3% of outbreaks. GII.1 was found between April and May 2010 and caused two outbreaks, 3% of the total. The GII.13 (HuIVi668/06/IND) genotype was the aetiological agent in 7% of outbreaks appearing in the first quarter of 2010.

In the most recent NoV season, July 2010–June 2011, there were three NoV genocusters circulating in Edinburgh, GII.1, GII.4 and GII.6. The GII.13 genotype was also isolated from a sporadic case in a children's hospital. Mainly, there were two GII.4 variants circulating; GII.4 2010 and GII.4 2006b. There were three new GII.4 2006b strains detected, carrying one (2006b v8) and two (v9, v10) nucleotide changes from Lincoln House/2006/UK. The NoV GII.4 2010 variant contained six new strains that differed from NoV variant GII.4 2010 (Hu/GII.4/New Orleans1805/2009/USA) by either

three (v23, v26, v27, v28), four (v25) or five (v24) nucleotides. In total, the GII.4 2010 variants caused over a third of the total outbreaks during that season. The remainder were largely due to the GII.4 2006b variant, in particular the v8 strain described above, which predominated during January and February 2011 when outbreaks numbers peaked. GII.1 and GII.6 emerged during December 2010 causing one outbreak each. One outbreak originating from a NoV 2006a variant was also detected in January 2011.

An analysis of the temporal distribution of the NoV outbreak strains showed the predominant strain changes over time (Fig. 2). Three peaks in norovirus activity could be observed between July 2008 and June 2011; between June 2008 and July 2009 the number of outbreaks per month peaked at 17 in February 2009 and the following two seasons saw peaks of 51 and 26 in January 2010 and January 2011, respectively. Linking this data with the incidence of NoV outbreaks in Edinburgh healthcare facilities during the study period showed that in all cases large increases in NoV activity coincided with the emergence of new strains, the most notable being the emergence of NoV variant GII.4 2006b v3 in January 2009, GII.4 2010 v1 in November 2009 and GII.4 2006b v8 in December 2010.

Both 2009/2010 and 2010/2011 were severe in terms of NoV gastroenteritis in Edinburgh. Between July 2008 and June 2009 there were 68 outbreaks. In 2009/2010 these figures more than doubled to 177 outbreaks and the 2010/2011 season was also severe with 110 outbreaks. The size of individual outbreaks does not differ significantly between epidemic and lull years but a reducing trend can be observed despite the peak in cases in 2009/2010 (Table 2).

A range of specialities were affected by outbreaks across all years, although the majority were associated with medicine of the elderly (geriatric medicine). In both 2008/2009 and 2009/2010 exactly 50% of outbreaks were located in medicine of the elderly rising to 62% in 2010/2011. Accordingly, the majority of infections were in those aged >66 years (90%); the 46–65, 19–45, 6–10 and 0–5 years age groups were associated with 5%, 2%, 0.6% and 3%, respectively (data not shown).

The 19 sporadic infections were caused by genotypes GII.2, GII.4 2006b (v3, v4, v5), GII.4 2007, GII.4 2010 (v1, v3, v5, v7, v8, v25), GII.6 and GII.13. Almost half (7/19) of the sporadic infections occurred in children, and 10% of the strains were not associated with outbreaks. Otherwise sporadic cases

Table 2. Total number of individuals involved and length of ward closure in laboratory-confirmed norovirus outbreaks in Edinburgh between July 2008 and June 2011

| | July 2008 to June 2009 | July 2009 to June 2010 | July 2010 to June 2011 |
|--|---------------------------|---------------------------|---------------------------|
| Total number of outbreaks | 68 | 177 | 110 |
| Average number of individuals in outbreak (95% CI) | 13 (11–16) | 12 (10–13) | 11 (8–13) |
| Length of ward closure in days (95% CI) | 7 (6–7) | 8 (7–8) | 8 (7–9) |

CI, Confidence interval.

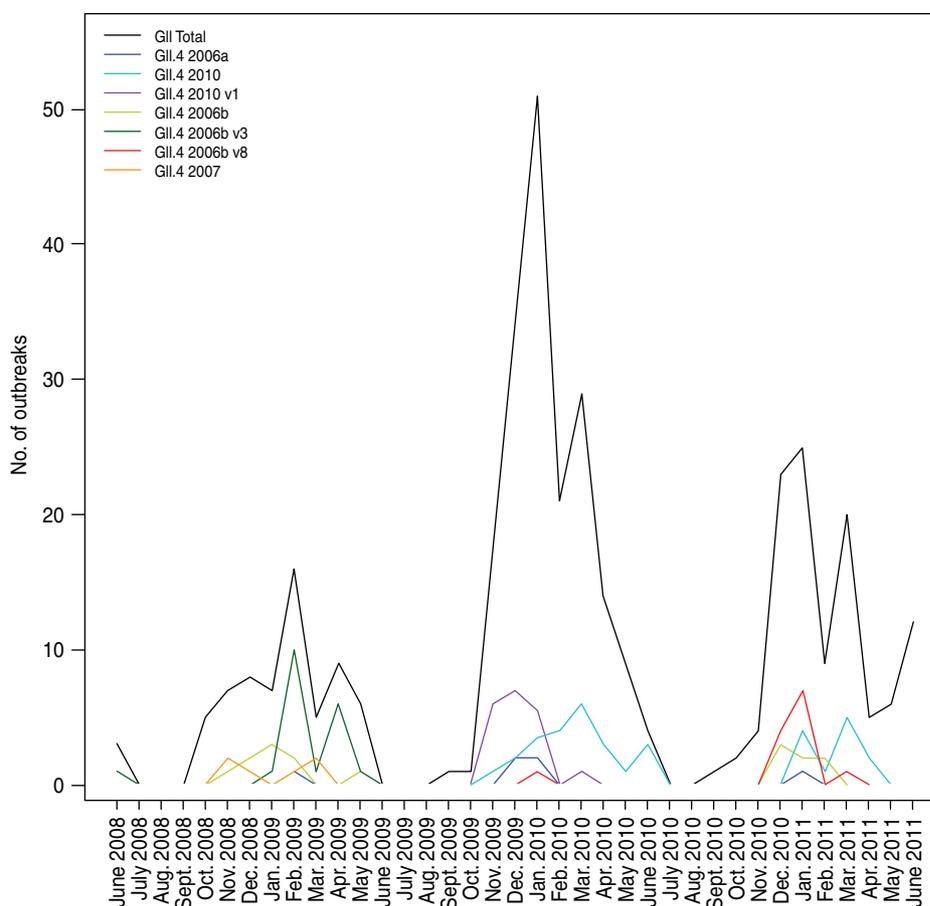


Fig. 2. Total number of laboratory-confirmed outbreaks of norovirus in Edinburgh tertiary care healthcare institutions between June 2008 and June 2011. The emergence of new NoV genotypes and variants over time are indicated by coloured lines.

were caused by strains that were currently in circulation in the healthcare facilities (data not shown).

DISCUSSION

In total, 355 outbreaks of NoV gastroenteritis were reported between June 2008 and July 2011. Analysis

of sequence data from the 5' end of the NoV capsid region for 38% of these outbreaks was successfully undertaken. Although this region is not the most heterogeneous part of the NoV genome, it was used to allow a conserved set of primers to enable the amplification of a wide range of GII genotypes. The genotyping rate using these primers for RT-PCR and

sequencing was 73%. This compares favourably with a multicentre study that reported a similar overall success rate (78%) for NoV genotyping of the same region compared to 52% for the more variable region in *orf2* [15].

A diverse range of GII genocusters were identified in our study with GII.4 being the predominant genotype. This is consistent with other recent molecular epidemiological and surveillance studies that have found GII.4 to be responsible for the majority of NoV infections and outbreaks worldwide [1, 14, 16, 17]. Overall, in our study, there were 51 strains of NoV variant GII.4 detected, which were responsible for 88% of outbreaks. A recent European study based on sequences submitted to the Food-Borne Viruses in Europe (FBVE) network reported that genogroup GII.4 was responsible for 87% of European outbreaks occurring between 2008 and mid-May 2011 [18].

Our analysis found that by March 2008 (data not shown) until May 2009, NoV variant 2006b was the prevalent circulating genotype in Edinburgh; 9% and 70% of NoV outbreaks during this period were due to NoV variants 2006a and 2006b, respectively, in our study. This reflects the worldwide situation during this period, where the NoV variant 2006b was reported as circulating with the NoV variant 2006a before going on to cause the majority of outbreaks during the winter of 2007/2008 [1]. During 2008/2009 a new NoV GII.4 variant, GII.4 2007 (also known as the Cairo variant), was also found in Edinburgh. In this study the first outbreak caused by NoV variant GII.4 2007 occurred in May 2008 (data not shown), with six further outbreaks between November 2008 and March 2009. The new genotype was identical in the 5' capsid region to strains isolated from children in Kolkata, India in 2009 [19], and highly related to a strain reported in Japan [20], Egypt [21], France [7], Belgium [22] and Canada [4]. Similar to our findings, Belliot *et al.* showed this new variant co-circulated with NoV GII.4 2006b variants in France, causing several outbreaks in the winter of 2008/2009, and suggested that NoV variant GII.4 2007 may have evolved from 2006b based on their close genetic similarity [7].

In November 2009, the NoV variant GII.4 2010 replaced the 2006b variant as the dominant circulating strain in Edinburgh healthcare institutions. Similar reports have been received from Asia and Europe [17, 23] with NoV GII.4 2010 variants being detected in France from February 2009 [7]. The NoV

GII.4 2010 variant predominated in Edinburgh healthcare institutions for much of 2009/2010. Early 2010 saw the emergence of six new NoV GII.4 2010 variants as well as several outbreaks due to a GII.13 strain. Over the 2009/2010 period 76% of outbreaks were caused by the GII.4 2010 variants. The remaining quarter of outbreaks were due to emergence of a new NoV 2006b variant (v8) along with outbreaks caused by the GII.1, GII.3, and GII.13 genotypes. Sequence comparison of the capsid region of the new GII.13 genotype suggested it was closely related to isolates from children in India, Hu/V1668/06/IND RdRp [19], and Jordan, Hu/GII/Irbid/JORD297/2007/Jordan [23]. Interestingly, our isolate also came from an outbreak in a children's hospital. The FBVE reported that, in Europe, NoV variant GII.4 2010 was the predominant circulating variant at the beginning of May 2011 [18]. Our study also identified NoV variant GII.4 2010 as the predominant circulating variant in April 2011, having been briefly displaced by a strain of GII.4 2006b in December 2010 and January 2011.

An interesting outcome of this work was the frequent emergence of new strains of NoV variants GII.4 2006b and GII.4 2010. The reason(s) why some strains are more successful than others is currently unclear but research to date suggests that a range of factors including virus stability, increased affinity for cellular receptors, herd immunity, environment and climate may all play a role [24–27]. The 2009/2010 season was a particularly severe season for NoV in Edinburgh with double the number of outbreaks of the preceding season. The peaks in activity in each season were linked to the emergence of new strains of virus, the most notable being the emergence of GII.4 2006b v3 in January 2009 leading to 17 outbreaks in February 2009, GII.4 2010 v1 in November 2009 and 2006b v8 in December 2010 leading to peaks of 51 outbreaks and 26 outbreaks in January 2010 and January 2011, respectively. In this study we have defined such strains by virtue of point mutations in 270 bp in the 5' region of the capsid gene. Although lineage defining amino-acid mutations have been reported in this region [2, 12, 28, 29], this is a relatively conserved region of the NoV genome and it is unclear whether these point mutations result in a phenotypic change in these viruses. Analysis of the P2 region of the genome in the outbreak-associated variants we have detected can provide further information on the homologous nature of the virus genomes and the selective advantages that they hold. Selective

advantages have been reported for viruses with nucleotide and amino-acid substitutions in these more variable, protruding regions of the capsid [30]. In particular, amino-acid changes in two exposed loops of the P2 domain have been shown to be responsible for virus escape mutants that have caused epidemics of gastroenteritis in the population [31, 32].

We found that the NoV variants GII.4 2006 and 2010, responsible for large increases in NoV gastroenteritis outbreaks, were circulating in Lothian healthcare facilities for at least 2–4 weeks preceding the peak of their activity, both the NoV variant GII.4 2006b v3 and v8 strains initially emerged in the season prior to their epidemic peaks. Gallimore *et al.* have reported that there is a greater mix of strains circulating at the start of the season compared to the end [28]. An active surveillance system could allow for the identification of variants that could become epidemic strains and certainly strains known to be associated with outbreaks elsewhere in the world.

The study also supports previous findings that, although NoV can infect all ages those most at risk are the elderly, the immunocompromised and young children. Outbreaks were more commonly associated with wards treating elderly patients and patients with immunosuppression, whereas, cases in children tended to be of a more sporadic nature. The average size of individual outbreaks in Edinburgh healthcare facilities may be decreasing over time, this trend appears to be independent of the overall severity of NoV infection in a season but may be inversely related to the length of ward closure. There may be additional infection control measures associated with this trend but there is need of a prospective analysis to ascertain the significance of this observation.

Additionally, the emergence of epidemic non-GII.4 strains has also been reported in recent literature. The winter of 2009/2010 saw the emergence of a GII.12 strain that was ultimately responsible for 16% of outbreaks in the USA that year [33]. Prior to 1991 non-GII.4 NoV strains had been shown to predominate [34]. Our study also found 14% of outbreaks in one season to be due to non-GII.4 genotypes. Most notable was the emergence of a GII.13 strain in January 2010 responsible for four outbreaks, 7% of the total, in the first quarter of 2010.

The emergence of new recombinants has also been observed in recent years [7, 20, 22]. The ORF1–ORF2 overlap is known to be a hotspot for NoV recombination [13, 22, 35, 36] and further analysis upstream of this region may also provide additional

information regarding the inclusion of recombinants in our isolates. Mathjis *et al.* have reported a number of novel norovirus recombinants isolated from food-borne outbreaks of gastroenteritis in Belgium in recent years [22], and it is unclear what significance these strains may play in the healthcare setting.

In conclusion, NoV outbreaks are a considerable burden in the NHS causing multiple ward closures, loss of resources, morbidity and possibly mortality in vulnerable populations. The present study revealed strains of genotype GII.4 2006b as the most recent predominant circulating variant in Edinburgh. The emergence of two new GII.4 strains, GII.4 2007 and GII.4 2010, was also detected. An important finding of this study was that outbreaks were linked to emergence of particularly successful variants of GII.4 strains. Although the reason for this is unknown it highlights the need for an active worldwide surveillance system to alert infection control teams to the detection of new and emerging strains and the need for further research into predictors of outbreak capacity in emerging variants.

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DECLARATION OF INTEREST

None.

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