



Use of F-Specific RNA Bacteriophage to Estimate Infectious Norovirus Levels in Oysters

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Abstract

Contamination of bivalve shellfish, particularly oysters, with norovirus is recognised as a significant food safety risk. Methods for quantification of norovirus in oysters using the quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) are well established, and various studies using RT-qPCR have detected norovirus in a considerable proportion of oyster samples, both in the UK and elsewhere. However, RT-qPCR detects viral genome, and by its nature is unable to discriminate between positive results caused by infectious viruses and those caused by non-infectious remnants including damaged virus particles and naked RNA. As a result, a number of alternative or complementary approaches to RT-qPCR testing have been proposed, including the use of infectious viral indicator organisms, most frequently F-specific RNA bacteriophage (F-RNA phage). In this study, we investigated the relationships between F-RNA phage and norovirus in digestive tissues from two sets of oyster samples, one randomly collected at retail (630 samples), and one linked to suspected norovirus illness outbreaks (nine samples). A positive association and correlation between PCR-detectable levels of genogroup II F-RNA bacteriophage (associated with human faecal contamination) and norovirus was found in both sets of samples, with more samples positive for genogroup II phage, at generally higher levels than norovirus. Levels of both viruses were higher in outbreak-related than retail samples. Infectious F-RNA phage was detected in 47.8% of all retail samples, and for a subset of 224 samples where characterisation of phage was carried out, infectious GII phage was detected in 30.4%. Infectious GII phage was detected in all outbreak-related samples. Determination of infectivity ratios by comparing levels of PCR-detectable (copies/g) and infectious GII phage (pfu/g) revealed that in the majority of cases less than 10% of virus detected by RT-qPCR was infectious. Application of these ratios to estimate infectious norovirus levels indicated that while 77.8% of outbreak-related samples contained > 5 estimated infectious norovirus/g, only 13.7% of retail samples did. Use of a combination of levels of PCR-detectable norovirus and infectious F-RNA phage showed that while only 7.0% of retail samples contained both > 100 copies/g norovirus and > 10 pfu/g F-RNA phage, these combined levels were present in 77.8% of outbreak-related samples, and 75.9% of retail samples with > 5 estimated infectious norovirus/g. We therefore suggest that combining RT-qPCR testing with a test for infectious F-RNA phage has the potential to better estimate health risks, and to better predict the presence of infectious norovirus than RT-qPCR testing alone.

Keywords Norovirus · Infectivity · F-specific RNA bacteriophage · Oysters · qRT-PCR

Introduction

Contamination of bivalve shellfish, particularly oysters, with norovirus is recognised as a food safety risk, with a considerable number of reports of outbreaks in literature (reviewed in Bellou et al. 2013).

Methods for quantification of norovirus in oysters using the quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) are well established, and an International Standard method using this technology has been

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published in 2013 (Anonymous 2013) with an updated version released in 2017 (Anonymous 2017). Various studies using RT-qPCR have detected norovirus in a considerable proportion (37.1–76.2%) of oyster samples, both in the UK (Lowther et al. 2012b, 2018) and elsewhere (Flannery et al. 2009; Polo et al. 2015; Suffredini et al. 2014).

However, RT-qPCR detects the viral genome, and by its nature is therefore unable to discriminate between positive results caused by infectious viruses and those caused by non-infectious remnants including damaged virus particles and potentially naked RNA (reviewed in Knight et al. 2013). Studies in our laboratory have shown both that elevated norovirus levels as detected by RT-qPCR are associated with increased human health risks (Lowther et al. 2010, 2012a) and also that naked RNA does not accumulate well in oyster tissues (Dancer et al. 2010). However, the possibility for RT-qPCR testing to overestimate the risks of norovirus in shellfish remains. Despite the high prevalence of norovirus RNA in tested samples, various studies have estimated the norovirus illness incidence per serving of shellfish at < 1% (Lowther et al. 2010; Hassard et al. 2017). If RT-qPCR testing is adopted into risk management measures without due consideration of this issue, overestimation of risk could cause unnecessary restrictions on shellfish producers without commensurate public health gains. As a result, a number of alternative or complementary approaches to RT-qPCR testing have been proposed. Direct culture methods for norovirus have been developed recently (Ettayebi et al. 2016; Jones et al. 2014), although, given their cost and complexity, seem unlikely to be suitable for routine food testing. As an alternative proxy, a number of methods have been developed that combine RT-qPCR with a pre-treatment step to enable discrimination between viruses with intact capsids and viruses without intact capsids (and which are therefore presumed to be non-infectious), or naked RNA (Dancho et al. 2012; Langlet et al. 2015; Nuanualsuwan and Cliver 2002; Randazzo et al. 2018).

Alternatively, the use of a viable viral indicator organism, most frequently F-specific RNA bacteriophage (F-RNA phage), has been proposed (Doré et al. 2000; Flannery et al. 2009; Hartard et al. 2016). In the United States, a threshold criterion for levels of F-RNA phage in shellfish flesh in production areas recovering from untreated sewage discharges is included in the National Shellfish Sanitation Program (Anonymous 2015). F-RNA phage are positive-sense, single-stranded RNA viruses (as is norovirus), belonging to the family *Leviviridae* (van Duin and Olsthoorn 2011). They are ubiquitous in sewage (Cole et al. 2003; Havelaar et al. 1985) and can be easily cultured (Havelaar and Hogeboom 1984). A number of different genogroups of F-RNA phage commonly circulate in the environment; however, genogroup II is most commonly associated with human faecal contamination (Cole et al. 2003), while genogroup I is more commonly

associated with animal waste. Infectious levels of different genogroups can be determined by nucleic acid hybridisation of phage isolates (Beekwilder et al. 1996). These various properties make F-RNA phage attractive as a viral indicator, particularly when compared with *Escherichia coli* as the currently used indicator of risk in oysters (Doré et al. 2000). However, the correlation between detection of F-RNA phage and norovirus is not absolute, and it has sometimes been proposed that it is therefore better for risk management purposes to test directly for norovirus as the pathogen of interest (Toze 1999; Miossec et al. 2001).

A possible compromise is to utilise the combined advantages of both norovirus detection by RT-qPCR (for pathogen detection) and F-RNA phage detection (for infectivity). In this study, we have investigated the combined application of both F-RNA phage and norovirus RT-qPCR testing to attempt to estimate infectious norovirus levels in oyster samples through the use of infectivity ratios, calculated by comparing levels of infectious genogroup II F-RNA phage with genogroup II F-RNA phage levels as detected by RT-qPCR. The established infectivity ratio was then applied to the norovirus RT-qPCR result to estimate the potentially infectious norovirus level. This approach was applied to two sets of oyster samples; one large set collected at the point of sale to the consumer using a randomised sampling plan, and a smaller one linked to suspected norovirus illness outbreaks.

Materials and Methods

Oyster Samples

Retail Samples

As described in Lowther et al. (2018), 630 samples of Pacific (*Crassostrea gigas*) and native oysters (*Ostrea edulis*) were collected from supermarkets, fishmongers, restaurants, online sales, and wholesalers across the United Kingdom during a 1-year survey (Mar 2015–Mar 2016). The randomised sampling plan for the survey was designed to provide a representative sample of product available to the UK consumer.

Outbreak-Related Samples

As part of its remit as the United Kingdom National Reference Laboratory and European Union Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Molluscs, the laboratory periodically receives samples of oysters and other shellfish to test as part of investigations into suspected norovirus illness outbreaks. Samples were included in this study if the following criteria were met:-

- Clinical confirmation of norovirus infection in at least one oyster consumer involved in the outbreak, or
- in the absence of testing of clinical samples for norovirus, symptoms in oyster consumers involved in the outbreak included diarrhoea and/or vomiting with an average time of onset of between 24 and 48 h, and
- sufficient stored shellfish material to enable the full suite of tests as described below available.

A total of nine samples, all Pacific oysters collected between 2012 and 2016, met these criteria and were included in the study (Table 1).

Detection and Quantification of Norovirus and Genogroup II Phage Using RT-qPCR

Virus and RNA extraction from all oyster samples was carried out as described in Lowther et al. (2018). Briefly, the digestive tissues of ten oysters were excised, pooled, and then finely chopped. A 2-g portion of the chopped tissues was subjected to a treatment with 100 µg/ml Proteinase K solution to release virus particles, with mengovirus vMCO tissue culture supernatant added as a within-sample virus/RNA extraction process control. RNA was then extracted from 500 µl of homogenate from the proteinase K treatment using NucliSENS® magnetic extraction reagents (BioMerieux, Marcy l’Etoile, France), eluting in 100 µl elution buffer.

For RT-qPCR for norovirus GI, QNIF4 and NV1LCR primers, and TM9 probe were used (da Silva et al. 2007; Hoehne and Schreier 2006; Svraka et al. 2007). For norovirus GII, QNIF2 and COG2R primers, and QNIFS probe were used (Kageyama et al. 2003; Loisy et al. 2005). For genogroup II phage, Genogroup II primers and probes (Wolf et al. 2008) were used. For mengovirus, mengo 110 (forward) and mengo 209 (reverse) primers, and mengo 147 probe were used (Costafreda et al. 2006). For norovirus and genogroup II phage assays, three aliquots of 5 µl sample RNA were tested in 25 µl total volume with one-step reaction mix prepared using the RNA UltraSense® one-step RT-qPCR system (Invitrogen) (final concentrations of 1× Reaction Mix, 500 nM forward and 900 nM reverse primers, and 250 nM probe, plus 0.5 µl Rox and 1.25 µl Enzyme Mix per reaction). For mengovirus, two aliquots of 5 µl cDNA were used. Amplification was performed using the following cycling parameters; 55 °C for 60 min, 95 °C for 5 min, and then 45 cycles of 95 °C for 15 s, 60 °C for 1 min and 65 °C for 1 min on an Mx3005P real-time PCR machine (Agilent, Santa Clara, CA, United States). Wells containing nuclease-free water and the above RT-qPCR reaction mixes were included on each plate as a negative control. Quantification of PCR-detectable norovirus and genogroup II phage followed the principles in the international standard method ISO 15216-1:2017 (Anonymous 2017). Log dilution

Table 1 Details of outbreak-related samples

Sample designation	No. of cases	Time of onset of symptoms	Clinical confirmation of norovirus?	PCR-detectable norovirus (copies/g)			Infectious F-RNA phage (pfu/g)		Infectivity ratio	Estimated infectious norovirus (virus/g)
				GI	GI/II	Total	Genogroup I ^b	Genogroup II ^b		
12-1123	36	8–48 h	No	194	361	556	38	30	8	9.6
13-374	15	28–50 h	Yes	1553	6316	7869	34	4	30	26.4
13-376	6	40 h	No	631	9398	10029	106	26	2	2.4
13-474	117	6–72 h	Yes	1469	5909	7377	104	0	104	988.7
14-280	6	48 h	No	259	2415	2674	78	3	75	61.8
14-350	8	24–48 h	No	59	1394	1453	105	0	105	105.9
15-360	15	24 h	Yes	264	1954	2218	1200	42	600	187.6
16-171	8	29–39 h	No	30	49	79	11	0	9	0.5
16-989	89	12–48 h	Yes	46	31	77	8	2	3	9.2

^aas determined by plaque assay

^bas determined by nucleic acid hybridisation

series (range 1×10^5 – 1×10^1 copies/ μ l) of linear dsDNA molecules carrying the respective target sequences were included on each RT-qPCR plate to generate a standard curve. For each RT-qPCR replicate for the sample under test, a concentration in copies/ μ l was determined using the corresponding standard curve. Negative replicates were ascribed a concentration of zero copies/ μ l. The average concentrations from the three replicates in each RT-qPCR assay were calculated to give an overall concentration in copies/ μ l for the extracted RNA, which was then converted to a quantity in detectable genome copies per g digestive tissues (copies/g) using the concentration factors inherent to the method. All samples were assessed for extraction efficiency by comparison of sample Ct values for mengovirus with a standard curve generated from the process control material. Samples with extraction efficiencies < 1% were retested. Quantitative results were not adjusted for losses during processing.

Quantification of Infectious F-RNA Phage by Plaque Assay

A double overlay plaque assay, using the genetically modified *Salmonella Typhimurium* WG49 host as described in Havelaar and Hogeboom (1984), with modifications to enable the use of oyster digestive tissues, was used to grow and enumerate total infectious F-RNA phage. Briefly, 1 g of chopped digestive tissues prepared as described above was homogenised in 2 ml peptone water using an ULTRA-TURRAX homogeniser (IKA, Staufen im Breisgau, Germany) then centrifuged at $3000 \times g$ for 5 min. The supernatant was decanted into a fresh tube; then, two replicate portions of 1 ml were added to separate 2.5 ml portions of molten 1% tryptone–yeast extract agar along with 1 ml of WG49 host culture and 100 μ l of calcium-glucose solution (3% CaCl_2 , 10% glucose w/v in dH_2O). The molten agar, sample, and host mixtures were mixed by inversion and poured onto previously prepared 2% tryptone–yeast extract–glucose agar plates. Positive and negative control plates were prepared in parallel. After incubation at 37 °C overnight, the number of plaques on each plate was counted, discounting large plaques with clear central lysis zones typical of somatic *Salmonella* phage. The total number of plaques across the two plates was multiplied by the sample volume and dilution factor to give a result in plaque forming units per g digestive tissues (pfu/g). The theoretical limit of detection of the assay (one plaque across two plates) corresponds to a concentration of 1.5 pfu/g.

Quantification of Infectious Genogroup I and Genogroup II F-RNA Phage by Nucleic Acid Hybridisation

Hybridisation probes for genogroup I and genogroup II phage were as described by Beekwilder et al. (1996), labelled at the 3' end with digoxigenin.

Positive plaque assay plates were briefly chilled in the refrigerator, then three membrane transfers per plate (one each for genogroup I and genogroup II phage, plus a spare for retesting) carried out by placing Hybond N + positively charged nylon membranes (Amersham, Little Chalfont, United Kingdom) onto the surface of the plate, then carefully peeling off after 1 min (for the first transfer) to 3 min (for the third transfer). The membranes were treated by immersion in denaturing solution (50 mM NaOH, 150 mM NaCl), then neutralising solution (100 mM sodium acetate, pH 6.0), then the viral genomes fixed by crosslinking by exposure to 120 mJ/cm^2 UV at 254 nm using a transilluminator.

Fixed membranes were washed at 37 °C for 30 min with 6 ml DIG Easy Hyb solution (Sigma Aldrich, Gillingham, UK), then hybridised at 37 °C for 60 min with 6 ml DIG Easy Hyb with 5 pmol/ml of either genogroup I or genogroup II hybridisation probe added. The membranes were washed and blocked using DIG Wash and Block buffers (Sigma Aldrich, Gillingham, UK), according to the manufacturer's instructions; then hybridised plaques were detected using the DIG Nucleic Acid Detection kit (Sigma Aldrich, Gillingham, UK), which utilises alkaline phosphatase-linked anti-digoxigenin antibodies, and a colorimetric substrate. For both genogroup I and genogroup II phage, positive plaques, appearing as dark purple areas on the membrane after completion of the detection process, were counted to give a result in pfu/g.

Calculation of Infectivity Ratios and Estimated Infectious Norovirus Levels

Infectivity ratios (R_I) were calculated for each sample as follows:-

$$R_I = \frac{GII_V}{GII_P}$$

where GII_V was the concentration of infectious genogroup II phage in pfu/g, and GII_P was the concentration of PCR-detectable genogroup II phage in copies/g. For those samples where no infectious genogroup II phage was detected, censored infectivity ratios were calculated by assuming an infectious genogroup II phage level of half the limit of detection of the assay (0.75 pfu/g), Where GII_V was greater than GII_P then R_I was capped at 1 (\equiv 100%).

Estimated infectious norovirus levels (N_I) in infectious virus per g digestive tissues (virus/g) were then determined as follows:-

$$N_I = N_p R_I,$$

where N_p was the concentration of PCR-detectable norovirus in copies/g.

As an example, a sample with 100 copies/g PCR-detectable norovirus, 200 copies/g PCR-detectable genogroup II phage, and 10 pfu/g infectious genogroup II phage would have an infectivity ratio of 5% (10/200) and an estimated infectious norovirus level of 5 virus/g (100×5%).

Statistical Analysis

For norovirus, calculated levels for genogroup I and genogroup II were combined to give an overall level. Relevant statistical analyses were carried out using the Minitab software package (Minitab, Inc., State College, PA, United States). Student's *t* test was used for comparison of the means of two datasets, Fisher's exact test was used as a test for association in 2×2 contingency tables while the χ^2 test was used for larger contingency tables. The test for significance of a correlation coefficient was used to test for correlation. For all tests, a significance level of 0.05 was used.

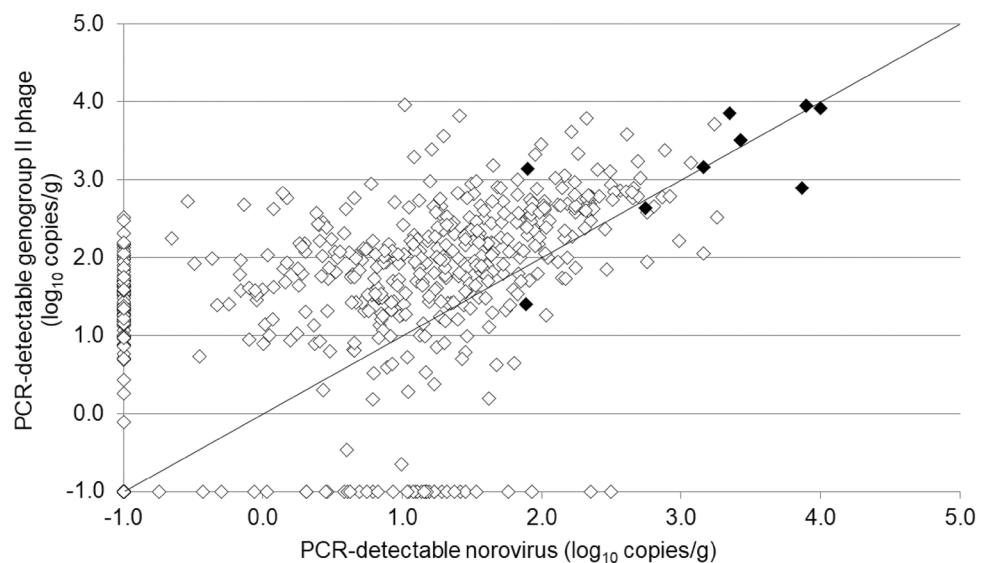
Results

Quantification of Norovirus and Genogroup II Phage by RT-qPCR

Retail Samples

As previously detailed (Lowther et al. 2018), 433 out of 630 retail samples (68.7%) tested positive for norovirus by RT-qPCR. Calculated quantities in positive samples ranged from 1 to 1805 copies/g, with four samples (0.6%) providing results over 1000 copies/g. The geometric mean for all positive results was 18 copies/g. Positive results for genogroup II phage by RT-qPCR were obtained for 492 retail samples (78.1%) with calculated quantities ranging from 1 to 9135 copies/g. Results over 1000 copies/g were found in 22 samples (3.5%) with the geometric mean for all positive results at 79 copies/g. A comparison of \log_{10} -transformed counts for norovirus and genogroup II phage is shown in Fig. 1. Both norovirus and genogroup II phage were detected by RT-qPCR in 391 samples (62.1%) while 96 were negative for both determinands. For samples positive for both, a statistically significant positive correlation in levels was found (for \log_{10} -transformed results, $r^2 = 0.2041$, $p < 0.00001$). For the 534 samples which were positive for either norovirus or genogroup II phage by RT-qPCR, detected levels of genogroup II phage were higher in 436 samples (81.6%). Levels of over 1000 copies/g were found for both norovirus and genogroup II phage in two samples (0.3%). A positive association between positivity for norovirus and genogroup II phage by PCR was found using Fisher's exact test ($p < 0.0001$).

Fig. 1 Comparison of PCR-detectable levels of norovirus and genogroup II phage in retail and outbreak-related samples. Retail samples are shown as open diamonds, outbreak-related samples as black diamonds. A line of equivalence is included and negative results for either determinand are shown at -1



Norovirus prevalence and levels in this set of samples showed a distinct seasonality as reported in Lowther et al. (2018), with more positive samples and higher levels in the winter. A similar seasonality was found for genogroup II phage, with 279 out of 325 samples (85.8%) collected during the winter months October to March testing positive, compared with 213 out of 305 samples (69.8%) collected from April to September. Geometric means of PCR-detectable genogroup II phage levels in positive samples were 110 copies/g and 52 copies/g for the winter and summer, respectively.

Outbreak-Related Samples

Nine outbreak-related samples passed the inclusion criteria and were included in the current study (Table 1).

All nine samples tested positive for both norovirus and genogroup II phage by RT-qPCR, with calculated quantities ranging from 77 to 10,029 copies/g for norovirus and 25 to 8935 copies/g for genogroup II phage. Levels of over 1000 copies/g were found for both norovirus and genogroup II phage in five samples (55.6%). Geometric mean levels of both norovirus (1369 copies/g) and genogroup II phage (1450 copies/g) were more than one log higher for outbreak-related samples than for positive retail samples. Differences between the mean levels between the two sample types were statistically significant for both viruses (Student's *t* test, $p < 0.0001$ in both cases).

Quantification of Infectious F-RNA Phage by Plaque Assay

Infectious F-RNA phage was detected in 301 retail samples (47.8%) and all nine outbreak-related samples. The maximum levels recorded in retail and outbreak-related samples were 684 and 1200 pfu/g, respectively. The geometric mean

levels were 8 pfu/g for positive retail samples and 61 pfu/g for outbreak-related samples. A statistically significant difference in mean levels between the two sample types was found (Student's *t* test, $p < 0.0001$).

Of retail samples collected during the winter months, 207 out of 305 samples (63.7%) tested positive for infectious F-RNA phage. For the summer months, 94 out of 305 samples (30.8%) tested positive. Geometric means of infectious F-RNA phage levels in positive samples were 9.3 pfu/g and 6.6 pfu/g in winter and summer, respectively.

A comparison of \log_{10} -transformed counts for infectious F-RNA phage and norovirus as detected by RT-qPCR is shown in Fig. 2. Both determinands were detected in 236 samples, while 132 samples were negative for both; a statistically significant positive association between them was found using Fisher's exact test ($p < 0.0001$), while a statistically significant positive correlation was found between the levels of the two determinands in positive samples (for \log_{10} -transformed results, $r^2 = 0.1216$, $p < 0.00001$).

A comparison of \log_{10} -transformed counts for infectious F-RNA phage and genogroup II phage as detected by RT-qPCR is shown in Fig. 3. Both determinands were detected in 258 samples while 95 samples were negative for both; a statistically significant positive association between them was found using Fisher's exact test ($p < 0.0001$), while a statistically significant positive correlation was found between levels of the two determinands in positive samples (for \log_{10} -transformed results, $r^2 = 0.1532$, $p < 0.00001$).

Quantification of Infectious Genogroup I and Genogroup II Phage by Nucleic Acid Hybridisation

A random sub-selection of 108 out of the 301 retail samples that had provided positive results for infectious F-RNA phage and all nine outbreak-related samples were subjected

Fig. 2 Comparison of levels of PCR-detectable norovirus and infectious F-RNA phage in retail and outbreak-related samples. Retail samples are shown as open diamonds, outbreak-related samples as black diamonds. Negative results for either determinand are shown at -1

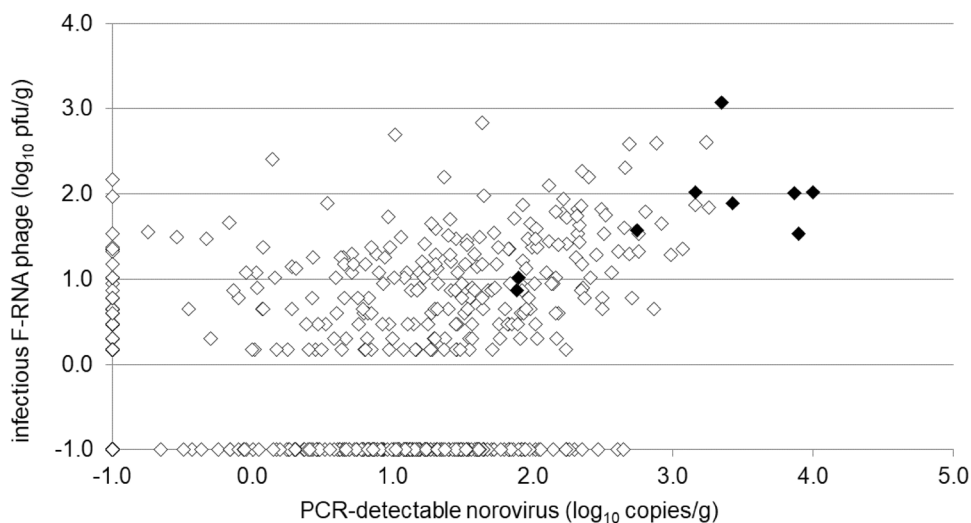
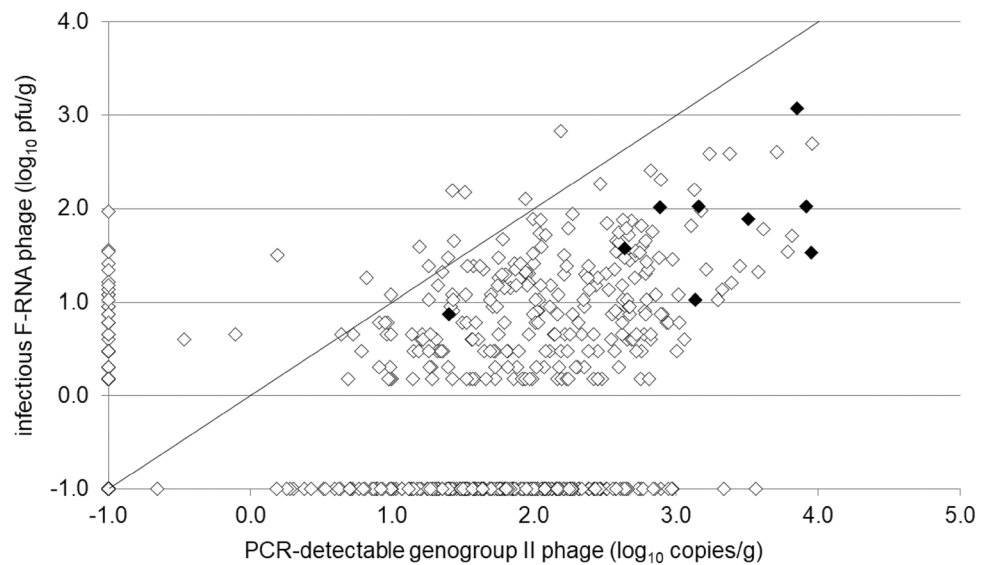


Fig. 3 Comparison of levels of PCR-detectable genogroup II phage and infectious F-RNA phage in retail and outbreak-related samples. Retail samples are shown as open diamonds, outbreak-related samples as black diamonds. A line of equivalence is included and negative results for either determinant are shown at -1



to partial characterisation of infectious F-RNA phage types using nucleic acid hybridisation to quantify infectious genogroup I and II phage. Positive hybridisation results were obtained for 94 retail samples, with 45 samples containing both genogroup I and genogroup II, 26 containing genogroup I only, and 23 containing genogroup II only. Six of the nine outbreak-related samples contained genogroup I phage, while all nine contained genogroup II. A majority of plaques were identified as genogroup I phage in 38 retail samples and one outbreak-related sample and genogroup II phage in 39 retail samples and five outbreak-related samples. For retail samples, 34.6% and 28.2% of all plaques subject to hybridisation analysis were identified as genogroup I and genogroup II phage, respectively, while for outbreak-related samples 6.3% and 55.6% of plaques were identified as genogroups I and II, respectively.

For statistical comparisons of retail and outbreak-related samples, 116 randomly selected infectious F-RNA phage negative retail samples (therefore by inference negative for infectious genogroup I and II phage) were combined with the 108 samples selected for testing by hybridisation to give an expanded dataset of 224 samples. This preserved the observed ratio ($\sim 48\%$) of infectious F-RNA phage positive samples in the overall dataset and avoided the introduction of statistical bias. Using this expanded set, the geometric mean of infectious genogroup II phage was considerably higher in outbreak-related samples (26 pfu/g) compared with positive retail samples (8 pfu/g), whereas for infectious genogroup I phage, levels were similar (8 pfu/g and 9 pfu/g for retail and outbreak-related samples, respectively). In addition, levels of infectious genogroup II phage were significantly higher in outbreak-related than in retail samples (Student's t test, $p < 0.0001$). Levels of infectious genogroup I phage were not

significantly different between the two sample types (Student's t test, $p = 0.610$).

Infectivity Ratios

For the expanded set of 224 samples with results for infectious genogroup II phage, infectivity ratios were calculated by comparing levels of infectious and PCR-detectable genogroup II phage. In 44 samples, no genogroup II phage was detected either by PCR or hybridisation and no ratio could be set. For those samples where no infectious genogroup II phage was detected (whether positive or negative for total infectious F-RNA phage, 112 samples in total), censored infectivity ratios were calculated by assuming an infectious genogroup II phage level of half the limit of detection of the assay (0.75 pfu/g). For one sample, infectious genogroup II phage levels were higher than PCR-detectable levels (which was not detected); in this case, the infectivity ratio was set at 100%. All nine outbreak-related samples contained detectable levels of infectious genogroup II phage that were lower than PCR-detectable levels, enabling an infectivity ratio to be directly calculated.

For retail samples, the infectivity ratio ranged from 0.02 to 100%. For outbreak-related samples, calculated ratios ranged from 0.02 to 13.4%. The number of samples with infectivity ratios in different range brackets (0–1%, 1–10%, 10–100%) for the different types of samples is shown in Table 2. There was no statistically significant difference between the different sample types in the distribution of samples in the different range brackets, whether infectious genogroup II phage negative retail samples with censored infectivity ratios were included (χ^2 test, $p = 0.8569$) or excluded from the analysis (χ^2 test, $p = 0.7493$). In both

Table 2 Number of retail and outbreak-related samples in different infectivity ratio brackets

Infectivity ratio	Retail samples		Outbreak-related samples
	a	b	
Not determined ^c	44	–	0
0–1%	73 (40.6%)	15 (22.1%)	3 (33.3%)
1–10%	78 (43.3%)	34 (50%)	4 (44.4%)
10–100%	29 (16.1%)	19 (27.9%)	2 (22.2%)

a—all samples. b—samples with censored and not determined infectivity ratios excluded

^csamples negative for both PCR-detectable and infectious genogroup II phage. Figures in parentheses are percentages of the total number of samples with determined infectivity ratios for each sample type

sample types, the infectivity ratio was less than 10% in the majority of samples.

Estimated Infectious Norovirus Levels

Estimated potentially infectious norovirus levels were calculated for the set of 180 samples with calculated infectivity ratios by applying the ratio to the level of PCR-detectable norovirus recorded for each sample. Estimated infectious norovirus levels are compared to PCR-detectable norovirus levels in Fig. 4 and the distribution of infectious levels in different brackets is shown in Table 3.

For retail samples, the highest estimated infectious norovirus level calculated was 125 virus/g (1805 copies/g PCR-detectable norovirus \times 6.94% infectivity ratio), although the majority of samples recorded estimated levels

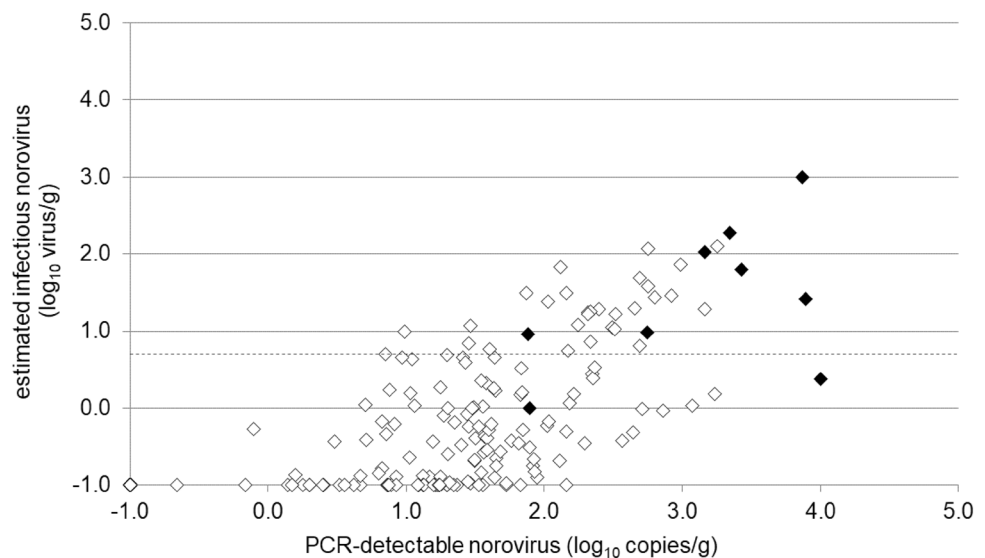
Table 3 Number of retail and outbreak-related samples with estimated infectious norovirus levels above and below 5 virus/g

Estimated infectious norovirus (virus/g)	Retail samples	Outbreak-related samples
Not determined ^a	13	0
0–5	182 (86.3%)	2 (22.2%)
> 5	29 (13.7%)	7 (77.8%)

^asamples positive for PCR-detectable norovirus and negative for both PCR-detectable and infectious genogroup II phage. Figures in parentheses are percentages of the total number of samples with estimated infectious norovirus levels for each sample type

of less than 1 virus/g. For outbreak-related samples, estimated infectious norovirus levels ranged from 0.5 virus/g (79 copies/g PCR-detectable norovirus \times 0.66% infectivity ratio) up to 989 virus/g (7377 copies/g PCR-detectable norovirus \times 13.40% infectivity ratio). Overall levels of estimated infectious norovirus in outbreak-related samples (geometric mean 24.8 virus/g) were markedly higher than retail samples (geometric mean of positive samples 0.5 virus/g); a statistically significant difference in the distribution of results between the two sample types was found (Student's *t* test, $p < 0.0001$). Seven out of nine outbreak-related samples had estimated infectious norovirus levels of > 5 virus/g, while only a minority (13.7%) of retail samples showed such levels; the distribution of numbers of the two types of samples above and below 5 virus/g was found to be significantly different (Fisher's exact test, $p < 0.0001$) indicating a possible association between these elevated levels of estimated infectious norovirus and human health risks.

Fig. 4 Comparison of levels of PCR-detectable and estimated infectious norovirus in retail and outbreak-related samples. Retail samples are shown as open diamonds, outbreak-related samples as black diamonds. A dashed line at five infectious virus/g is included. Negative results for either determinand are shown at -1



Analysis of the Combination of PCR-Detectable Norovirus and Infectious F-RNA Phage Results

As shown, a significant difference in levels of both PCR-detectable norovirus and infectious F-RNA phage was found between retail and outbreak-related samples. We therefore investigated the application of different risk thresholds using these two determinands both singly and in combination to the different sample types (Table 4). Retail samples with an estimated infectious norovirus level of > 5 virus/g (and which therefore may have posed a greater risk to human health) were also compared to the different risk thresholds.

Simple risk thresholds based on positivity for either PCR-detectable norovirus or F-RNA phage resulted in large proportions of retail samples (68.7% for norovirus and 47.8% for F-RNA phage) and all outbreak-related samples exceeding the threshold. Increasingly high combined thresholds (up to > 500 copies/g PCR-detectable norovirus and > 10 pfu/g infectious F-RNA phage) significantly reduced the number of retail samples exceeding the threshold, while still identifying the majority of outbreak-related samples. Compared with outbreak-related samples, similar proportions of retail samples with > 5 virus/g estimated infectious norovirus exceeded most threshold combinations, with the exception of combinations including > 500 copies/g PCR-detectable norovirus, where markedly lower proportions of retail samples with > 5 virus/g estimated infectious norovirus exceeded the threshold.

Table 4 Application of risk thresholds using combinations of PCR-detectable norovirus and infectious F-RNA phage results

Combined risk threshold applied		Number of samples above threshold applied		
PCR-detectable norovirus result	Infectious F-RNA phage result	Retail samples		Outbreak-related samples
		a	b	
Positive	Any	433 (68.7%)	29 (100%)	9 (100%)
> 100 copies/g	Any	73 (11.6%)	23 (79.3%)	7 (77.8%)
> 500 copies/g	Any	12 (1.9%)	7 (24.1%)	7 (77.8%)
Any	Positive	301 (47.8%)	28 (96.6%)	9 (100%)
Any	> 10 pfu/g	127 (20.2%)	26 (89.7%)	8 (88.9%)
Positive	Positive	236 (37.5%)	28 (96.6%)	9 (100%)
> 100 copies/g	Positive	60 (9.5%)	23 (79.3%)	7 (77.8%)
> 500 copies/g	Positive	12 (1.9%)	7 (24.1%)	7 (77.8%)
Positive	> 10 pfu/g	114 (18.1%)	26 (89.7%)	8 (88.9%)
> 100 copies/g	> 10 pfu/g	44 (7.0%)	22 (75.9%)	7 (77.8%)
> 500 copies/g	> 10 pfu/g	10 (1.6%)	7 (24.1%)	7 (77.8%)

a—all samples. b—samples with > 5 virus/g estimated infectious norovirus. Figures in parentheses are percentages of the total number of samples for each sample type

Discussion

The results obtained in our study show that F-RNA phage, particularly genogroup II, has many of the important properties classically required by an indicator organism (National Research Council 2004), being commonly present in test samples, and correlated with the target organism while generally being at higher levels than the pathogen. In common with norovirus, F-RNA phage showed a distinct winter seasonality as previously reported (Doré et al. 2003.) Comparing results obtained by RT-qPCR, genogroup II F-RNA phage was present in 90.3% of retail samples where norovirus was detected, and levels of genogroup II F-RNA phage were higher than norovirus in 83.1% of these samples. A statistically significant association in the presence/absence and correlation in levels of the two viruses as determined by RT-qPCR was found. Although infectious F-RNA phage (both total and genogroup II) was detected only in a minority of samples, its presence was more frequent in norovirus positive samples.

Although retail samples were tested immediately upon receipt at the laboratory, or after short periods of frozen storage consistent with the international standard method ISO 15216-1:2017 (Anonymous 2017), by necessity outbreak samples were stored frozen for longer periods (up to 3 years). This might have been expected to impact detectable levels of PCR-detectable and particularly viable virus; however, in all cases higher levels were detected in outbreak samples despite the prolonged storage indicating that any effects were relatively minor. The association observed with higher levels of norovirus and outbreak-related samples has been observed previously (Lowther et al. 2012a). However, in addition, in this study, the outbreak-related samples also contained higher levels of F-RNA phage, both PCR-detectable and infectious. Where hybridisation was carried out, a significantly higher proportion of plaques were found to contain genogroup II phage in outbreak-related samples compared with retail samples, supporting the association between this genogroup and human faecal contamination (Cole et al. 2003).

Comparison of genogroup II F-RNA phage levels as determined by RT-qPCR and plaque assay/hybridisation revealed a significant discrepancy between total PCR-detectable and infectious levels of this virus. Of RT-qPCR-positive retail samples subjected to infectious phage characterisation, the majority (62.6%) provided negative results for infectious genogroup II F-RNA phage. Direct calculation of infectivity ratios using samples that were positive by both RT-qPCR and hybridisation showed that in the majority of retail samples (72.1%), less than 10% of the PCR-detectable genogroup II F-RNA phage was infectious. The highest ratio determined was 70.8%,

indicating that in some cases a high degree of congruence between PCR and infectivity results can be expected. Interestingly, there was no apparent difference between the range of infectivity ratios observed in retail and outbreak-related samples. In addition, it is important to note that we observed a wide range of infectivity ratios in both retail and outbreak samples. This suggests that the application of a generic percentage of infectious virus, for example, determined experimentally using a single stock of virus, would not be effective. Infectivity ratios vary widely from sample to sample, presumably reflecting the individual history of virus particles contaminating each sample. Factors such as different sewage treatment regimes (Hewitt et al. 2011), the amount of sun exposure (Lytle and Sagripanti 2005), and the duration of residence in the environment (Bosch et al. 2006) could all influence this ratio in oyster samples.

In our study, we have trialled the novel approach of using infectivity ratios calculated from genogroup II F-RNA phage results in order to estimate potentially infectious norovirus in oysters. This method has some limitations. In a significant proportion of samples, ratios were calculated using very low analytical results. Our laboratory has experimentally determined a limit of quantification for the norovirus RT-qPCR method at 100 copies/g; however, no equivalent figures for either PCR-detectable or infectious genogroup II F-RNA phage have been determined, although the figure for the RT-qPCR method is likely to be broadly similar. As a result, in this study, no consideration of limits of quantification was made with results used as obtained; however, for routine application of such a method, it would be necessary to make allowances for such areas of uncertainty. The use of infectivity ratios and estimated viable norovirus levels calculated using levels below the analytical limit of quantification could also raise questions about the conclusions we have drawn from these data—however, it is important to note that in the majority of both outbreak-related samples, and retail samples with estimated infectious norovirus of > 5 virus/g, results for PCR-detectable norovirus and genogroup II F-RNA phage and for infectious F-RNA phage were at moderate to high levels (e.g. > 100 copies/g for RT-qPCR assays, > 10 pfu/g for infectivity). The conclusions drawn from these samples will have been minimally affected by this type of method uncertainty and should be considered robust therefore.

An additional complication is that we cannot be certain whether infectivity ratios calculated for F-RNA phage would correlate with actual infectivity ratios for norovirus (which cannot currently be directly determined) in the same samples. However, the two viruses both possess single-stranded RNA genomes and are of a similar size and morphology (Hartard et al. 2016) and could be expected to possess similar characteristics with regards to infectivity. In addition,

genogroup II F-RNA phage and norovirus are both associated strongly with human faecal contamination, and the inactivation stresses they will be subject to en route from the human gut through the environment to shellfish are likely to be very similar therefore. The development of direct culture methods for norovirus (Ettayebi et al. 2016; Jones et al. 2014) may enable experimental work investigating the relationship between infectivity in norovirus and F-RNA phage; however, the question of different environmental resistance and survival rates applies in all cases where the use of indicator organisms is considered (National Research Council 2004) and it is certainly the case that the characteristics of norovirus are likely to be more similar to those of F-RNA phage than those of *E. coli*, the current regulatory indicator.

Supporting the possible use of F-RNA phage and norovirus testing in combination we show that, as could be expected by the established dose–response relationships (Teunis et al. 2008), estimated infectious norovirus levels were notably higher in outbreak-related samples than retail samples. For example, the geometric mean of estimated infectious norovirus in outbreak-related samples was more than 40× higher than in retail samples, and an association was noted between outbreak-related samples and estimated infectious norovirus levels of > 5 virus/g digestive tissues indicating the potential of this approach for monitoring and managing risk.

In practical terms, F-RNA phage characterisation using nucleic acid hybridisation is a relatively labour-intensive method, with long assay times, and only a small number of samples can be assayed simultaneously, limiting the usefulness of this type of calculation in routine settings. We therefore investigated the use of a simpler risk monitoring system using various possible levels of norovirus RT-qPCR and infectious total F-RNA phage results alone or in combination. This approach also shows promise, with, for example, a result combination of > 100 copies/g PCR-detectable norovirus and > 10 pfu/g infectious total F-RNA phage including the majority of both outbreak-related samples and retail samples with estimated infectious norovirus of > 5 virus/g but excluding the majority (> 90%) of total retail samples. A further benefit of using this combination of action levels for both PCR-detectable norovirus and infectious F-RNA phage is that it should circumvent any concerns about limits of quantification raised by the method for estimation of infectious norovirus as described above. We would therefore recommend further evaluation of the potential for combining norovirus and infectious F-RNA phage testing to obtain optimum risk management criteria that succeed in identifying potentially hazardous food without imposing disproportional restrictions on shellfish producers.

In conclusion, we suggest that although norovirus RT-qPCR testing alone could reduce human health risks associated with norovirus contamination of bivalve shellfish

(European Food Safety Authority 2012), especially if compared with the use of bacterial indicators of faecal contamination as currently applied in legislation in both the European Union (Anonymous 2004) and the United States (Anonymous 2015), combining RT-qPCR testing with the infectivity test for F-RNA phage has the potential to better estimate actual health risks, to better predict the presence of infectious norovirus, and to avoid unproductive restrictions on producers.

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References

- Anonymous (2004). Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of European Communities*, L226, 83–127.
- Anonymous (2013). Microbiology of food and animal feed -- Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR -- Part 1: Method for quantification. In *ISO/TS 15216-1:2013*. Geneva: International Organization for Standardization.
- Anonymous, 2015. National Shellfish Sanitation Program. Guide for the Control of Molluscan Shellfish, 2015 Revision. <https://www.fda.gov/food/guidanceregulation/federalstatefoodprograms/ucm2006754.htm>.
- Anonymous (2017). Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR -- Part 1: Method for quantification. In *ISO 15216-1:2017*. Geneva: International Organization for Standardization.
- Beekwilder, J., Nieuwenhuizen, R., Havelaar, A. H., & van Duin, J. (1996). An oligonucleotide hybridization assay for the identification and enumeration of F-specific RNA phages in surface water. *Journal of Applied Bacteriology*, 80, 179–186.
- Bellou, M., Kokkinos, P., & Vantarakis, A. (2013). Shellfish-borne viral outbreaks: A systematic review. *Food and Environmental Virology*, 5, 13–23.
- Bosch, A., Pintó, R. M., & Abad, F. X. (2006). Survival and transport of enteric viruses in the environment. In S. M. Goyal (Ed.), *Viruses in foods. food microbiology and food safety* (pp. 151–187). Boston: Springer.
- Cole, D., Long, S. C., & Sobsey, M. D. (2003). Evaluation of F + RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. *Applied and Environmental Microbiology*, 69, 6507–6514.
- Costafreda, M. I., Bosch, A., & Pintó, R. M. (2006). Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and Environmental Microbiology*, 72, 3846–3855.
- da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. *Applied and Environmental Microbiology*, 73, 7891–7897.
- Dancer, D., Rangdale, R. E., Lowther, J. A., & Lees, D. N. (2010). Human norovirus RNA persists in seawater under simulated winter conditions but does not bioaccumulate efficiently in Pacific oysters (*Crassostrea gigas*). *Journal of Food Protection*, 73, 2123–2127.
- Dancho, B. A., Chen, H., & Kingsley, D. H. (2012). Discrimination between infectious and non-infectious human norovirus using porcine gastric mucin. *International Journal of Food Microbiology*, 155, 222–226.
- Doré, W. J., Henshilwood, K., & Lees, D. N. (2000). Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. *Applied and Environmental Microbiology*, 66, 1280–1285.
- Doré, W. J., Mackie, M., & Lees, D. N. (2003). Levels of male-specific RNA bacteriophage and *Escherichia coli* in molluscan bivalve shellfish from commercial harvesting areas. *Letters Applied Microbiology*, 36, 92–96.
- Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R., Neill, F. H., Blutt, S. E., Zeng, X. L., Qu, L., Kou, B., Opekun, A. R., Burrin, D., Graham, D. Y., Ramani, S., Atmar, R. L., & Estes, M. K. (2016). Replication of human noroviruses in stem cell-derived human enteroids. *Science*, 353, 1387–1393.
- European Food Safety Authority Panel on Biological Hazards. (2012). Scientific Opinion on Norovirus (NoV) in oysters: methods, limits and control options. *EFSA Journal*, 10, 2500.
- Flannery, J., Keaveney, S., & Doré, W. (2009). Use of FRNA bacteriophages to indicate the risk of norovirus contamination in Irish oysters. *Journal of Food Protection*, 72, 2358–2362.
- Hartard, C., Banas, S., Loutreul, J., Rincé, A., Benoit, F., Boudaud, N., & Gantzer, C. (2016). Relevance of F-specific RNA bacteriophages in assessing human norovirus risk in shellfish and environmental waters. *Applied and Environmental Microbiology*, 82, 5709–5719.
- Hassard, F., Sharp, J. H., Taft, H., LeVay, L., Harris, J. P., McDonald, J. E., Tuson, K., Wilson, J., Jones, D. L., & Malham, S. K. (2017). Critical Review on the Public Health Impact of Norovirus Contamination in Shellfish and the Environment: A UK Perspective. *Food and Environmental Virology*, 9, 123–141.
- Havelaar, A. H., & Hogeboom, W. M. (1984). A method for the enumeration of male-specific bacteriophages in sewage. *Journal of Applied Bacteriology*, 56, 439–447.
- Havelaar, A. H., Hogeboom, W. M., & Pot, R. (1985). F specific RNA bacteriophages in sewage: Methodology and occurrence. *Water Science and Technology*, 17, 645–655.
- Hewitt, J., Leonard, M., Greening, G. E., & Lewis, G. D. (2011). Influence of wastewater treatment process and the population size on human virus profiles in wastewater. *Water Research*, 45, 6267–6276.
- Hoehne, M., & Schreier, E. (2006). Detection of Norovirus genogroup I and II by multiplex real-time RT-PCR using a 3'-minor groove binder-DNA probe. *BMC Infectious Diseases*, 6, 69.
- Jones, M. K., Watanabe, M., Zhu, S., Graves, C. L., Keyes, L. R., Grau, K. R., Gonzalez-Hernandez, M. B., Iovine, N. M., Wobus, C. E., Vinjé, J., Tibbetts, S. A., Wallet, S. M., & Karst, S. M. (2014). Enteric bacteria promote human and mouse norovirus infection of B cells. *Science*, 346, 755–759.
- Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F., Takeda, N., & Katayama, K. (2003). Broadly reactive

- and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology*, *41*, 1548–1557.
- Knight, A., Li, D., Uyttendaele, M., & Jaykus, L. A. (2013). A critical review of methods for detecting human noroviruses and predicting their infectivity. *Critical Reviews in Microbiology*, *39*, 295–309.
- Langlet, J., Kaas, L., & Greening, G. (2015). Binding-based RT-qPCR assay to assess binding patterns of noroviruses to shellfish. *Food and Environmental Virology*, *7*, 88–95.
- Loisy, F., Atmar, R. L., Guillon, P., Le Cann, P., Pommepuy, M., & Le Guyader, F. S. (2005). Real-time RT-PCR for norovirus screening in shellfish. *Journal of Virological Methods*, *123*, 1–7.
- Lowther, J. A., Avant, J. M., Gizynski, K., Rangdale, R. E., & Lees, D. N. (2010). Comparison between quantitative real-time reverse transcription PCR results for norovirus in oysters and self-reported gastroenteric illness in restaurant customers. *Journal of Food Protection*, *73*, 305–311.
- Lowther, J. A., Gustar, N. E., Hartnell, R. E., & Lees, D. N. (2012a). Comparison of norovirus RNA levels in outbreak-related oysters with background environmental levels. *Journal of Food Protection*, *75*, 389–393.
- Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E., & Lees, D. N. (2012b). Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Applied and Environmental Microbiology*, *78*, 5812–5817.
- Lowther, J. A., Gustar, N. E., Powell, A. L., O'Brien, S., & Lees, D. N. (2018). A One Year Survey of Norovirus in UK Oysters Collected at the Point of Sale. *Food and Environmental Virology*, *10*, 278–287.
- Lytle, C. D., & Sagripanti, J. L. (2005). Predicted inactivation of viruses of relevance to biodefense by solar radiation. *Journal of Virology*, *79*, 14244–14252.
- Miossec, L., Le Guyader, F., Pelletier, D., Haugarreau, L., Caprais, M. P., & Pommepuy, M. (2001). Validity of *Escherichia coli*, enterovirus, and F-specific RNA bacteriophages as indicators of viral shellfish contamination. *Journal of Shellfish Research*, *20*, 1223–1227.
- National Research Council (US) Committee on indicators for waterborne pathogens. 2004. Indicators for waterborne pathogens. National Academies Press. Washington.
- Nuanualsuwan, S., & Cliver, D. O. (2002). Pretreatment to avoid positive RT-PCR results with inactivated viruses. *Journal of Virological Methods*, *104*, 217–225.
- Polo, D., Varela, M. F., & Romalde, J. L. (2015). Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. *International Journal of Food Microbiology*, *193*, 43–50.
- Randazzo, W., Khezri, M., Ollivier, J., Le Guyader, F. S., Rodríguez-Díaz, J., Aznar, R., & Sánchez, G. (2018). Optimization of PMAxx pretreatment to distinguish between human norovirus with intact and altered capsids in shellfish and sewage samples. *International Journal of Food Microbiology*, *266*, 1–7.
- Suffredini, E., Lanni, L., Arcangeli, G., Pepe, T., Mazzette, R., Ciccaglioni, G., & Croci, L. (2014). Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy. *International Journal of Food Microbiology*, *184*, 21–26.
- Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteyn, B., & Koopmans, M. (2007). Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *Journal of Clinical Microbiology*, *45*, 1389–1394.
- Teunis, P. F., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baric, R. S., Le Pendu, J., & Calderon, R. L. (2008). Norwalk virus: how infectious is it? *Journal of Medical Virology*, *80*, 1468–1476.
- Toze, S. (1999). PCR and the detection of microbial pathogens in water and wastewater. *Water Research*, *33*, 3545–3556.
- van Duin, J., & Olsthoorn, R. C. L. (2011). Family—Leviviridae. In A. M. Q. King, M. J. Adams, E. B. Carstens & E. J. Lefkowitz (Eds.), *Virus taxonomy—Ninth report of the international committee on taxonomy of viruses* (pp. 1035–1043). Amsterdam: Elsevier.
- Wolf, S., Hewitt, J., Rivera-Aban, M., & Greening, G. E. (2008). Detection and characterization of F + RNA bacteriophages in water and shellfish: Application of a multiplex real-time reverse transcription PCR. *Journal of Virological Methods*, *149*, 123–128.

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