

## **Molecular screening of blue mussels indicated high mid-summer prevalence of human genogroup II Noroviruses, including the pandemic “GII.4 2012” variants in UK coastal waters during 2013**

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### **ABSTRACT**

This molecular study is the first report, to the best of our knowledge, on identification of norovirus, NoV GII.4 Sydney 2012 variants, from blue mussels collected from UK coastal waters. Blue mussels (three pooled samples from twelve mussels) collected during the 2013 summer months from UK coastal sites were screened by RT-PCR assays. PCR products of *RdRP* gene for noroviruses were purified, sequenced and subjected to phylogenetic analysis. All the samples tested positive for NoVs. Sequencing revealed that the NoV partial *RdRP* gene sequences from two pooled samples clustered with the pandemic “GII.4 Sydney variants” whilst the other pooled sample clustered with the NoV GII.2 variants. This molecular study indicated mussel contamination with pathogenic NoVs even during mid-summer in UK coastal waters which posed potential risk of NoV outbreaks irrespective of season. As the detection of Sydney 2012 NoV from our preliminary study of natural coastal mussels interestingly corroborated with NoV outbreaks in nearby areas during the same period, it emphasizes the importance of environmental surveillance work for forecast of high risk zones of NoV outbreaks.

## Introduction

Blue mussels (*Mytilus* spp) are filter-feeders and are widespread in European coastal waters, which are often in close proximity to urban sewage treatment units.<sup>1, 2</sup> Filter-feeders may retain particles at 4 µm with 100% efficiency.<sup>3</sup> Faecal contamination of water (via sewage discharge) poses a serious threat to human health as bivalves (mussels and oysters) which filter large volumes of water through their gills as part of their feeding activities, bio-accumulate pathogenic microbes, including enteric bacteria and viruses. *In situ* studies with bio-accumulation of a virus indicator in oysters have shown that they can effectively concentrate viruses (up to 99-fold) compared to the surrounding water.<sup>4</sup> An adult blue mussel may filter ~ 72 litres of water on an average per day<sup>5</sup> and have been proposed as bio-samplers for assessment of faecal contamination in recreational waters.<sup>6</sup> Consumption of bivalves as food is often reported to be the cause of disease outbreaks by enteric viruses. Though primary infection results from ingestion of faecal contaminated food or water, unintentional ingestion of contaminated recreational waters can also lead to gastrointestinal illness.<sup>7</sup> Norovirus outbreaks have a distinct seasonality linked to winter months.<sup>8, 9</sup> NoV infections are called “gastric flu” for its similar seasonality & lack of effective therapeutics like influenza viruses, and also for its high infectivity and rapid evolution.<sup>10, 11</sup>

Noroviruses (NoVs) are a non-enveloped positive sense single-stranded RNA virus of the *Caliciviridae* family. NoVs have a significant impact on human health as they are highly infectious and cause acute gastroenteritis in all age groups accounting for >200,000 deaths worldwide each year, especially in children.<sup>12</sup> The organism is highly contagious and problematic as a hospital-acquired infection. According to WHO, <10 virions are sufficient to cause infection in adults and NoV outbreaks often lead to closure of entire hospital wards every year affecting both patients and staff. Despite attempts to control via ‘deep cleaning’ and implementing hygiene measures outbreaks can cause considerable inconvenience and

economic losses.<sup>13-15</sup> NoVs cannot be cultured on cells and detection and diagnosis increasingly relies solely on molecular methods such as reverse transcriptase-PCR (RT-PCR).<sup>16</sup> Diversity of NoVs both genetically and antigenically were demonstrated through RT-PCR and genomic sequencing.<sup>17</sup> NoVs are divided into six genogroups (GI-GVI) and genogroups are further subdivided into around forty genetic clusters or genotypes.<sup>18</sup> NoV genogroups which infect humans are I (NoV GI), II (NoV GII) and rarely IV (NoV GIV). NoV GII.4 is responsible for 80% of the disease outbreaks<sup>19</sup> and is currently the most virulent strain circulating in the UK.<sup>20</sup> Since it has a faster mutation rate than other NoVs<sup>21</sup> new variants emerge every 2-3 years and is also responsible for 60–80% of outbreaks worldwide.<sup>22</sup> The GII.4 variant named Sydney 2012 has progressively replaced the predecessor GII.4 New Orleans 2009 variant globally.<sup>23, 24</sup> GII.4 outbreaks occurred preferentially during winter months<sup>25</sup> and most works have been conducted with samples collected during winter months.<sup>26</sup> Thus the aim of the present study was to screen for NoV contamination/retention in bivalves during the summer months in the UK coastal seawaters. This work contributes to the development of a working methodology for the routine surveillance of mussels round the year for identification and genetic characterization of enteric pathogenic viruses accumulated within them.

## **Materials and Methods**

### **Collection and Processing of Samples**

Eight wild blue mussels (numbered 1-8) were collected in April 2013; they were attached to a nylon rope fragment on the metal supporting girders under Mumbles Pier at The Mumbles, Swansea, UK. Another four mussels (numbered 9-12) were obtained in June, 2013 from the Royal Dock at Grimsby, UK. Hepatopancreas, gills and gastrointestinal tissues were dissected out from these mussels. Tissues (~3.0 g) from mussels 1-4, 5-8 and 9-12 constituted

the three pooled samples 1, 2 and 3 respectively. Mussel tissues were triturated; digested with proteinase K and centrifuged at 3000g to collect ~5 ml supernatant.<sup>27</sup> The supernatant was filtered to remove tissue and other debris including unwanted microbes (like bacteria and fungi) using 0.45 µM filter (Anachem-Supatop, UK) for further downstream applications.

### **Viral RNA Extraction, Reverse Transcription and Nested PCR**

Following processing of the samples, viral RNA was extracted using QIAamp Viral RNA Mini kit (Qiagen, Germany) according to the instructions of the manufacturer. Extracted RNA (in 60µl water) was used for reverse transcription to cDNA using BioScript RT-PCR Kit (Bioline, UK) and NoV-specific reverse primer, 1422.<sup>28</sup>

The cDNA was amplified using NoV GI-specific (PCR nos. 443, 446) and GII-specific (PCR nos. 443, 444) semi-nested PCRs (Expand High Fidelity PCR kit, Roche, Germany) according to previously published protocols.<sup>29</sup> These PCRs target part of the NoV RNA polymerase, *RdRP* gene. The PCR numbering has been adopted from the numbering of the PCR protocols as previously published.<sup>28</sup>

The target amplicon sizes were 327 bp for the first round PCR (no. 443) for both NoV GI and GII; 188 bp for the second round PCR in case of NoV GI and 237 bp for NoVGII. Positive PCR-amplified products (electrophoresed on 1.5% agarose gel) were purified and custom-sequenced for both strands.

The sequencing primers were the same forward and reverse primers of the respective PCRs that resulted in visible bands on the gel (e.g. primers for PCR no. 446 for GI NoV or 444 for GII NoV).

Armoured RNA (Cells-to-cDNA™ II Kit, Ambion, UK) was used as positive control by spiking extracted RNA from mussels with the armoured RNA to test for RT and PCR inhibitors in the mussel RNA concentrate.

Samples 1-3 were further screened for NoV GI and NoV GII capsid genes using nested PCRs (PCR nos. 475, 476) and (PCR nos. 437, 438) respectively and also for HAV (PCR nos. 675, 676) following previously published protocols.<sup>28</sup>

All three samples were also screened for NoV GIV (PCR nos. 603, 612; 546, 542) and COG4F, G4SKR and G4SKF.<sup>28, 30</sup> For NoV GIV detection by PCR nos. 603 and 612, the reverse primer 1565 used for cDNA synthesis had the sequence as described earlier<sup>31</sup> and not as described in La Rosa et al.<sup>28</sup> The latter sequence appears to be incorrect. Samples 2 and 3 were further tested for HEV (PCR nos. 711, 712) and astrovirus (PCR nos. 696, 697).

## **Sequencing**

Nucleotide (nt) sequences, confirmed by bi-directional sequencing of the PCR products (PCRs 446, 444) were subject to NCBI BLAST for determining genetic matches with sequences available in the database. They were then aligned using Clustal W with other closely related NoV sequences identified from the BLAST search. A phylogenetic tree was generated by the neighbour-joining method using MEGA 6<sup>32</sup> on 169 nt of *RdRP* gene (pertaining to nt positions 4326-4494 as in JX459908 NoV GII.4 Sydney variant) to characterize the NoV strains and study their genetic distances with other closely related NoV strains.

## **Results**

### **Sequencing of NoVs**

All three samples were positive for NoV GII-specific PCR (no. 444) (Fig. 1a, b). Samples 2 and 3 were also positive for NoV GI-specific PCR (no. 446) (Fig.1b, sample 2 is shown) and produced PCR products of expected band length (188 bp). All samples were negative for GIV NoV. Samples screened for other enteric viruses were also negative (within the limits of

detection of the PCRs performed) except a PCR product of expected size (PCR no. 712; 457 bp) observed for HEV in case of sample 3. However, sequencing pooled product from three such bands resulted in non-HEV sequences as also in case of non-NoV sequences observed for the 188 bp band from the NoV GI-specific PCR (data not shown). GI & GII NoV PCRs targeting NoV capsid genes (PCR nos. 476, 438) were negative.

### **Relationships with other NoVs**

Phylogenetic analysis of aligned sequences revealed that samples 1 and 2 from Swansea clustered closely with several pandemic NoV GII.4 2012 variants. Both samples, however, had differences at nucleotide (nt) sequence levels. NoV from sample 1 was named GII.4 UK 2013a and that from sample 2 was named GII.4 UK 2013b. The GII.4 Sydney variants of NoVs from mussels in the present study clustered with NoVs of Chinese and Taiwanese origin (Fig. 1).

The sample 3 from Grimsby clustered closely with other NoV GII.2 strains and this NoV was named GII.2 UK 2013 (Fig. 2). NoV GI-specific PCR products from samples 2 and 3 produced non-NoV sequences which could not be matched with any known sequence using BLAST.

Though both samples contained NoV GII.4, the predominant strains as preferentially amplified by PCRs were different as evident from nucleotide differences in their *RdRP* gene sequences and reflected in phylogenetic clustering (Fig. 2). In the tree, prototype sequence from a NoV GII obtained from GenBank (accession number X86557) and an outgroup sequence from a NoV GI (GQ845370) were included. Prototype for NoV GII.4 included in the tree was the Sydney 2012 variant (accession number JX459907) which interestingly caused high incidence of NoV outbreaks in the UK during 2012. Both the NoV GII.4 variants from Swansea grouped closely with the 2012 NoV GII.4 human strains, including the

“Sydney 2012” variants as detected earlier.<sup>24</sup> The most interesting finding of this study is the fact that NovGII.4 Sydney variants were found even in a pilot scale of samples which advocates for the high prevalence of these pathogenic genotypes in the mussels of UK coastal waters.

**Figure. 1.**

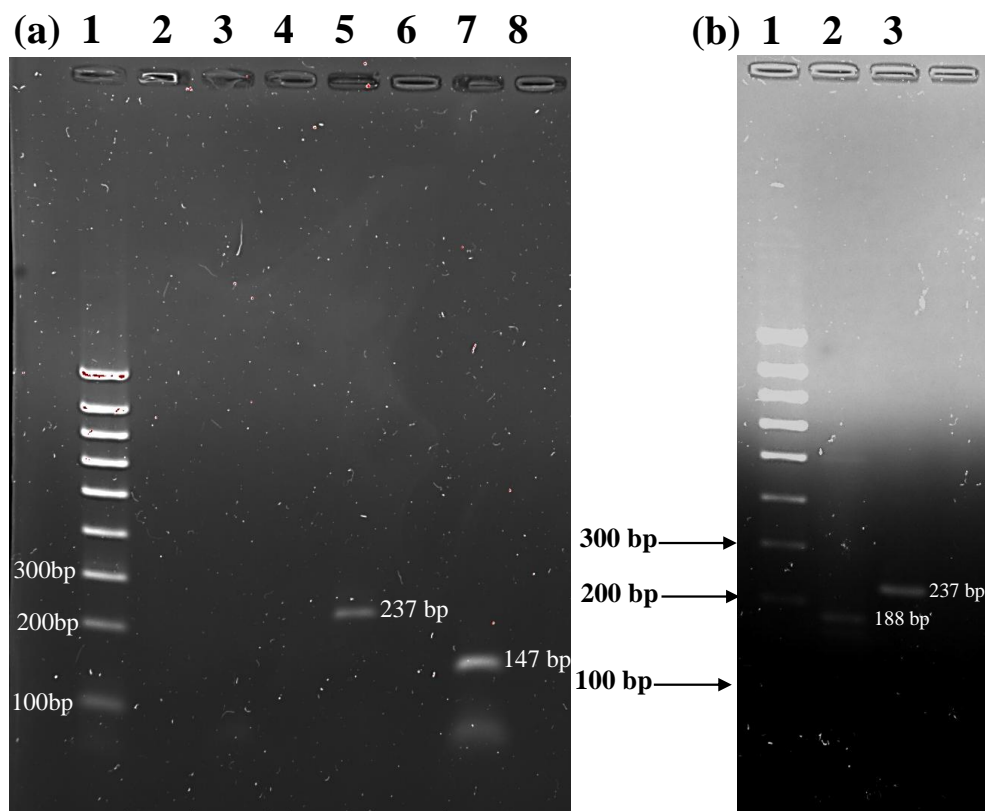


Fig. 1: Representative gel electrophoresis gels, for NoV GI (PCR 446) and NoV GII (PCR444) from mussel samples 1 and 2.

a) Mussel sample 1 PCR Lane 1: Hyperladder IV (Bioline, UK); Lane 3: Negative control for NoV GII-specific PCR (PCR nos. 443, 444; nuclease-free water as template for both PCRs); Product for PCR no. 444 was run on gel, Lane 5: NoV GII PCR product (237bp); Lane 7: Positive control for PCR inhibitors in mussels (147bp).

b) Mussel sample 2 PCR Lane 1: Hyperladder IV; Lane 2: NoV GI PCR product (188bp); Lane 3: NoV GII PCR product (237bp).

**Figure. 2.**

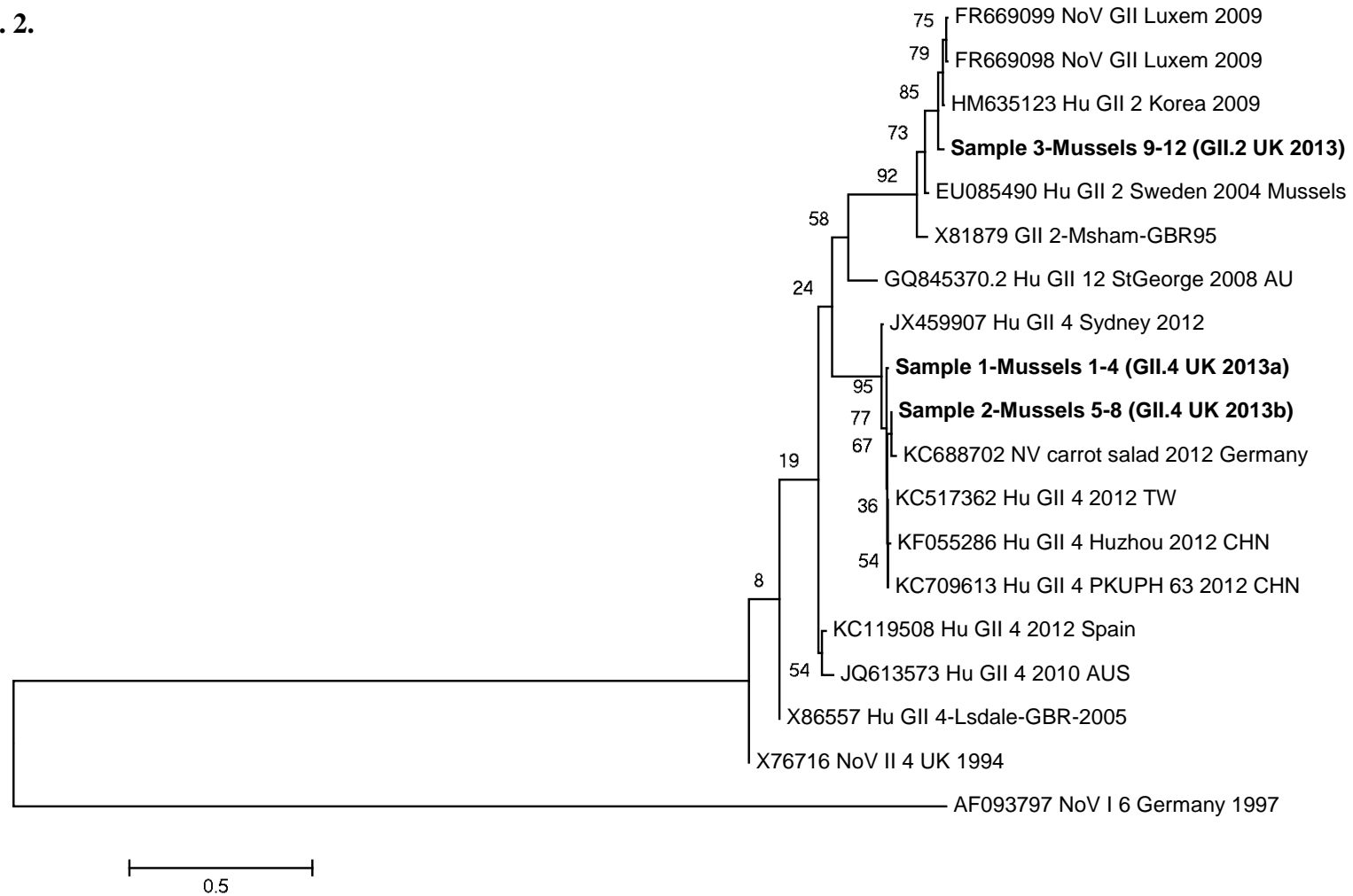


Fig. 2: Phylogenetic analysis of the norovirus RNA-dependent RNA polymerase gene (partial) of several NoV genogroups and genotypes. NV sequences, retrieved from Genbank for comparing with our NoV strains in the mussels are identified by their Genbank accession numbers on the figure. The scale bar indicates genetic distance in terms of nucleotide substitution/site/year.



## Discussion

NoVs are most common cause of outbreaks of food-borne illnesses and pose serious health risks to humans. In the present small-scale study, mussel samples obtained from seawater near Swansea were found to contain two GII.4 variants. In February 2012, four wards were closed in a nearby public hospital due to NoV outbreak (<http://www.bbc.com/news/uk-wales-south-west-wales-17110220> accessed on 14.10.2015). However, if the GII.4 virus strains identified in the mussels were genetically identical to the NoV strains responsible for the Swansea outbreaks, it would demonstrate that these viruses probably survive the sewage treatment process and concentrate in mussels. This could not be analysed in the present study as no sequence data on the outbreak of NoV strains from Swansea in 2012 were available in the public domain. In this context, it is worth mentioning that the most commonly detected GII.4 strain in the healthcare settings in UK in the season (2013-2015) was Sydney 2012 (PHE Monthly National Norovirus and Rotavirus Report, 14 April, 2016.<sup>33</sup> NoV capsid PCRs were negative which could have resulted from primer mismatch as capsid proteins are exposed to higher level of immune pressure and therefore, immune selection compared to more conserved non-structural proteins like virus RNA-dependent RNA polymerase. Consequently, the capsid genes are more prone to higher rates of mutation. As humans are mostly infected by the GII strains, the present work indicates the need for a larger study of surveillance of enteric viruses (e.g. NoV, HAV virus and HEV, rotaviruses and others) that contaminate coastal waters of the UK and become bio-accumulated in mussels, clams and oysters<sup>34</sup> confirming their role as a food chain source of infection.

NoV GI and GII have been detected in oyster samples harvested from bays and estuaries worldwide<sup>35, 36</sup> and also from human samples.<sup>25</sup> However, this is the first report, to the best of our knowledge, on NoV GII.4 Sydney 2012 variant detected in blue mussels collected from UK coastal waters. It suggests that choosing noroviruses as a parameter would be

worthy enough if viral testing of mussels is introduced on a routine basis. It was also interesting to note that norovirus is highly detectable even in a small scale sampling of mussels during summer months, suggesting the possible prevalence of pathogenic NoV strains contaminating UK coastal waters throughout the year.

It had been reported earlier that NoV outbreaks are more likely to occur during winter<sup>4</sup> as cold water temperatures and reduced ultraviolet light increase their survival.<sup>37</sup> The present work detected NoVs to a great extent even in summer months, so it may be proposed that mussels act as reservoirs to retain and shelter the viruses round the year so that they can contribute to the flux of viable NoVs contamination of sea waters during winter and potentiate greater spread to cause severe outbreaks of NoV infections during the winter months.

One limitation of the present work was that the sample size was not large but still comparable to that reported earlier.<sup>28</sup> Despite pilot level sample size, the interesting and important observation was that different pathogenic NOV GII, including GII.4 Sydney variants (as evident from sequence variations in the *RdRp* gene) could be detected from potentially all the blue mussels collected. This suggests substantial environmental contamination which perhaps explains why Sydney 2012 variant was isolated from most UK outbreaks during 2013-14.

One possible explanation for the clustering of the GII.4 Sydney variants of NoVs from mussels with NoVs of Chinese and Taiwanese origin could be that the mussel collection sites in the present study were places where year-round maritime activities take place and coastal water contamination from ships and vessels from faraway places could not be ruled out.

Surveillance and control of NoV outbreaks related to mussel and other shellfish consumption is therefore crucial to prevent spread of infection in the population. We believe that this work will be an important addendum to the ongoing research in NoV epidemiology in the UK.<sup>38-40</sup>

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