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4
5   **Methods for ascertaining norovirus disease burdens**
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13

14   **Introduction**

15   The discovery of norovirus in 1972[1], changed the understanding of the aetiology of gastroenteritis,
16   making it the virus to be identified as an agent of gastroenteritis in humans.

17   Today, norovirus is recognised as one of the commonest human infections and estimated to be
18   associated with 125 million cases and 35,000 deaths worldwide in 2010[2]. Better epidemiological
19   surveillance and outbreak investigations[3], coupled with wider implementation of molecular-based
20   laboratory diagnostics[4] are leading to better estimates of the burden of norovirus infections as well as
21   improved outbreak control.

22   Data from challenge studies of prototype norovirus vaccines[5, 6] demonstrated that protection against
23   infection and disease can be achieved, however there remain significant challenges to development of
24   a norovirus vaccine. Recent advances in cell culture systems for norovirus[7, 8] and current research
25   investigating the distribution of norovirus-associated disease in the population, for whom the disease
26   burden is greatest, understanding host susceptibility factors, how to deploy novel technologies detecting
27   norovirus in food and environmental matrices, and methodologies for ascertaining cases, are important
28   in increasing our understanding of norovirus. Answers to these will help design strategies for vaccine
29   and antiviral development, and how these might be best deployed to control norovirus infection.

30

31   **Norovirus virology**

32   At the time of discovery, the virus was referred to as the Norwalk agent, but as other related viruses
33   were described in association with gastroenteritis, they became known as Norwalk-like viruses (NLVs),
34   or – based on their morphology by electron microscopy – small, round, structured viruses (SLSVs).
35   Following the cloning and sequencing of the Norwalk agent genome in 1993[9], and subsequently other
36   NLVs[10], defining the genetic relatedness of these viruses led to their reclassification in the *Caliciviridae*
37   family of viruses under the genus *Norovirus* in 2002[11].

38 Classification of this genetically diverse group of viruses[12, 13] has described six established[14]
39 genogroups (GI-GVI), and a proposed seventh[15, 16]. Two genogroups (GI and GII) are important
40 pathogens of humans (GII also contains pathogens of animals, but there is no evidence of zoonotic
41 transmission[17]) and genogroups are further subdivided into genotypes: nine GI and twenty-two GII
42 genotypes have been described[12-14]. Norovirus names are presented as genotypes, e.g. genogroup II-
43 genotype 4 (GII.4) and strains are named for the place and year of their first description: e.g. GII.4/New
44 Orleans 2009[18] or GII.4/Sydney 2012[19].

45 The norovirus genome is a single strand of positive-sense ribonucleic acid (+ssRNA) that is ~7700
46 nucleotides in length, organised as three open reading frames (ORF1-3)[20]. The 5'-proximal ORF1
47 encodes a polyprotein that is post-translationally processed by the virus-encoded protease into six non-
48 structural proteins, including a genome-linked protein (VPg/NS5), protease (Pro/NS6) and an RNA-
49 dependent RNA polymerase (RdRp/NS7). Both ORF2 and ORF3 encode a single protein each, VP1
50 and VP2 respectively, that are structural proteins involved in formation and stabilisation of the virus
51 particle[21].

52 Norovirus particles are 27-35nm in diameter and comprised of 180 copies of the VP1 protein, which
53 itself is organised into three main domains: N-terminal (N), shell (S) and protruding (P), which is further
54 arranged as P1 (subdivided as P1.1 and P1.2) and P2[21]. In the mature infectious virus particle, the N
55 domain is internal, and the P2 domain is the most external part of the virus, making it highly surface-
56 exposed and placed to coordinate many of the interactions between norovirus and its host environment.

57 The primary host cell receptor for human norovirus is unknown, but can interact with histo-blood group
58 antigens (HBGAs)[22]; these are glycans expressed on epithelial cells and in mucosal secretions, which
59 determine ABO blood type groups. Norovirus strains may use HBGAs as attachment factors or co-
60 receptors[23], and sites in the VP1 P2 domain have been identified as HBGA binding sites[24-29]. Synthesis
61 of HBGAs occurs by sequential modification of a precursor, and the process is controlled by glycosyl-
62 transferase enzymes from several genetic loci that exhibit polymorphism throughout the human
63 population. The ABH and Lewis antigens are relevant to norovirus binding, and as such the phenotype
64 of an individual for secretory ABH and Lewis antigens is a host susceptibility factor for norovirus
65 infection. Specifically, individuals with a non-functional *FUT2* gene, which encodes an α1,2-
66 fucosyltrasnferase, have a 'non-secretor' phenotype, and are more resistant to norovirus infection that
67 'secretor' individuals[30, 31]. Polymorphism at this locus may modulate susceptibility to other causes of
68 diarrhoeal disease[32].

69 Public health laboratory surveillance worldwide has demonstrated dominance of GII.4 viruses[33-36],
70 however other norovirus genotypes circulate consistently, if at a lower level, in the population. The GII.4
71 cluster of norovirus strains have been the most commonly detected noroviruses circulating worldwide
72 since the mid-1990s, over time, distinct variants of the GII.4 virus evolve, emerge, and then recede to
73 be replaced by a new variant[33]. Emergence of a new GII.4 variant is associated with higher levels of
74 infection and illness in the population and increased numbers of outbreaks[37], although severity of
75 disease does not necessarily increase. These emergence events may be geographically contained (e.g.

76 a 2003 variant emerged in Asia, and a 2006 variant spread in Europe)[38], or may be global, with new

77 variants emerging and spreading worldwide over the course of a single year (as seen in 2002[39], 2006[40],

78 2009[18] and 2012[19]).

79 Noroviruses have been shown to have high evolutionary rates, up to 10^{-2} substitutions/site/year in the

80 VP1 protein[41], due to the error-prone nature of the virus-encoded RdRp[42]. The rate of evolution is fastest

81 in the P2 domain, which interacts with the host immune system. Immune response to norovirus infection

82 appears to target this region of the virus capsid, and epitopes in this domain have been identified as

83 important in defining the antigenic profile of GII.4 norovirus strains[43-47], leading to the emergence of

84 antigenically distinct viruses in the population, associated with epidemic/pandemic waves of

85 gastroenteritis[33, 37, 48, 49].

86 The emergence of variant GII.4 strains is associated with mutations occurring in the virus at epitope A

87 (VP1 amino acid positions 296-298, 368 & 372) and D (VP1 amino acid positions 393-395)[44, 50].

88 Mutations, particularly in epitopes A and D, will be selected for in the virus population if the mutations

89 are such that existing immunity in the host population is evaded by the mutated virus, but the virus is

90 otherwise not disadvantaged. Because much of the human population is exposed to antigenically similar

91 noroviruses at a similar time, virus-specific immunity is likely similar between many people. As a result,

92 the variant norovirus is advantaged, being more likely to successfully evade existing immunity, and

93 subsequently establish more infections and be transmitted. In this way, the virus can spread quickly

94 through the population. Eventually infections generate new immunological responses, which ultimately

95 limit the success of this variant in the population, but in turn creates an ecological niche favourable for

96 a new variant, and the process cycles again. This process has been observed for GII.4 noroviruses

97 throughout the 1990s, 2000s and 2010s[19, 33, 37, 45, 47].

98

99 **Norovirus surveillance**

100 Surveillance of norovirus is complicated because most people do not contact medical services when

101 they are ill. In the UK, it is suggested that for each laboratory report of norovirus around 300 cases go

102 unreported[51]. This is largely related to the nature of the illness itself. The virus is highly infectious with

103 an estimated infectious dose of around 10-100 virus particles (virions) needed to cause infection[52], with

104 a high probability of infection from ingesting a single particle[53]. It has a short incubation period,

105 anywhere between 12 and 72 hours, and symptoms typically last for around 24- 48 hours[54]. Despite

106 these difficulties it is still recognised as the commonest cause of gastrointestinal disease, not just in the

107 UK, but worldwide[55]. In the UK it is estimated between 3 and 4 million cases occur annually[51, 56], at a

108 cost of £106 million to patients and the health care services. In the USA this estimate is around 21

109 million domestically acquired cases[57]. Infections with norovirus occur in all age groups, however, the

110 highest incidence is in children aged less than five years[56, 58].

111

112 The illness is often described as generally mild and self-limiting. The description of a mild infection can
113 trivialise the effect of the illness; in England it has been estimated that 3000 admissions occur annually
114 as a result of norovirus infection in adults[59] or 0.3% of emergency admissions in those aged over 65,
115 and 0.1% in adults aged 16-64 years. The consequences of infection are also greater in vulnerable
116 populations. In a study in the county of Avon, UK, hospital patients were ill for longer than care home
117 residents and staff working in the hospitals or care home, with around 10% of inpatients affected still
118 showing symptoms 7 days after becoming ill[60]. There is also evidence that norovirus can contribute to
119 mortality in the elderly. Modelling of deaths suggests that norovirus is associated with 20% in those
120 aged over 65 years who died of infectious intestinal disease, and that 13% of deaths caused by non-
121 infectious intestinal disease[61].

122 Public Health England have conducted surveillance of gastrointestinal disease outbreaks since 1992[62].
123 Analysis of the first nine years of data highlighted the importance of norovirus outbreaks in hospitals;
124 over 80% of all reported outbreaks in hospitals were suspected or confirmed as norovirus, and 25% of
125 all general outbreaks occurred in hospitals[63].

126

Recent developments

128

Surveillance

130 Since the recognition of the importance of norovirus as a cause of GI disease a more detailed online
131 surveillance system was set up in 2009[63]. The online system increased ascertainment of outbreaks in
132 hospitals, with more outbreaks reported in the first year than the whole of the preceding system[63]. Both
133 systems highlighted the increased activity during the winter months, and the considerable burden it
134 places on NHS hospitals in England. The online system suggests around 13000 patients and 3000 staff
135 are affected each year, moreover, almost 9000 bed days are lost because of restrictions to admissions
136 during outbreaks[63].

137 The key to surveillance of norovirus is allying the epidemiology with surveillance of virology. It is often
138 difficult to achieve this. Recording the number of outbreaks, and laboratory reports indicates levels of
139 infection, but they cannot directly ally this knowledge of circulating strains of the virus. The activity
140 recorded in both Public Health England's hospital outbreak reporting scheme and laboratory reports
141 suggests that seasonal activity varies considerably. The reasons for changes in seasonal activity need
142 unpicking and modelling of changes in the circulating strains of norovirus against laboratory reporting
143 provided evidence that modifications within the virus itself leads to changes in the epidemiology. In the
144 autumn/winter of 2012 PHE recorded increased levels of norovirus activity; later attributed to the
145 emergence of the Sydney 2012 strain[37]. However, other reasons have been proposed, such as changes
146 in winter conditions such as falling temperature[64].

147 Given the difficulty in surveillance of norovirus infections from direct sources, other developments need
148 to be explored. For example, social media could provide early indications of increasing activity. There

149   are a number of publications looking at the use of internet search and social media postings to provide

150   information on increased disease activity[65-67]. Other forms of syndromic surveillance have been used

151   such as the use of telephone helpline data to map diarrhoea and vomiting[68], difficulties with this

152   approach fall mainly on disentangling the causes of the illness from syndromes (diarrhoea and

153   vomiting). Norovirus is not the only cause of D&V and has a seasonality similar to that of rotavirus,

154   similarly sapovirus has similar illness characteristics to norovirus.

155

156   *Virus culture systems*

157   Understanding the interactions of norovirus with host cells has been limited by the lack of an *in vitro*

158   laboratory cell culture system. Attempts to establish conventional cell culture approaches were

159   unsuccessful[69], after which alternative approaches were developed[70, 71], however, these were limited in

160   their usefulness.

161   More recently, progress has been made towards development of laboratory culture systems for human

162   norovirus. Two systems have been described: one describes human norovirus replication in B cells[8],

163   and a second which describes human norovirus replication supported by stem cell-derived human

164   enteroids[7]. These systems present exciting new opportunities to understand how norovirus interacts

165   both with the host cell and with the host environment.

166   The system using human enteroids[7] provides a model for processes of norovirus replication such as

167   attachment/entry, genome replication, and virus assembly/release can be interrogated in a biologically

168   relevant cell type. Advances in these areas will be crucial for identifying targets for virus-specific

169   interventions, and evaluating how effective different antiviral therapies can limit norovirus replication.

170   Further insights into virus entry and egress will enhance understanding of the interactions between virus

171   and host receptors and identify novel interactions between virus and host that serve as intervention

172   targets, for example antibodies which interfere with attachment or release processes, thus neutralising

173   free virus.

174   The second system, in which norovirus replication is supported in B cells, uses commensal bacteria

175   that express HBGAs to facilitate virus replication in this model[8, 72]. Analysis of norovirus replication in

176   this system could enhance understanding of the interaction between norovirus and HBGAs – and

177   identify how these interactions might be disrupted– but also what interactions might occur with, and

178   what role might be played by, the microbiome during norovirus infection[73].

179

180   **Norovirus vaccines**

181   Modelling studies have shown that norovirus vaccination would offer healthcare and economic benefit[74].

182   These could help control and prevent the large-scale and often protracted outbreaks often seen in

183   healthcare settings[75] and other settings such as in the military[76, 77].

184 Until very recently, development of candidate vaccines focussed on recombinant protein systems;
185 expression of the norovirus capsid protein VP1 *in vitro* leads to self-assembly of the protein into virus-
186 like particles (VLPs) that are antigenically and morphologically identical to infectious virus, but lacking
187 a genome, VLPs are entirely non-infectious[78].

188 Early clinical studies of responses in humans to immunisation with VLPs demonstrated they were
189 immunogenic when delivered orally[79, 80] or intranasally[81]. A randomised, double-blind placebo-
190 controlled trial conducted in healthy, susceptible adult volunteers investigated the safety and efficacy
191 of vaccination using norovirus VLPs, followed by challenge with a homologous norovirus strain[6]. This
192 trial demonstrated 70% of vaccine recipients had a virus-specific IgA response, and vaccination reduced
193 the frequency of both infection and disease between placebo control group and vaccine recipients[6].

194 However, the prototype vaccine (and challenge strain) used in this trial was based on a single norovirus
195 strain – the prototype GI.1 Norwalk virus/1968 – which is uncommon, detected in <1% of norovirus
196 strains characterised per year in surveillance programmes in developed countries. As the most
197 significant disease burden is associated with the GII.4 genocluster, any candidate vaccine would need
198 to elicit immunity to GII.4 norovirus strains, and cross-react to antigenically distinct GII.4 variants. A
199 chimeric VLP was developed incorporating epitopes from antigenically distinct GII.4 viruses[82], and
200 induced broadly-reactive antibody responses[83].

201 A subsequent trial incorporated the chimeric VLP into a GI.1/GII.4 bivalent vaccine formulation and
202 demonstrated vaccine induced seroconversion in 90% of vaccine recipients, and reduced
203 gastroenteritis following challenge[5]. However, the predefined primary endpoints were not achieved in
204 this study, and further studies are necessary to assess how effective this candidate vaccine would be
205 in the general population, and specifically in paediatric and elderly populations. Furthermore, studies
206 must address both the duration of and the inter-/intra-genotype breadth of protection.

207

208 **Perspectives**
209 Clearly, significant progress has been made in understanding the virology and epidemiology of
210 norovirus in humans: but there remain significant gaps in our knowledge, important for development of
211 therapeutic and preventative interventions, and ascertaining norovirus disease burden to understand
212 how these should be utilised, and to measure their effectiveness. This is true in all economic settings,
213 but especially in low economic settings.

214 One key question is to understand the emergence of norovirus strains. With no animal reservoir[17], the
215 virus must be sustained – and continuously evolve – in the human population. Using genomics
216 approaches to measure and monitor virus diversity among circulating strains, and to characterise and
217 measure whether observed genetic changes induce phenotypic changes will be crucial in developing
218 the systems needed to understand and monitor emergence events, particularly those that lead to rapid
219 pandemic spread of norovirus strains. There is increasing evidence that children may act as important
220 reservoirs of norovirus, and the virus may exploit the more naïve immunological background in children

221   to explore antigenic diversity, ultimately leading to virus diversification and subsequent emergence of

222   novel strains[84].

223   Second, more detailed understanding of the burden of the disease, transmission dynamics and

224   pathogenesis in risk groups, both those at risk of more severe disease (immunocompromised[85],

225   elderly[61]), and those more likely to come into contact with or are at higher risk of transmitting the virus

226   (food-handlers[86], healthcare workers[87], military personnel[76]) is needed. There are complex

227   epidemiological and virological questions relating to the distribution of norovirus-associated disease in

228   the population, for whom the disease burden is greatest, as well as understanding host susceptibility

229   factors. Integrated laboratory and epidemiological studies are crucial to investigate how norovirus is

230   transmitted, disease attribution via different transmission pathways, how infections can be tracked in

231   the population and during outbreaks, and what role susceptibility factors such as HBGA phenotype or

232   the individual microbiome composition may play in norovirus infection, development of disease and

233   outcomes.

234   Third, alongside data on the direct burden of disease, enhanced data are needed to understand where

235   interventions may alleviate transmission and disease overall, as many settings are interlinked. For

236   example, administering a norovirus vaccine to patients in long term care homes might help prevent

237   outbreaks in this environment, but might have limited effects on the population as a whole. However, it

238   may be a worthwhile strategy if vaccination in care homes subsequently prevents outbreaks in hospitals

239   and reduces bed blocking.

240   With the recent advances in laboratory culture systems for norovirus[7, 88], next generation sequencing

241   technologies[89], improved diagnostics[4] and measuring phenotypic characteristics of noroviruses[90], there

242   are new opportunities to advance understanding of this common and important human pathogen.

243

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