# Digital PCR for Quantifying Norovirus in Oysters Implicated in Outbreaks, France

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Using samples from oysters clearly implicated in human disease, we quantified norovirus levels by using digital PCR. Concentrations varied from 43 to 1,170 RNA copies/oyster. The analysis of frozen samples from the production area showed the presence of norovirus 2 weeks before consumption.

Shellfish have a long history as vectors of human enteric viruses; this relationship is particularly apparent with oysters and norovirus (*1*,*2*). Specific norovirus ligands found in oyster tissues facilitate the persistence of viral particles for several weeks, resistance to depuration, and strain selectivity by the oyster (*1*,*3*). Although advances have been made in virus detection in shellfish, quantification of norovirus in oysters associated with outbreaks still presents a challenge. More accurate quantification is essential for risk analysis and to understand the exact role played by shellfish in norovirus transmission, as this will be important to support the implementation of norovirus regulations.

Norovirus reference materials are essential for quantification by real-time PCR, but they are not widely available for inclusion in standard curves; however, this limitation may be overcome by using digital PCR (dPCR) (*4*). This technology is based on partitioning of the sample into thousands of individual PCRs that contain, in theory, 1 or no copies of the nucleic acid target. After amplification, the total number of target molecules is calculated, with no need for external reference standards (*4*). The partitioning of samples into large numbers of subsamples may also decrease the impact of enzyme inhibitors possibly linked to matrix-type components. This partitioning may be particularly advantageous for the detection of viruses in food and environmental samples, which tend to be complex, with a large variety of inhibitory compounds but relatively low numbers of viruses (*4*,*5*). Norovirus-specific primers and probes targeting the open reading frame 1–2 region used for the real-time reverse transcription PCR were used in a microfluidic-based dPCR to enable norovirus quantification in oyster samples associated with outbreaks.

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DOI: http://dx.doi.org/10.3201/eid2212.160841

## The Study

In France, medical doctors who diagnose norovirus gastroenteritis in ≥2 persons who shared a common meal are required to declare a suspected foodborne illness outbreak. All meal participants then receive a standardized questionnaire that addresses the foods consumed, the symptoms, and the timing of illness, allowing the calculation of the relative risk and its 95% CI. Information on outbreaks to laboratories must be transmitted quickly to enable collection of samples that are directly linked to the clinical cases.

Eight outbreaks were considered for this study on the basis of the following criteria: clinical diagnosis of norovirus in sick consumers; epidemiologic confirmation that oysters were implicated; and rapid notification of responsible oyster production areas. The outbreaks occurred during the winter months in private houses except for 1 that occurred in a nursing home (outbreak 8) (Table). The attack rate varied from 43% to 100%, with median incubation times between 0.5 and 2 days. Fecal samples available for 2 outbreaks (1 for outbreak 6 and 3 for outbreak 8) confirmed the presence of norovirus (National Reference Center for Enteric Viruses, Dijon, France, pers. comm.). Eight shellfish samples were collected from batches that were directly implicated, and 1 sample was taken from leftovers in the nursing home's refrigerator, increasing the likelihood that the samples were representative of consumed oysters. An additional 16 samples were collected from implicated production areas located along different coasts of France, including frozen samples (during the winter and spring months, Ifremer laboratories doing official control monitoring of shellfish for *Escherichia coli* routinely freeze leftover samples). Viruses were eluted from oyster digestive tissues by using the reference method (*6*), and then quantified using the QuantStudio 3D Digital PCR system (Thermo Fisher, Villebon, France) (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/12/16-0841-Techapp1.pdf).

No norovirus was detected in 1 oyster sample; norovirus genogroups GI, GII, or both were detected in 9, 11, and 4 samples, respectively (Table). Overall, norovirus concentrations ranged from 43 to 1,170 RNA copies/oyster; the

**Table.** Characteristics of outbreaks of norovirus infection associated with consumption of oysters, analyzed oyster samples, and virus concentrations obtained by digital PCR*

| | Epidemiology information | | | | Samples analyzed | | Viral RNA copies/oyster | |
|---|---|---|---|---|---|---|---|---|
| Outbreak no. | Date of consumption | No. sick/ no. exposed | Days to illness onset† | Sample no. | Days to sampling‡ | Sample location§ | Genogroup GI | Genogroup GII |
| 1 | 2014 Feb 23 | 7/16 | 0.5 | 3486 | 4 | Same batch | ND | $1.09 \times 10^2$ |
| | | | | 3488 | 7 | Prod area | ND | ND |
| | | | | 3489 | 7 | Prod area | ND | $2.96 \times 10^2$ |
| 2 | 2014 Feb 27 | 3/4 | 2 | 3498 | 5 | Same batch | ND | $3.72 \times 10^2$ |
| 3 | 2014 Mar 16 | 4/4 | 1 | 3519 | 2 | Prod area | ND | $2.02 \times 10^2$ |
| | | | | 3517 | 5 | Prod area | ND | $6.81 \times 10^2$ |
| | | | | 3518 | 2 | Prod area | $3.80 \times 10^2$ | $6.15 \times 10^2$ |
| | | | | 3531 | 15 | Prod area | $1.08 \times 10^3$ | ND |
| | | | | 3532 | 15 | Prod area | $3.88 \times 10^2$ | ND |
| 4 | 2014 Dec 12 | 3/3 | No data | 3703 | 6 | Same batch | $1.18 \times 10^2$ | ND |
| | | | | 3694 | –4 | Prod area | ND | $1.70 \times 10^2$ |
| 5 | 2014 Dec 14 | 2/2 | 1.5 | 3704 | 3 | Same batch | ND | $1.21 \times 10^2$ |
| | | | | 3705 | 3 | Same batch | $2.74 \times 10^2$ | 44.1 |
| | | | | 3695 | –6 | Prod area | ND | 43.2 |
| | | | | 3698 | –6 | Prod area | $1.18 \times 10^2$ | ND |
| | | | | 3700 | –6 | Prod area | $1.1 \times 10^3$ | $9.50 \times 10^2$ |
| 6 | 2014 Dec 27 | 3/6 | 0.5 | 3733 | 6 | Same batch | ND | $1.26 \times 10^2$ |
| 7 | 2015 Jan 9 | 3/4 | 1.5 | 3740 | 3 | Same batch | $9.20 \times 10^2$ | ND |
| | | | | 3738 | –3 | Prod area | $1.17 \times 10^3$ | ND |
| | | | | 3739 | –3 | Prod area | $6.38 \times 10^2$ | 53.4 |
| 8 | 2015 Mar 29 | 16/36 | 2 | 3816 | 3 | Consumed | ND | 82.1 |
| | | | | 3817 | 3 | Same batch | $1.85 \times 10^2$ | ND |
| | | | | 3791 | –19 | Prod area | ND | $1.87 \times 10^2$ |
| | | | | 3792 | –19 | Prod area | $8.28 \times 10^2$ | ND |
| | | | | 3822 | 10 | Prod area | $1.28 \times 10^2$ | ND |

*ND, not detected; Prod, production.
†Median days to onset of vomiting or diarrhea.
‡Days from date of consumption to sample collection.
§Same batch = samples from batch of oysters consumed.

highest concentrations detected were GI. For outbreak 8, in which a leftover sample from the implicated meal was obtained, norovirus GII was detected at a concentration of 82 RNA copies/oyster, whereas norovirus GI was detected at a concentration of 185 RNA copies/oyster in the same batch collected from the oyster farm.

In a previous dose–response model for norovirus GI and GII based on outbreak investigations, differences were observed between consumers with the secretor phenotype, for which infection and disease probability were high at low doses compared with nonsecretor phenotypes (*7*). Although method sensitivity may need to be improved, the concentrations reported here are consistent with observed illness in dose–response studies to date (*8*). Norovirus GI and GII were detected in oyster samples from the production area and in 4 fecal samples (National Reference Center for Enteric Viruses, pers. comm.).

Because oyster contamination occurs through the filtration of seawater contaminated by human sewage, many contamination events involving both norovirus genogroups and different strains have been described worldwide; this study provides additional evidence of the diversity of contamination (*1*). In contrast to person-to-person transmission in which GII strains dominate, oysters favor the transmission of some specific GI strains, a major consideration for the global epidemiology of norovirus (*1*,*3*). Thus, identifying if oysters implicated in outbreaks are contaminated with norovirus GI or GII is important, because genetic susceptibility means that some consumers do not become infected with certain GI or GII strains; this affects the disease and favors the distribution of some norovirus strains. Such a comprehensive approach will provide information for risk analysis and assist in understanding norovirus infections (*7*,*9*).

Although we obtained some norovirus sequences from 6 implicated batches, confirming the specificity of the dPCR, we believe that the development of technology such as next-generation sequencing will provide more detailed information on the full range of strains present in samples. Obtaining more accurate information on strain diversity and quantification will be valuable for molecular epidemiology studies and management.

In France, oysters are a popular dish, especially during December–April, when they are in the optimal low-fat condition for consumption. They are opened just before consumption and eaten raw; intravalvular seawater is tipped out, thus eliminating food handler contamination. Because this is the highest period for potential contamination by norovirus, samples are kept frozen by laboratories in France for analysis in case of outbreaks. In the current case, this was useful because it demonstrated the presence of norovirus up to 19 days before the shellfish were marketed. This detection in samples collected 2 weeks before an outbreak suggests that illness could have been prevented. Control shellfish samples from different production areas were analyzed at the same time and were negative for norovirus (data not shown), correlating well with the estimated NoV prevalence of less than 10% in France (*10*).

## Conclusions

This study demonstrates that outbreaks could be prevented by performing shellfish analysis at times of the year at which norovirus risk is elevated, such as the winter season, and following microbial alert events such as sewage overflows and heavy rainfall. Application of dPCR to shellfish implicated in outbreaks will provide accurate quantification, which is useful for further risk analysis studies. This application will help to improve regulations and enhance the safety of products on the market, keeping in mind that the sanitary quality of coastal areas is of primary concern.

### Acknowledgments

We acknowledge the help of Laboratoire Environnement Ressource and Direction Departementale pour la Protection des Populations colleagues for sampling, and colleagues from the National Reference Center for Enteric Viruses, Dijon, France, for sharing the fecal test results. We are grateful to Mathias Bruyand (French National Public Health Agency) for critical review of the manuscript.

This study was supported by the Food Standards Agency (NovProtOy, FS101068), and by Ifremer (Direction Scientifique) through financial support for DP, by Direction Générale de l'Alimentation (French government DGAL, convention LNR), and by EU-H2020 grant no. 643476 Compare.

Dr. Polo is a postdoctoral researcher at the IFREMER Mmicrobiology laboratory. His interests include the study of shellfish-borne norovirus transmission, surveillance, and the development of new prevention strategies.

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Article DOI: http://dx.doi.org/10.3201/eid2212.160841

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## Technical Appendix

### Oyster Sample Analysis

Oyster samples were sent to the laboratory by the veterinary service for the leftover sample and the 8 batch samples, and by Ifremer technicians for the 16 samples collected from the implicated production areas. Oysters, maintained at 4°C during shipment (or frozen for the 8 samples collected before outbreaks occurred), were washed, shucked, and immediately dissected to recover the digestive tissues. Viruses were eluted from 2 g of digestive tissues by using the proteinase K protocol and nucleic acids (NAs) were extracted by using the NucliSens kit (bioMérieux, Lyon, France) (*1*). Extraction efficiency was verified using with Mengovirus added to the tissue as specified in the reference method.

### Amplification Conditions

Mengovirus amplification was performed using primers, probe, and cycling conditions described in the reference method (*1*), and the extraction efficiency was calculated for each sample. As specified in the reference method, only samples with an extraction efficiency above 1% were considered acceptable.

For norovirus detection, primers and probes targeting the open reading frame 1–2 region were used. The sequences were as follows: For GI, QNIF4 (FW) (5′-CGC TGG ATG CGN TTC CAT-3′ with N: A/C/G/T), NV1LCR (REV) (5′-CCT TAG ACG CCA TCA TCA TTT AC-3′) and the probe NVGG1p (5′-TGG ACA GGA GAY CGC RAT CT-3′ with Y: C/T and R: A/G). For GII, QNIF2 (FW) (5′- ATG TTC AGR TGG ATG AGR TTC TCW GA-3′ with R: A/G and W: A/T), COG2R (REV) (5′-TCG ACG CCA TCT TCA TTC ACA-3′), and the probe QNIFs (5′-AGC ACG TGG GAG GGC GAT CG-3′). Probes were labeled with ZEN-Iowa BlackFQ double-quenched (*2*).

cDNA was obtained using the SuperScript III Reverse Transcription System (Invitrogen Thermo Fisher, Villebon, France). Reverse transcription conditions were 30 min at 55°C and 15 min at 70°C. Five µL of cDNA was then amplified with the QuantStudio 3D Digital PCR Master Mix (Invitrogen Thermo Fisher), using the primers and probes at the concentrations recommended for the real-time reverse transcription PCR (rRT-PCR) (ISO/DIS 15216). Samples were loaded onto the QuantStudio 3D Digital PCR 20K chips with 20,000 partitions of 865 pL each, and then loaded into the QuantStudio 3D Digital PCR system (Thermo Fisher, France). After 10 min at 96°C, 45 amplification cycles were performed.

#### Quantification

Following amplification, endpoint fluorescence of each partition was analyzed with the QuantStudio 3D AnalysisSuite Cloud Software (version 3.0.3; Invitrogen Thermo Fisher) to statistically estimate the number of copies of target DNA. Final quantification data were provided by the software through counting the number of positive chambers (H) out of the total number of chambers (C) per chip. Then, the Poisson distribution was used to estimate the average number of molecules per partition (λ), so $\lambda = -\ln(1 - H/C)$. A no-template control consisting of water instead of NA extract was included in each run. The final result is expressed as cDNA copies per microliter.

#### Concentration per Oyster

The concentration per oyster was back-calculated using an efficiency of 100% for cDNA production (checked by real-time RT-PCR, data not shown), and the volume of NA extract analyzed. First, the NoV concentration was calculated per g of DT, and then per oyster based on the total weight of one animal.

#### Typing

Positive samples that were collected from batches implicated in the outbreaks were sequenced (except three samples due to lack of material). NA extracts were amplified by the standard RT-PCR method with the same reverse transcription  and platinum *Taq* polymeraseenzymes. Primers targeting the polymerase and the capsid regions were used in a 2-step semi-nested format, and 40 cycles of amplification were performed (using dedicated rooms and all precautions to avoid cross-contamination). Amplicons from positive samples were purified and sequenced directly (*3*).

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**Technical Appendix Table.** Norovirus typing in samples collected in some batches implicated in outbreaks, France*

| Sample | NoV GI | NoV GII |
|---|---|---|
| 3498 | | GII.6 (cap) |
| 3704 | | GII.17 (pol) |
| 3705 | | GII.3 (cap) |
| 3733 | | GII.4 Syd (cap) |
| 3740 | GI.5 (cap) | |
| 3817 | GI.4 (cap) | |

*NoV sequence was confirmed after amplification of a fragment targeting the polymerase (pol) or capsid (cap) region.