

LONDON
SCHOOL of
HYGIENE
& TROPICAL
MEDICINE



LSHTM Research Online

Smith, KC; Inns, T; Decraene, V; Fox, A; Allen, DJ; Shah, A; (2017) An outbreak of norovirus GI-6 infection following a wedding in North West England. *Epidemiology and infection*, 145 (6). pp. 1239-1245. ISSN 0950-2688 DOI: <https://doi.org/10.1017/S0950268816003368>

Downloaded from: <http://researchonline.lshtm.ac.uk/3439432/>

DOI: <https://doi.org/10.1017/S0950268816003368>

Usage Guidelines:

Please refer to usage guidelines at <http://researchonline.lshtm.ac.uk/policies.html> or alternatively contact researchonline@lshtm.ac.uk.

Available under license: <http://creativecommons.org/licenses/by-nc-nd/2.5/>

<https://researchonline.lshtm.ac.uk>

Title Page

An outbreak of norovirus GI-6 following a wedding in North West England

K. C. SMITH^{1,2}, T. INNS^{3,4}, V. DECRAENE³, A. FOX⁵, D. J. ALLEN^{4,6}, A. SHAH¹.

¹Health Protection Team, Public Health England, North West, United Kingdom

²School of Public Health, Health Education North West, Liverpool, United Kingdom

³Field Epidemiology Service, National Infections Service, Public Health England, Liverpool, United Kingdom

⁴NIHR Health Protection Research Unit in Gastrointestinal Infections, UK

⁵Food Water and Environmental Microbiology Laboratory, Public Health England, Preston, United Kingdom

⁶Virus Reference Department, National Infections Service, Public Health England, Colindale, London, United Kingdom

Corresponding Author: Dr Anjila Shah, Health Protection Team, Public Health England, North West, United Kingdom. Email: Anjila.Shah@phe.gov.uk

1 **Summary**

2 In August 2015 a gastroenteritis outbreak occurred following a wedding. An outbreak investigation
3 was undertaken and a cohort study was conducted using an online survey. Of 140 guests, 134
4 received the survey and 113 responded (84.3% response rate). 70 respondents met the case
5 definition of vomiting and/or diarrhoea within 72 hours of the wedding (61.9% attack rate). Fifteen
6 exposures were associated with illness; on stratification, all were confounded by the ham hock
7 starter. Multivariable analysis showed a significant association with exposure to ham hock (RR 6.62,
8 95% CI: 2.19-20.03). Eight guests and two catering staff submitted stool samples. All tested positive
9 for norovirus GI-6, including a food-handler who had vomiting less than 48 hours before the
10 wedding. A single genotype was detected amongst all samples, suggesting a single source of
11 contamination. The transmission pattern suggested point-source exposure. The most plausible cause
12 of the outbreak was transmission from an infected food-handler via contaminated food. This
13 highlights the importance of appropriate exclusions for symptomatic food-handlers. Additionally, the
14 food-handler's stool sample was submitted 7 days after symptom resolution. The potential for
15 extended viral excretion, and the extremely low infective dose of norovirus, may mean that current
16 exclusion guidelines are not of sufficient duration.

17

18 **Introduction**

19 Norovirus is a highly infectious viral pathogen of the *Caliciviridae* family that is a common cause of
20 gastroenteritis in adults and children [1]. Norovirus was found to be the most common cause of
21 infectious intestinal disease in the UK during the IID2 longitudinal study [2]. Additionally, using 2008-
22 2009 prices, norovirus was estimated to have a total annual cost to patients and the health service
23 of £81 million (95% CI: £63 million - £106 million), which, based on the same study, was a larger
24 economic burden than that of campylobacter and rotavirus combined [3].

25 Infected individuals excrete the virus in faeces and vomit, and continue to do so for several days,
26 before, during and after their symptoms. Asymptomatic infection also occurs, and these individuals
27 can also shed the virus, posing a particular challenge to infection control [4] [5] [6]. Humans are the
28 only known reservoir for human norovirus infections, but contamination of food, water and the
29 environment is possible, so indirect transmission of norovirus also occurs [4] [7] [8] [9].

30 This paper describes an investigation into a food-associated norovirus outbreak linked to a wedding
31 in the North West. In August 2015, Public Health England (PHE) were contacted about a wedding,
32 with reports that over 50 of the 140 guests were experiencing symptoms of illness including
33 diarrhoea, vomiting and headache. An outbreak was declared and an Outbreak Control Team (OCT)
34 was convened to undertake an outbreak investigation, identify the source of the outbreak and
35 ensure appropriate control measures were implemented to prevent further transmission.

36

37 **Methods**

38 **Epidemiological Investigations**

39 Cases were defined as any person who attended the wedding and experienced vomiting and/or
40 diarrhoea with an onset date in the 72 hours following the wedding.

41 A retrospective cohort study was carried out to support the outbreak investigation, using a list of
42 food and drink items provided by the caterers. A web-based survey was sent to people who
43 attended the wedding, and was first emailed to the bride's family for circulation six days after the
44 wedding. Respondents could complete the survey on behalf of other guests, such as young children,
45 and were asked to indicate if they had done so. Staff who worked at the wedding were excluded
46 from the cohort as their exposures were believed to be systematically different from guests.

47 Demographics and exposure amongst cases and non-cases were described and compared.
48 Univariable and multivariable analysis was undertaken. Poisson regression with robust error
49 variance was used to quantify associations between exposures and illness (Risk Ratios (RR) with 95%
50 Confidence Interval (95% CI)).

51 For each item with a p value <0.05 on univariable analysis, univariable regression was conducted to
52 explore if any of the associations showed a dose-response relationship. For canapés, respondents
53 were asked to select from none, 1-2 or 3+. For other food items, respondents were asked to select
54 from none, a taste or a portion. Exposure to drink items and the chocolate truffles was not
55 quantified in the survey questions.

56 The multivariable analysis was undertaken using forward stepwise modelling. Only those items
57 which significantly improved model parsimony, as measured using the Akaike information criterion
58 (AIC), were included in the final model.

59 All analyses were undertaken using Stata v12.1.

60 **Microbiological Investigations**

61 Stool specimens were submitted by two catering staff and eight wedding guests. The eight wedding
62 guests came from four different households. The 10 samples were collected between 5 and 13 days
63 after the wedding. All 10 samples were tested for bacteria and enteric viruses. Environmental swabs
64 were also taken at the caterer's premises and the wedding venue, and were tested for hygiene

65 indicator organisms (including enterobacteriaceae, E.coli and coagulase positive Staphylococci) and
66 norovirus.

67 For faecal specimens, total nucleic acid was obtained using QIAextractor automated nucleic acid
68 extraction platform (QIAGEN). For environmental specimens, total nucleic acid was extracted using a
69 GTC–silica method performed as previously described [10]. From total nucleic acid, RNA was
70 converted to cDNA in a random-primed reverse transcription reaction and norovirus detected using
71 real-time PCR methods as previously described [11].

72 Noroviruses were genotyped through nucleic acid sequence analysis of a region encoding the S-
73 domain of the capsid (region C) [12] [13]. Sequence analysis was performed using Bionumerics v6.1
74 (Applied Maths, Kortrijk, Belgium).

75 **Environmental Investigations**

76 Environmental Health Officers (EHOs) conducted environmental inspections of the caterer's
77 premises and the wedding venue, along with review of the caterer's Food Safety Management
78 System and discussions with staff from both companies. No food samples were submitted as none
79 were remaining from the event.

80

81 **Results**

82 **Epidemiological Investigations**

83 The survey was sent to 134 of 140 wedding guests. In total, 113 (83.4%) individuals responded to the
84 survey, of whom 60 (53.1%) were male. Age was reported by 89 respondents, with an average age of
85 39, ranging from 4-88.

86 Of the survey respondents, 76 (67.3%) reported illness. However, one gave an onset date greater
87 than 72 hours after the event and 5 reported illness but did not have symptoms of vomiting or
88 diarrhoea, so were not included as cases. Therefore, of the 113 respondents, 70 (61.9%) met the
89 case definition.

90 Of the 70 cases, 50 (71.4%) experienced vomiting, and 64 (91.4%) experienced diarrhoea. Nausea
91 was reported by 65 (92.9%) and abdominal pain was reported by 51 (72.9%) people. Over half of
92 cases also experienced fever (54.3%), and half reported headache (50.0%), with a small number
93 (7.1%) reporting bloody diarrhoea. The most commonly reported symptom duration was 2 days
94 (48.6%), with duration ranging from less than one day to five or more.

95 There were no significant differences between the cases and non-cases by age or gender. Individuals
96 who attended the event during the whole day, which included the canape reception and wedding
97 breakfast as well as the evening buffet, had an estimated relative risk (RR) of 4.42 (95% CI 1.49-
98 11.99) of becoming a case when compared with those who attended only the evening buffet.

99 The analytical study identified 15 items where there was strong evidence of association between
100 exposure and being a case, as shown in Table 1. At a univariable level, the highest RRs were for the
101 ham hock starter (RR 7.63, 95% CI 2.60-22.32), the meat sausages served to adults (RR 6.91, 95% CI
102 2.37-20.15), and the mashed potato (RR 5.35, 95% CI 2.15-13.31) served as an accompaniment to all
103 of the main courses. Of the 70 cases, 67 (95.7%) ate the ham hock and meat sausages and 65
104 (92.8%) ate the mashed potatoes.

105 For the dose-response analysis the canapés either showed a lower effect at a higher dose, or did not
106 have strong evidence for a dose-response relationship. The ham hock starter, meat sausages, and
107 leeks all showed strong dose-response relationships. The mashed potato, peas and lemon meringue
108 all showed some dose-response. The bread basket showed no evidence of a dose-response
109 relationship.

110 The mashed potato, peas, leeks, bread basket, lemon meringue, chocolate truffles and all of the
111 canapés were confounded by the ham hock. The champagne was also confounded by the ham hock
112 with some evidence of effect modification. The mineral water showed possible confounding.

113 Due to strong collinearity between the ham hock and the meat sausages it was not possible to assess
114 confounding. Consequently, for subsequent modelling, the ham hock was used and the meat
115 sausages were excluded in view of the higher univariable risk associated with the ham hock.

116 The final multivariable model is shown in Table 2; inclusion of the leeks, the chicken skewers, the
117 cheese risotto, and the mineral water significantly improved the model. When adjusted for these
118 items, there was still a strongly significant relationship between becoming a case and having been
119 exposed to the ham hock (adjusted RR 6.62, 95% CI: 2.19-20.03).

120 In addition to the quantitative analysis, qualitative comments were also received via the survey. Of
121 45 text responses, ten people (22.2%) mentioned the ham hock, with comments including concerns
122 about the serving temperature, the smell, the taste, the texture and the meat being undercooked.
123 One person (2.2%) commented that the lemon meringue was sour, but no other comments were
124 received about specific food items.

125 **Microbiological Investigations**

126 **Stool Samples**

127 The 8 specimens submitted from wedding guests for testing were negative for any pathogenic
128 bacteria, however, genogroup I norovirus was detected in all eight samples.

129 Two staff members who catered the event submitted specimens and genogroup I norovirus was
130 detected in both. One of these individuals reported symptoms prior to the event, and the other
131 reported symptoms that began on the day of the event. A third symptomatic staff member did not
132 submit a specimen.

133 **Environmental Samples**

134 Of the 17 swabs taken from the caterers' premises, 7 were tested for hygiene indicator organisms
135 and 10 were tested for norovirus. A hygiene indicator sample from the chiller handle tested positive
136 for Enterobacteriaceae.

137 Twenty swabs were taken from the venue and tested for norovirus. A genogroup II norovirus was
138 detected in one swab from the ladies' toilet and one from the chiller handle. The venue had not
139 been open to the public between the wedding and the sampling. However, venue staff had access to
140 the chiller, which they used to store milk, and the public toilets in the intervening period.

141 **Additional Testing**

142 All of the positive samples were sent to the national reference laboratory for further typing. Further
143 analysis was conducted for all 10 stool samples (8 guests and 2 staff members). The genogroup I
144 virus was characterised as genotype 6 (GI-6); all sequences were identical. No further analysis was
145 possible on the environmental swabs.

146 **Environmental Investigations**

147 Wedding Venue

148 An EHO visit did not identify any areas of concern. The venue staff were not involved with catering
149 and none reported being unwell before, during or after the wedding. The norovirus identified on
150 environmental swabs was different to that involved in the outbreak; however, in view of its presence
151 the EHO provided reminders of handwashing advice to venue staff.

152 Caterer

153 The investigation identified a number of areas of concern. The chef who provided catering on the
154 wedding day had vomiting which had resolved less than 48 hours earlier, and a GI-6 norovirus was
155 detected in a faecal specimen submitted 5 days after the wedding. Two staff were ill at the wedding

156 and were quarantined at the venue until the end of the event; one submitted a specimen in which
157 GI-6 norovirus was detected.

158 The ham hock starter was cooked two days prior to the wedding by a chef who did not attend the
159 event and did not report illness. It was transported to the venue in moulds on the wedding day,
160 when it was plated and garnished, without further cooking, by the chef who subsequently tested
161 positive for norovirus. There were no records of control checks to assess storage, transport, hot
162 holding or service temperatures. A hand wash basin at the caterer's premises was next to a raw
163 meat preparation area.

164 As a result of the findings of the outbreak investigation, a warning letter was issued by the local
165 authority environmental health team.

166

167 **Discussion**

168 Our investigation concluded that this outbreak of norovirus gastroenteritis was associated with a
169 ham hock dish that had been prepared by a food-handler who had vomiting less than 48 hours
170 before the wedding. A GI-6 norovirus was detected in the faeces of all cases and food handlers
171 tested. The food-handler who had vomiting less than 48 hours before the wedding submitted a
172 specimen five days after the wedding in which a GI-6 virus was detected, having had no further
173 symptoms since the vomiting resolved.

174 The epidemiological analysis indicated a strong association between the ham hock and becoming a
175 case. The collinearity between the ham hock and the meat sausages meant that the sausages were
176 excluded from further analysis, and this is a limitation of the study. It was also noted that three of
177 the cases did not report consuming the ham hock. The lack of any food samples for testing also
178 meant that it was not possible to prove that any items were contaminated with norovirus. However,

179 the epidemiological analysis, including the qualitative feedback in the survey, alongside the findings
180 of the environmental investigation, lead to the conclusion that the ham hock was the most likely
181 vehicle of transmission. Furthermore, the positive stool sample in a food-handler who was
182 symptomatic prior to the wedding provided a plausible source of contamination of the food.

183 Although GI-6 norovirus strains are detected in the UK each year, over the last 10 years they have
184 only accounted for 1.0-1.5% of all norovirus strains identified (DJA personal communication). A
185 paper on diversity of norovirus in the North of England included two outbreaks which were
186 designated as GI-6 [13]. Publications have also reported outbreaks linked with GI-6 in Hesse,
187 Germany [14], the USA [15] and Australia [16].

188 The Centers for Disease Control and Prevention in the USA, reported in 2013 that there had been an
189 increase in the proportion of GI-6 outbreaks in recent years, with 7.7% of 2012 outbreaks being due
190 to GI-6 compared with 1.4% in 2010. Additionally, it was found that GI-6 outbreaks were more likely
191 to be associated with food, and had summer seasonality, when compared with non-GI-6 outbreaks
192 [17]. This correlates with the timing and mode of transmission of the outbreak investigations
193 detailed in this paper.

194 The IID2 study has identified that the burden of norovirus in the UK is likely to be much higher than
195 that identified by national surveillance. It is estimated that for every norovirus case identified
196 through surveillance, there were 12.7 GP consultations (95% CI 8.8-18.3), and 288 cases in the
197 community (95% CI 239-346) [2]. Furthermore, as GI-6 outbreaks have been shown to be less likely
198 to occur in healthcare settings than non-GI-6 outbreaks [17], it may be that there is an element of
199 underreporting when compared with the strains that commonly occur in healthcare settings, due to
200 a decreased likelihood of symptom reporting and sample submission.

201 The food-associated transmission in this outbreak was associated with contamination by an infected
202 food handler, who was not appropriately excluded for 48 hours following the resolution of

203 symptoms. There have been multiple papers outlining the role of post-symptomatic food handlers in
204 the transmission of norovirus and subsequent outbreaks [8, 6, 7]. This outbreak further highlights
205 the importance of appropriate exclusions for symptomatic food handlers.

206 Whilst the food handler returned to work less than 48 hours after symptom resolution, the positive
207 stool sample 7 days after reported resolution of symptoms also reinforces the potential for extended
208 viral excretion in those infected. It has been demonstrated in that post-symptomatic viral shedding
209 of norovirus may last longer than the typical 48 hour exclusion period [18, 19]. Particularly in view of
210 the low infective dose of norovirus, this extended, post-symptomatic excretion, could well be an
211 ongoing risk for transmission. Therefore, as previously highlighted, in food handlers with
212 gastroenteritis symptoms, there should be further consideration of whether the existing exclusion
213 guidelines are sufficient [5].

214

215 **Conclusions**

216 This study found that this norovirus outbreak was food-associated transmission of norovirus (GI-6),
217 associated with inappropriate exclusion of an infected food-handler. The summer seasonality and
218 mode of transmission are in keeping with broader trends related to GI-6 in the US. The extended
219 post-symptomatic viral excretion in the food handler highlights the need for further consideration of
220 appropriate duration of exclusion in this group.

221 **Acknowledgements**

222 The authors would like to thank the OCT, particularly the EHOs and laboratory staff, for their
223 significant contribution to the outbreak investigation.

224

225 **Financial Support**

226 This research received no specific grant from any funding agency, commercial or not-for-profit
227 sectors.

228 The research was funded by the National Institute for Health Research Health Protection Research
229 Unit (NIHR HPRU) in Gastrointestinal Infections at University of Liverpool in partnership with Public
230 Health England (PHE), in collaboration with University of East Anglia, University of Oxford and the
231 Institute of Food Research. DJA is based at Public Health England. The views expressed are those of
232 the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health or Public
233 Health England.

234

235 **Conflict of Interest**

236 None

237

238 **References**

239 1. **Norovirus Working Group.** Guidance for the management of norovirus infection in cruise
240 ships. London: Health Protection Agency, 2007.

- 241 2. **Tam CC, et al.** Longitudinal study of infectious intestinal disease in the UK (IID2 study):
242 incidence in the community and presenting to general practice. *Gut* 2012; **61**: 69-77.
- 243 3. **Tam CC, O'Brien SJ.** Economic cost of campylobacter, norovirus and rotavirus disease in the
244 United Kingdom. *PLOS One*. Published online: 1 February 2016.
245 doi:10.1371/journal.pone.0138526.
- 246 4. **Hawker J, et al.** *Communicable Disease Control and Health Protection Handbook*, 3rd edn.
247 Chichester: Wiley-Blackwell, 2012.
- 248 5. **Barrabeig I, et al.** Foodborne norovirus outbreak: the role of an asymptomatic food handler.
249 *BMC Infectious Diseases*. Published online: 15 September 2010.
250 doi:10.1186/1471-2334-10-269.
- 251 6. **Franck KT, et al.** Sources of calicivirus contamination in foodborne outbreaks in Denmark,
252 2005-2011 – the role of the asymptomatic food handler. *The Journal of Infectious Diseases*
253 2015; **211**: 563-570.
- 254 7. **Patterson T, Hutchings P, Palmer S.** Outbreak of SRSV gastroenteritis at an international
255 conference traced to food handled by a post-symptomatic caterer. *Epidemiology and*
256 *Infection* 1993; **111**: 157-162.
- 257 8. **Thornley CN, et al.** Multiple outbreaks of a novel norovirus GII.4 linked to an infected
258 post-symptomatic food handler. *Epidemiology and Infection* 2013; **141**: 1585-1597
- 259 9. **Einöder-Moreno M, et al.** Non-heat-treated frozen raspberries the most likely vehicle of a
260 norovirus outbreak in Oslo, Norway, November 2013. *Epidemiology and Infection*. Published
261 online: 16 February 2016. doi:10.1017/S0950268816000194.
- 262 10. **Boom R, et al.** Rapid and simple method for purification of nucleic acids. *Journal of Clinical*
263 *Microbiology* 1990; **28**: 495-503.
- 264 11. **Kageyama T, et al.** Broadly reactive and highly sensitive assay for Norwalk-like viruses based
265 on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology* 2003;
266 **41**: 1458-1557.

- 267 12. **Kojima S, et al.** Genogroup-specific PCR primers for detection of Norwalk-like viruses.
268 *Journal of Virological Methods* 2002; **100**: 107-114.
- 269 13. **Gallimore CI, et al.** Diversity of noroviruses cocirculating in the North of England from 1998
270 to 2001. *Journal of Clinical Microbiology* 2004; **42**: 1396-1401.
- 271 14. **Maunula L, Miettinen IT, von Bonsdorff CH.** Norovirus outbreaks from drinking water.
272 *Emerging Infectious Diseases* 2005; **11**: 1716-1721.
- 273 15. **Xerry J, et al.** Genetic characterization of genogroup I norovirus in outbreaks of
274 gastroenteritis. *Journal of Clinical Microbiology* 2010; **48**: 2560-2562.
- 275 16. **Bruggink LD, Dunbar NL, Marshall JA.** Norovirus genotype diversity in community-based
276 sporadic gastroenteritis incidents: a five-year study. *Journal of Medical Virology* 2015; **87**:
277 961-969.
- 278 17. **Leshem E, et al.** Genotype GI.6 norovirus, United States, 2010-2012. *Emerging Infectious*
279 *Diseases* 2013; **19**: 1317-1320.
- 280 18. **Rockx B, et al.** Natural history of human calicivirus infection: a prospective cohort study.
281 *Clinical Infectious Diseases* 2002; **35**: 246-253.
- 282 19. **Atmar RL, et al.** Norwalk virus shedding after experimental human infection. *Emerging*
283 *Infectious Diseases* 2008; **15**: 1553-1557.
- 284 20. **Tamura K, et al.** MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular*
285 *Biology and Evolution* 2013; **30**: 2725-2729

Table 1 – Univariable Analysis

Exposure	Exposed			Unexposed			Risk Ratio [95% CI]	p Value
	Total	Case	AR%	Total	Case	AR%		
Ham Hock	82	67	81.7	28	3	10.7	7.63 [2.60-22.32]	<0.0001
Meat Sausages	84	67	79.8	26	3	11.5	6.91 [2.37-20.15]	<0.0001
Mashed Potato	82	65	79.3	27	4	14.8	5.35 [2.15-13.31]	<0.0001
Lemon Meringue	80	63	78.8	31	7	22.6	3.49 [1.80-6.76]	<0.0001
Peas	69	56	81.2	38	11	29.0	2.80 [1.68-4.67]	<0.0001
Leeks	59	49	83.1	44	15	34.1	2.44 [1.59-3.73]	<0.0001
Chicken Skewers	59	48	81.4	47	19	40.4	2.01 [1.39-2.91]	<0.0001
Champagne	67	53	79.1	40	16	40.0	1.98 [1.33-2.95]	<0.0001
Bread Basket	66	51	77.3	41	16	39.0	1.98 [1.32-2.97]	<0.0001
Cheese Risotto Balls	57	45	79.0	47	19	40.4	1.95 [1.35-2.83]	<0.0001
Tandoori Chicken	52	42	80.7	55	25	55.5	1.78 [1.29-2.44]	<0.0001
Spiced Lamb	54	41	75.9	54	27	50.0	1.52 [1.12-2.06]	0.009
Mushroom Risotto	42	33	78.6	63	33	52.4	1.50 [1.13-1.99]	0.008
Mineral Water	39	29	74.4	60	30	50.0	1.49 [1.09-2.03]	0.021
Chocolate Truffles	42	32	76.2	68	38	55.9	1.36 [1.04-1.79]	0.041

Table 2 – Multivariable Model

Exposure	Adjusted Risk Ratio [95% CI]	P
Ham Hock	6.62 [2.19 - 20.03]	0.001
Leeks	1.15 [0.84 - 1.57]	0.397
Chicken Skewers	0.91 [0.69 - 1.20]	0.912
Cheese Risotto Balls	1.01 [0.77 - 1.33]	0.943
Mineral Water	1.27 [0.98 - 1.64]	0.071

Figure 1: Epidemic curve showing onset date of illness in persons associated with the wedding,

August, 2015

286

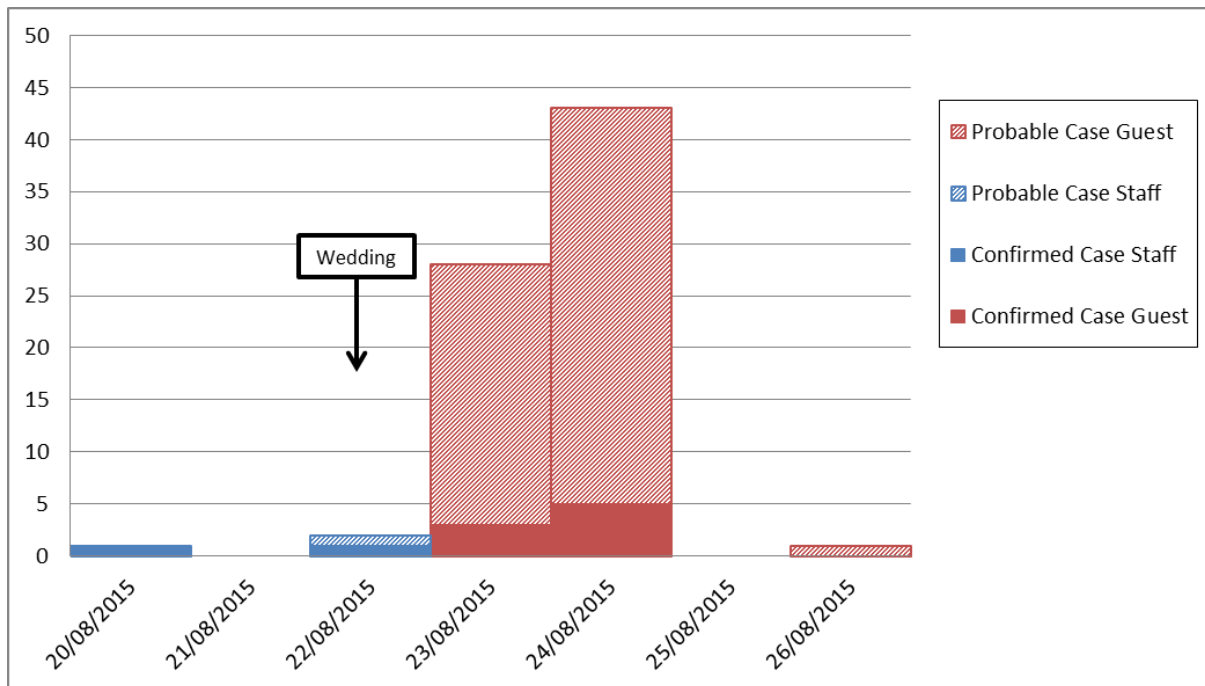


Figure 2: Phylogenetic tree derived from 8 norovirus partial capsid (ORF2) sequences from 8 specimens collected from symptomatic guests. The tree was constructed using the Maximum Likelihood method in MEGA6 [20]. Branch length represents number of substitutions per site. Specimens from cases are marked with blue triangles; reference sequences obtained from GenBank are marked with grey squares. Numbers on branches show bootstrap values >80% (1000 replicates).

