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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

Subhajit Biswas[*,1], *Philippa Jackson*, *Rebecca Shannon*, *Katherine Dulwich*, *Soumi Sukla*[1], *Ronald A. Dixon*

University of Lincoln, School of Life Sciences, Brayford Pool, Lincoln, Lincolnshire, United Kingdom

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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.[1,2] Filter-feeders may retain particles at 4 μm with 100% efficiency.[3] 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

* *Corresponding author.*
E-mail: subhajit.biswas@iicb.res.in (S. Biswas).
[1] Current address: CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata, West Bengal 700032, India.

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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.[4] An adult blue mussel may filter ~72 L of water on an average per day[5] and have been proposed as bio-samplers for assessment of faecal contamination in recreational waters.[6] 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.[7] Norovirus outbreaks have a distinct seasonality linked to winter months.[8,9] 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.[10,11]

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.[12] 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.[13–15] NoVs cannot be cultured on cells and detection and diagnosis increasingly relies solely on molecular methods such as reverse transcriptase-PCR (RT-PCR).[16] Diversity of NoVs both genetically and antigenically were demonstrated through RT-PCR and genomic sequencing.[17] NoVs are divided into six genogroups (GI-GVI) and genogroups are further subdivided into around forty genetic clusters or genotypes.[18] 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[19] and is currently the most virulent strain circulating in the UK.[20] Since it has a faster mutation rate than other NoVs[21] new variants emerge every 2–3 years and is also responsible for 60–80% of outbreaks worldwide.[22] The GII.4 variant named Sydney 2012 has progressively replaced the predecessor GII.4 New Orleans 2009 variant globally.[23,24]

GII.4 outbreaks occurred preferentially during winter months[25] and most works have been conducted with samples collected during winter months.[26] 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 3000 × *g* to collect ~5 mL supernatant.[27] 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 60 μL water) was used for reverse transcription to cDNA using BioScript RT-PCR Kit (Bioline, UK) and NoV-specific reverse primer, 1422.[28]

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.[29] 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.[28]

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.[28]

All three samples were also screened for NoV GIV (PCR Nos. 603, 612; 546, 542) and COG4F, G4SKR and G4SKF.[28,30] For NoV GIV detection by PCR nos. 603 and 612, the reverse primer 1565 used for cDNA synthesis had the sequence as described earlier[31] and not as described in La Rosa et al.[28] 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

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