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Current state of methods for detection of infectious foodborne viruses

Report of the Foodborne viruses National Reference Laboratory

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Contents

Glossary

CIA	Capside integrity assay
EMA	Ethidium monoazide
EU	European Union
FSA	Food Standards Agency
HAV	Hepatitis A virus
HEV	Hepatitis E virus
HIE	Human intestinal enteroids
ISO	International Organisation for Standardisation
PGM	Porcine gastric mucin
PMA	Propidium monoazide
NASBA	Nucleic acid-based sequence amplification
NRL	National Reference Laboratory
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
TBEV	Tick-borne encephalitis virus

1. Introduction

Contamination of foods, including bivalve shellfish and fresh produce, with viruses including norovirus, hepatitis A virus (HAV) and hepatitis E virus (HEV) is widely recognised as a food safety risk, with a considerable number of reports of outbreaks in the literature (reviewed in Bellou et al, 2013, Callejon et al, 2015).

Methods for quantification of norovirus and HAV in foods using the quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) are well established, and an international standard method using this technology was published in 2013 (ISO, 2013) with an updated version including validation data released in 2017 (ISO, 2017). Studies using RT-qPCR have detected norovirus RNA in the majority of UK oyster samples, both in samples taken from the production area (Lowther et al, 2012b) and at the point-of-sale to the consumer (Lowther et al, 2018), while other studies have shown the presence of norovirus RNA in fresh produce (Cook et al, 2019) and HEV RNA in bivalves available to the UK consumer (O'Hara et al, 2018), albeit at much lower frequency (<10%).

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 free RNA. As a result, there is a possibility that RT-qPCR testing can overestimate the risks of foodborne viruses.

A number of detection methodologies and modifications to the RT-qPCR have been developed that aim to better represent infectious virus levels (methods for norovirus reviewed by Knight et al, 2013, Manuel et al, 2018, Liu and Moore, 2020). Broadly these include:

- methods to directly culture infectious virus from test samples
- pre-treatment methods that aim to increase the ratio of intact to damaged (presumed non-infectious) virus particles in the sample (either by selective purification of intact virus or removal of damaged particles and free RNA)
- methods that aim to detect non-fragmented RNA rather than short RNA fragments (presumed non-infectious)
- methods that aim to estimate target virus infectivity by extrapolating from a culturable indicator virus

This review, requested from the National Reference Laboratory for Virus in Foods by the Food Standards Agency, aims to summarise the current state-of-the art of such methods. The principal focus is on methods for norovirus, particularly as applied to bivalve shellfish samples; this food/virus combination is one of the most significant viral food safety issues in the UK, and in addition, the high prevalence of detection means that this issue is more difficult to manage using RT-qPCR detection alone as a trigger for interventions, whereas for less frequently detected food/virus combinations the precautionary principle may be

more practicable. However, application of norovirus infectivity methods to other foodstuffs and application of infectivity methods to other viruses are also considered.

2. Direct culture methods

Norovirus has historically proved very resistant to development of *in vitro* culture methods. The first reports (within the last 15 years) of successful culture using 3-D organoids (Straub et al, 2007) and B cells (Jones et al, 2015) have proven either impossible to replicate in other laboratories or have had limited utility due to their optimisation for particular virus strains.

More recently however, a method for norovirus culture using human intestinal enteroids (HIE) has been published (Ettayebi et al, 2016) that has proven more versatile and durable. HIEs are generated from stem cells isolated from intestinal crypts in human intestinal tissues; they are multicellular, differentiated cultures containing multiple intestinal epithelial cell types (enterocytes, goblet, enteroendocrine and Paneth cells) and can be grown either as 3-D cultures or as monolayers. To ensure replication of diverse norovirus strains it is important that cell donors are selected to provide the appropriate expression of histo-blood group antigens (norovirus receptors) on the cell surfaces, and in addition culture additives such as bile may be required to ensure replication of some norovirus strains and genotypes (Ettayebi et al, 2016).

In this system, viral replication is demonstrated by comparing levels of norovirus RNA in cell culture wells (as detected by RT-qPCR) at 1 hour post inoculation (at which point no replication is assumed to have taken place), with levels after a longer period (usually 24-96 hours). The result is expressed as a $\log_{10}$ or fold-increase between the two timepoints, for example twofold (Davis et al, 2020) fivefold (Overbey et al, 2021) or tenfold increases (Chan et al, 2019) have been considered as a positive result for replication.

This methodology has been used in developments such as research into fundamental virus/host interactions (Haga et al, 2020) and demonstration of inactivation of a variety of strains and genotypes of norovirus by disinfectants including chlorine and alcohol (Costantini et al, 2018) and green tea extract (Randazzo et al, 2020). However, to date no successful application of the enteroid system to the detection of infectious virus in food or environmental samples has been reported.

Overbey et al (2021) have identified critical parameters for enteroid experiments in order to maximise the robustness of the system with the aim of applying these methods to environmental samples. These critical parameters include the age of the HIE cells used to seed monolayers and the media used in this procedure. It is hoped that continuing improvements to this new technology will enable its application to food samples in the

medium term. However, there are significant obstacles to their use for determining levels of infectious virus in foods. At present, viral replication is highly strain dependent, with for example successful replication reported only for certain GII genotypes, and no GI strains by Constantini et al (2018). Successful virus replication requires high initial inputs of RT-qPCR detectable virus, with ID_{50} values of $4.4x10^2$ – $4.0x10^3$ genome copies per 100µl well reported for 3 different GII virus strains (Costantini et al, 2018) and successful replication generally associated with input levels of 10^4 copies per well as reported by Overbey et al (2021). These concentrations of RT-qPCR detectable virus are orders of magnitude higher than those found in oyster samples associated with norovirus illness outbreaks (Lowther et al, 2012a), so application of HIE methods to detection of infectious noroviruses in bivalve shellfish would require either significant improvements in sensitivity, or development of appropriate virus concentration methods for bivalve samples. Finally, although higher input levels of virus are associated with higher -fold increases, this relationship is not linear (Costantini et al, 2018, Overbey et al, 2021) and the HIE method is not at present considered quantitative.

For HAV, culture models using primate cells (e.g., FRhK-4 cell line) have been developed, but usually require cell-culture adapted HAV strains (Binn et al, 1984) and are limited to experimental use. Successful culture of wild-type HAV has been rarely reported (Konduru and Kaplan, 2006) but cannot be considered established and to date has not been applied to detection of HAV in food samples, which has relied on application of molecular detection methods.

3. Pre-treatments

3.1. Porcine Gastric Mucin and other affinity reagents

Molecules that selectively bind virus capsid proteins can be used as affinity reagents in the extraction of viruses from samples prior to detection of viral RNA by RT-qPCR. In this way the sample is enriched for intact virus particles, while damaged virus particles (presumed to be non-infectious) and free RNA are removed. RT-qPCR detection will in theory then correlate more closely with presence of infectious viruses than in methods where viral RNA is extracted without employing an affinity reagent. Affinity reagents are normally applied as a coating to magnetic beads to facilitate separation of the virus particles from the remainder of the sample and the removal of impurities though the various wash steps of the purification process.

Early methodological developments utilised affinity reagents including antibodies raised against Norwalk virus (the prototype norovirus – now described as genotype GI.1) (Gilpatrick et al, 2000) and histo-blood group antigens (Cannon and Vinje, 2008) however

the most commonly used affinity reagent in recent years is porcine gastric mucin (PGM; carbohydrate molecules from the surface of pig gastric cells). Pigs are susceptible to GII noroviruses (Sugieda and Nakajima, 2002) and PGM shares many molecular characteristics with the histo-blood group antigens that are found on the surface of human epithelial cells and which act as receptors for norovirus particles in the human gut.

Binding of human norovirus to PGM was first demonstrated by Tian et al (2005) and the same group subsequently developed a PGM pre-treatment which enabled successful detection of norovirus spiked into various food samples (strawberries, romaine lettuce, 0.25% (w/v) Pacific oyster digestive tissue homogenate) at levels which were undetectable using the unmodified (no pre-treatment applied) viral RNA extraction method only (Tian et al, 2008). It was, however, unclear if the improved detection resulted from the specific binding of virus particles to the PGM-coated beads or the improved removal of RT-qPCR inhibitors using the PGM pre-treatment compared with the unmodified method.

In subsequent years, the PGM affinity binding pre-treatment has been used to investigate norovirus inactivation by high pressure processing in oyster and clam homogenates spiked with faecal material (Ye et al, 2014, Ye et al, 2015). Reductions in RT-qPCR detectable norovirus of >4 $\log_{10}$ were observed following treatments at high pressure and low temperature. However, comparative data using viral RNA extractions without PGM pre-treatment was not generated and therefore it was not possible to determine whether reductions were all down to a loss of PGM binding ability by the virus particles, or if other changes (e.g., denaturation of food matrix protein leading to reductions in extraction efficiency) may have contributed. PGM pre-treatment methods have also been used to investigate the efficacy of high-pressure processing to inactivate norovirus on blueberries (Li et al, 2013). This study demonstrated that immersion in water substantially increased susceptibility of norovirus genotype GI.1 to high pressure, with treatments at 600 MPa for 2 min at 1 and 21 °C resulting in < 1 $\log_{10}$ reductions while a 2.7 $\log_{10}$ reduction was achieved by a treatment at 500 MPa for 2 min at 1 °C when blueberries were immersed in water.

Farkas et al (2018) applied the PGM pre-treatment method to assess the presence of infectious norovirus in a variety of different environmental waters (influent and effluent wastewater, sea, estuarine and river waters, and sediment). A large majority (83%) of wastewater samples that were positive for norovirus using the unmodified viral RNA extraction remained positive when tested using the PGM pre-treatment (albeit at lower levels in most cases), suggesting a high prevalence of intact viruses in these types of samples. Fewer norovirus-positive surface water samples (33%) tested positive with the PGM pre-treatment, however levels determined using the unmodified method were often close to the limit of detection of the assay.

Despite application of the PGM affinity pre-treatment to artificially contaminated food samples to investigate virus inactivation, and to naturally contaminated environmental

samples, to date no publications demonstrating its application to naturally contaminated bivalve shellfish or other foods are available.

3.2. RNase protection methods

In contrast to methods using affinity reagents that aim to preferentially purify intact viruses, a second class of pre-treatments exist that aim to degrade or otherwise make unavailable for amplification free viral RNA and viral RNA in damaged virus particles (presumed to be non-infectious). The first of this type of pre-treatments applied to viruses utilises RNase; this enzyme degrades free RNA and depending on the type and extent of damage, is able to penetrate damaged virus particles and degrade the RNA within but is not able to penetrate intact virus capsids.

This method was first developed by Nuanualaswan and Cliver (2002), who used it to demonstrate inactivation of HAV, poliovirus and feline calicivirus (as a culturable norovirus surrogate) by ultraviolet light, hypochlorite and heating at 72°C. Lamhoujeb et al (2008) applied the method (as a pre-treatment before detection by nucleic acid-based sequence amplification - NASBA - rather than RT-qPCR) to demonstrate heat inactivation of GII norovirus; this showed that feline calicivirus was more sensitive to heat than norovirus and therefore not a good surrogate for inactivation experiments. They also examined persistence of GII norovirus on the surface of refrigerated foods (lettuce and cooked turkey slices) using methods with and without RNase pre-treatment in parallel. This demonstrated that potentially infectious virus persisted for up to 10 days on the surface of foods, although levels did decrease over time. In addition, results were similar both with and without RNase pre-treatment, indicating that at least in the case of slow viral inactivation at low temperatures, nucleic acid detection alone does not necessarily overestimate infectious virus. The persistence of potentially infectious GII norovirus in different foodstuffs (frozen foods, sauces, ready-to-eat salads, mincemeat, fruit, and vegetables) was subsequently measured using a method including an RNase pre-treatment for a wide variety of processes used by the food industry to preserve or disinfect food (heating, cryoconservation, acidification, cooling; Mormann et al, 2010). This demonstrated that although heating was often effective in removing potentially infectious norovirus, other processes had little impact on virus levels.

It has been suggested that the proteinase K-based virus extraction method for bivalve shellfish mandated in the international standard method for foodborne viruses (ISO, 2017) is incompatible with measurement of viral integrity using RNase pre-treatment, as the proteinase itself damages the capsid of infectious virus particles, rendering the RNA of viruses extracted in this way artificially susceptible to RNase degradation and potentially leading to underestimation of infectious virus levels (Langlet et al, 2018).

This complicates the routine application of this type of method to bivalve shellfish and necessitates the use of an alternative virus extraction method that preserves capsid

integrity. Nevertheless, RNase pre-treatments have been applied to both artificially and naturally contaminated bivalve samples in a small number of studies. In an unusual example of a study combining different pre-treatment technologies, Ye et al (2014 and 2015) combined RNase pre-treatment with PGM affinity purification to investigate norovirus inactivation by high pressure processing in oyster and clam homogenates spiked with faecal material. In a study of norovirus genetic diversity in oysters collected from production areas in Japan (Imamura et al, 2017), the authors applied an RNase pre-treatment to the samples before deep sequencing of PCR products amplified from the norovirus capsid gene using Illumina Miseq technology, with the stated aim to "avoid false-positive polymerase chain reaction (PCR) results derived from non-infectious virus particles". Despite the pre-treatment, norovirus was amplified from a considerable proportion of the oyster samples (e.g., 20.6% of samples contained GII norovirus) and considerable diversity of norovirus strains across the samples was identified (8 different GI genotypes and 5 different GII genotypes) indicating a significant prevalence and diversity of potentially infectious norovirus in Japanese oysters.

During the UK Norovirus Attribution Study (NoVAS consortium, 2019) an alternative RNase-based pre-treatment (dubbed the Capsid Integrity Assay - CIA) was elaborated as part of Work Package 2 led by Leatherhead Food Research. In this method, samples are subjected to heat treatment prior to addition of RNase. Free RNA, and RNA from both infectious virus and certain types of damaged virus particles are assumed to be susceptible to degradation following heat treatment, with residual signal due to a heat+RNase resistant type of non-infectious particle called a ribonucleoprotein complex (RNP). In this way, where standard RNase protection methods (with no heat treatment) assume that RT-qPCR signal following pre-treatment is indicative of the presence of infectious virus, under the CIA it is anticipated that samples containing significant proportions of infectious virus will show a reduction of signal after pre-treatment. Experiments with faecal samples and spiked oyster homogenates identified a number of difficulties with the application of the CIA to bivalves; differences in heat sensitivity between different norovirus strains and significant matrix effects on heat sensitivity were observed. Both observations compounded interpretation of detection of heat+RNase resistant particles. The method also exhibited reduced sensitivity compared with the international standard method (ISO, 2017) resulting from the need to use a different RNA extraction method with a reduced starting volume due to problematic interactions between precipitates formed after heat treatment with the magnetic beads used in the international standard.

Nevertheless, the CIA was trialled with 11 naturally contaminated oyster samples collected at the point-of-sale to the consumer, all of which had tested positive at comparatively high levels (>500 copies/g) using RT-qPCR. For 4 samples, an estimate of the percentage of heat+RNase sensitive (and potentially infectious) GII norovirus could be determined by comparing results for the untreated subsample with results for heat plus RNase treated subsamples (for the other 7 samples this was not possible due to high Ct values or

negative replicates in the untreated subsamples). In each case the estimate was >38% indicating significant levels of potentially infectious norovirus in these samples. The CIA was also applied to fresh produce samples in this study and although fewer technical issues were experienced in experimentally contaminated samples, low sensitivity was a problem (in the same way as for oysters) for naturally contaminated fresh produce samples collected at the point-of-sale. Of 9 samples (7 lettuce, 2 raspberries) that had tested positive using RT-qPCR and were subjected to the CIA, an estimate of ~40% potentially infectious virus was obtained for a single lettuce sample (estimates were not possible for the other samples due to high Ct values or negative replicates in the untreated subsamples).

3.3. Monoazides and other nucleic acid binding compounds

In contrast with RNase methods which aim to destroy exposed viral RNA, a variety of pre-treatments have been developed that add nucleic acid binding compounds to the sample. Where free RNA, or unprotected RNA in damaged virus particles is present, this is bound covalently by the compound; this covalent bonding renders the RNA incompatible with amplification by RT-qPCR. The most commonly applied compounds to date have been monoazide dyes. These dyes are photo-inducible, requiring exposure to bright light in order to activate covalent bonding with nucleic acid; the success of the method is therefore dependent on the ability of light to penetrate the sample matrix. Monoazides were initially applied to discriminate between infectious and non-infectious bacterial cells. The first compound used was ethidium monoazide (EMA; Nogva et al, 2003), which was later replaced by propidium monoazide (PMA; Nocker et al, 2006) and its derivatives, due to EMA's disadvantage of being able to penetrate live cells of certain bacterial species.

The first application of PMA to virus testing was described in Parshionikar et al (2010). A number of different enteric viruses including GI norovirus were inactivated using heat treatments and hypochlorite, then detected using a method including a PMA pre-treatment. The method at this stage showed promise with some viruses although results for norovirus were inconsistent; virus that was assumed to be completely heat inactivated was still detectable by RT-qPCR after PMA treatment although conventional (agarose gel-based) RT-PCR signal disappeared.

A number of subsequent publications further investigated and refined the methods for discrimination of infectious virus. Sánchez et al (2012) compared pre-treatments using PMA and RNase for discrimination of viable HAV in suspensions. RT-qPCR signal from HAV inactivated for 5 minutes at 99°C was reduced by >2.40 $\log_{10}$ following PMA pre-treatment, whereas RNase pre-treatment only produced a 0.55 $\log_{10}$ reduction, indicating the greater suitability of PMA at least for monitoring this particular virus/inactivation combination. The use of surfactants in monoazide pre-treatments for viruses including

HAV was investigated by Coudray-Meunier et al (2013), demonstrating that the addition of chemicals such as Triton X-100 to the reaction improved the ability of the method to remove signal from damaged virus particles, presumably by facilitating penetration of the damaged capsids by the dye. Triton X-100 is now routinely used as an additional reagent in monoazide pre-treatments. Other publications indicated limitations of the pre-treatments for virus infectivity discrimination, for example it was suggested that the PMA method was less effective where virus was aggregated (virus particles bound together in clumps) rather than monodispersed (virus particles not bound together; Escudero-Abarca et al, 2014).

The first application of PMA pre-treatment to viruses in food samples, including bivalve shellfish, was reported by Moreno et al (2015). Samples of a number of different foodstuffs including lettuce, parsley, spinach, cockles and coquina clams were artificially inoculated with heat inactivated HAV and subjected to PMA pre-treatment followed by RNA extraction and RT-qPCR, or RNA extraction and RT-qPCR alone. The bivalve test samples (cockle and coquina clam) comprised proteinase K homogenates produced using the ISO 15216 procedure (ISO, 2013). Although the treatment was successful at discriminating infectious and non-infectious virus in the absence of food matrix and somewhat successful at discriminating in vegetable concentrates, in undiluted bivalve homogenates there was almost no difference in RT-qPCR detection in the presence and absence of PMA pre-treatment (<0.5 $\log_{10}$ compared with >3.45 $\log_{10}$ reduction in infectivity as judged by cell culture). However, in 1/10 diluted homogenates reductions of 1.1 to >2.14 $\log_{10}$ were seen using PMA pre-treatment. These results strongly suggested that the turbidity of bivalve homogenates may prevent photo-induction of PMA, however subsequent publications (detailed below) have suggested that a more recently developed monoazide dye PMAxx may be less sensitive to issues with turbidity in bivalve samples. This dye was initially shown to be more effective than EMA, PMA and another newly developed alternative dye named PEMAX for discrimination of heat inactivated GI and GII norovirus (Randazzo et al, 2016). Subsequently Randazzo et al (2018b) used PMAxx pre-treatment for discrimination of infectious and heat inactivated HAV inoculated into lettuce, spinach, mussel, and oyster samples. This study demonstrated that while the PMAxx RT-qPCR method gave results that closely matched infectivity assays when applied to vegetable samples, for bivalve shellfish samples (proteinase K homogenates), reductions in signal were smaller than measured reductions in infectivity, in particular where inoculation levels were high, resulting in overestimations in infectious HAV levels in bivalve samples using the PMAxx method.

Nevertheless, reductions in RT-qPCR signal were larger using the PMAxx pre-treatment than equivalent results with PMA (Moreno et al, 2015), indicating the newer dye's greater suitability for use with bivalve shellfish samples. This study (Randazzo et al, 2018b) also demonstrated a better correlation between PMAxx RT-qPCR and infectivity results with heat inactivation at high temperatures (72-90°C) compared to inactivation at 60°C, possibly indicating that loss of viral infectivity due to "gentler" treatments may not be sufficient to produce changes in capsid conformation sufficient to allow permeation of

PMAxx. This observation of lower losses in PMAxx RT-qPCR signal after treatment at 60°C was also reported for norovirus (Randazzo et al, 2018a). This study further optimised the PMAxx pre-treatment for the bivalve shellfish matrix and applied it to artificially contaminated, bioaccumulated and naturally contaminated bivalve samples. When applied to artificially contaminated oyster, mussel and cockle homogenates, PMAxx pre-treatment always reduced detection of heat-inactivated norovirus GI and GII by RT-qPCR but the extent of reduction was somewhat erratic and dependent on the exact combination of genogroup and bivalve species. In bioaccumulated oysters only partial utility of the PMAxx pre-treatment was observed with some small signal reductions (~1 $\log_{10}$) observed when inactivation using the highest temperatures (95°C) was applied. Finally, the PMAxx pre-treatment was also applied to 5 naturally contaminated oyster samples; in all cases these samples showed reduction in norovirus GI or GII signal of no more than 0.72 $\log_{10}$ following PMAxx pre-treatment. Where these naturally contaminated oyster samples were heat-treated to thermally inactivate noroviruses prior to testing, the PMAxx pre-treatment resulted in generally greater reductions in RT-qPCR signals. This appeared to indicate that the lack of significant signal reductions seen in non-heat-treated samples was not necessarily an artefact caused by technical limitations of the PMAxx method as applied to bivalve shellfish samples but may have resulted from genuinely significant levels of undamaged and potentially infectious norovirus in the 5 samples tested.

In recent years PMA or PMAxx pre-treatments have been applied to bivalve samples in a small number of additional studies. In the majority of cases these studies have used artificially contaminated or bioaccumulated bivalve shellfish samples to e.g., investigate heat inactivation of norovirus and HAV in clams (Fuentes et al, 2021), or the inactivation of norovirus using dielectric barrier discharge plasma treatment on oysters (Choi et al, 2020). Razafimahefa et al (2021) attempted to further optimise the PMAxx pre-treatment method using mussels bioaccumulated with murine norovirus (GV), by for example replacing the proteinase K-based virus extraction method (ISO, 2017) with a method using anionic polymer-coated magnetic beads. In addition, however, another recent study (Sarmento et al, 2020) applied a PMAxx pre-treatment to 16 naturally contaminated norovirus positive jewelbox clam and mussel samples collected from growing areas in Brazil. Individual samples showed variable reductions in RT-qPCR signal with pre-treatment of up to 3 $\log_{10}$, and a statistically significant difference between levels in treated and untreated samples was observed with median values 1.08 $\log_{10}$ lower.

The authors concluded that their results demonstrated that “a large fraction of the detected norovirus corresponded to non-infectious particles”; however, norovirus was notably still detected in all samples after PMAxx pre-treatment, indicating that the results were “still of a concern for potential infection for shellfish consumers.”

While nucleic acid binding infectivity pre-treatments have overwhelmingly used monoazide dyes, a small number of alternative compounds have been investigated, including platinum and palladium compounds that can be chelated by nucleic acids, rendering them

unavailable for amplification as with azide dyes. These compounds were originally used to discriminate between infectious and non-infectious bacterial cells (Soejima et al, 2016) but have been successfully applied to viruses including norovirus (Fraisse et al, 2018), HAV and HEV (Randazzo et al, 2018c) in laboratory-constructed samples. These compounds have the benefit of being cheaper and simpler to apply than monoazides, and do not require photo-induction to bind nucleic acids, and both cited studies (Fraisse et al, 2018, Randazzo et al, 2018c) showed better discrimination using platinum chloride ($PtCl_4$) compared to PMAxx. Platinum compounds carry associated health risks however (Leifels et al, 2020), and to date no studies applying such compounds to virus detection in foods have been published.

3.4. Limitations of pre-treatment methods

Due to a number of factors including incomplete removal of non-infectious materials owing to technical difficulties with method application to bivalve shellfish and other food matrix samples and the inability of methods to discriminate infectious virus and virus inactivated in subtle ways rather than wholesale capsid damage, the possibility that levels of infectious virus may be overestimated by the different pre-treatments described above is often cited by the authors of studies using such methods (Moreno et al., 2015, Randazzo et al, 2016, Rönnqvist et al., 2014). The impact of such overestimation was modelled mathematically by Walker et al (2019), who showed that, particularly in samples where the true proportion of viable virus is very low, moderately effective pre-treatments may still result in significant $\log_{10}$ overestimations after RT-qPCR.

Nevertheless, it is true that even in the case of only partially efficient pre-treatments, positive results ought to better represent detection of infectious virus than in methods using RT-qPCR without viability pre-treatment. However, with the current state of pre-treatment methodologies, results obtained cannot be considered absolute measures of infectious virus. They would ideally need to be assessed in the context of background data on baseline levels of viruses in foods, and levels associated with illness in consumers. Such background data is available for some food/virus combinations using RT-qPCR methods without pre-treatments (Cook et al, 2019, European Food Safety Authority, 2019, Lowther et al, 2012a, Lowther et al, 2012b, Lowther et al, 2018), however to date has not been collected using methods with infectivity pre-treatments.

4. Molecular methods for detection of intact genomes

The pre-treatment methods described above are designed to detect damage to the virus capsid but are unable to detect damage to the viral genome e.g., fragmentation or other modification that may also result in a loss of infectivity. Stressors that damage the genome (e.g., germicidal UV light) do so by modifying bases or causing breaks in the RNA strand (fragmentation) in a random manner (i.e., such modifications or fragmentation can occur at any point in the genome). This type of damage leads to non-infectivity of the affected virus but will only render the virus genome non-detectable by PCR where damage occurs in the RT-qPCR target region. Where the RT-qPCR target region is significantly shorter than the genome it is possible that inactivatory genome damage can occur that doesn't impact PCR detection to a great extent (as only that proportion of genomes in the population which suffer damage in the RT-qPCR target region will become non-detectable). Where the target is much longer, ideally full length, reductions in infectivity and PCR detection should correlate more closely. For the RT-qPCR assays described in the international standard method (ISO, 2017) the target regions are very short; 86, 89 and ~170 bases (depending on the strain) for norovirus GI, GII and HAV respectively, compared to total genomes of >7500 bases. Some molecular methods for detection of longer genome stretches (sometimes approaching full length) have been developed in order to help assess virus infectivity, notably long-range RT-qPCR. In standard RT-qPCR for foodborne viruses (ISO, 2017), the reverse transcription (RT) of the viral RNA is primed by the reverse PCR primer, carried out in the same reaction as the qPCR (one-step RT-qPCR), and is thus insensitive to any genome damage outside of the qPCR target region. In long-range RT-qPCR however, the RT is primed using a primer that binds significantly downstream of the qPCR target region, in a separate reaction, before the addition of the qPCR primers (two-step RT-qPCR). For norovirus and other RNA viruses, the most common priming site for long-range RT is the poly-A tail at the extreme 3’ end of the genome (the RT primer used is therefore a polymeric string of deoxythymidine residues). For some viruses, e.g., HAV or murine norovirus (GV), by combining this RT priming site with a qPCR target region close to the 5’ end of the genome it is possible in theory to detect only genomes that are virtually full length. For human norovirus this is however not possible; due to considerable genomic heterogeneity between human norovirus strains in both GI and GII, only very limited regions of the genome can be used to design broadly reactive qPCR assays. The region targeted by the international standard method (ISO,2017) and virtually all alternative norovirus qPCR assays is the junction of ORF1 and ORF2, which is closer to the 3’ end of the genome than the 5’ end. Nevertheless, combination of this qPCR target region with long-range RT targeting the poly-A tail in theory guarantees that positive results correspond to detection of RNA molecules of >2300 bases.

Long-range RT-qPCR for norovirus infectivity assessment was first investigated by Wolf et al (2009). They first compared long-range and short-range assays for murine norovirus

(GV) as a culturable surrogate for human norovirus; in both cases the RT primer targeted the poly-A tail, while the qPCR target regions were either at a distance of 4600 bases (long-range) or 142 bases from the RT site (short-range). Using long-range RT-qPCR, the signal dropped off significantly following UV treatment (while still overestimating to some extent infectious levels as determined by plaque assay) whereas using the short-range assay, the signal was barely affected by UV treatment. For heat treatment however, neither long- or short-range RT-qPCR was able to model the reduction in infectivity, presumably indicating that this mode of inactivation does not significantly damage the genome. Long-range and short-range RT-qPCR assays for UV-inactivated human norovirus GI and GII were also investigated in this study; the approach was necessarily different than for murine norovirus. Rather than fixing the RT priming site and changing the qPCR target region, for human norovirus the qPCR target region was fixed (ORF1-ORF2 junction) and the RT was either primed using the poly-A tail (long range; 2300 bases remote from the qPCR target region), or the PCR reverse primer (short-range). As with murine norovirus, following UV treatment, qPCR signal reduced considerably using long-range RT-qPCR, but was essentially unchanged using the short-range assay.

In contrast to this study that showed the apparent utility of the long-range RT-qPCR approach for discriminating between infectious and non-infectious virus in some circumstances, a second study applying the same long-range and short-range assays to murine and human norovirus inactivated using UV, heat, and ethanol (Li et al, 2014) found no significant differences in reductions of signal for the two assays. Levels detected by long-range RT-qPCR were however always ~1 $\log_{10}$ lower than short-range RT-qPCR, even using virus that was not inactivated, indicating that the long-range RT priming strategy reduces the overall sensitivity of the method. The GI and GII long- and short-range RT-qPCR assays were also applied in this study to 8 raspberry and 4 oyster samples that had previously tested positive for contamination with human norovirus. There were fewer positive detections using the long-range assay (14/24) compared with the short-range assay (23/24) and the levels detected were generally lower, however considering the reduced overall sensitivity of the long-range assay this is perhaps unsurprising, and the authors considered that the frequent detection of norovirus using the long-range assay indicated the “abundant presence of intact [norovirus] particles”.

In subsequent years, few applications of long-range RT-qPCR assays to discrimination of infectious virus have been reported, however Razafimahefa et al (2021) applied a number of different long-range RT-qPCR assays to murine norovirus inactivated with UV or heat. Long-range assays were generally less sensitive than short-range assays when applied to infectious virus, with the reduction in sensitivity increasing with the remoteness of the RT-priming site from the qPCR target region.

All long-range assays showed significantly greater signal reduction following UV inactivation of virus than equivalent short-range assays, with the differences increasing with the remoteness of the RT-priming site from the qPCR target region, but differences in

signal reduction between long-range and short-range assays was more modest following heat inactivation. Interestingly, this study also applied long-range assays in combination with PMAxx pre-treatment. Signal reductions were significantly larger for PMAxx-long-range assays after both heat and UV treatments than for short-range assays (without PMAxx pre-treatment). For heat treatment the majority of the difference was explained by the use of PMAxx, while for UV treatment most of the difference was explained by the use of long-range RT-qPCR, indicating that combining these approaches in a single assay may reduce detection of virus that has been inactivated due to either capsid or genome damage.

With the increasing development of single RNA molecule sequencing techniques (Garalde et al, 2018) there is a prospect of identifying full length viral genomes using such methods. Batista et al (2020) used nanopore-based sequencing to determine the whole genome sequence of HAV in tissue culture supernatant. In this study they were able to demonstrate the presence in the sample of intact genomes with reads of up to 7667 bases although the large majority of sequencing reads were <2000 bases; it is not clear whether this was a reflection of genuine fragmentation of the genome, or an artefact of the sequencing method. Even under the best circumstances however, whole genome sequencing of viruses contaminating food samples is very difficult due to extremely low target levels, and generation of sequence data is normally dependent on amplification of short fragments of the genome by PCR (Desdouits et al, 2020). Therefore, the use of single molecule sequencing methods for infectivity discrimination does not seem to be a realistic short-term possibility.

5. Estimation of infectivity using culturable indicator viruses

Culturable enteric viruses have frequently been proposed as indicators of pathogenic virus contamination in foods, particularly bivalve shellfish. Amongst the most frequently proposed indicator is F-specific RNA bacteriophage (F-RNA phage; Flannery et al, 2009, Hartard et al, 2016) a group of related RNA viruses belonging to the genera *Emesvirus* and *Qubevirus* and infecting coliform bacteria. Lowther et al (2019) elaborated a method to estimate levels of infectious norovirus in oyster samples by applying an infectivity ratio (determined by comparing levels of infectious and RT-qPCR detectable genogroup II F-RNA phage) to levels of norovirus as determined by RT-qPCR. Calculated infectivity ratios were <10% in the majority of oyster samples (83.4%) however estimated infectious norovirus levels were significantly higher in outbreak-related oyster samples (geomean of 24.8 infectious norovirus/g) compared with non outbreak-related samples collected at the point-of-sale to the consumer (geomean in positive samples of 0.5 infectious norovirus/g). Infectious GII F-RNA phage was found in 30.4% of non outbreak-related samples

(compared with RT-qPCR detection in 78.1% of samples) and 37.1% of non outbreak-related samples that were positive for GII F-RNA phage by RT-qPCR. Infectious GII F-RNA phage was found in all outbreak-related samples. Despite the apparent correlation between estimated infectious norovirus and health risks found in this study, limitations of this approach include the need to carry out parallel infectivity and RT-qPCR tests for F-RNA phage in all samples, and the assumption that infectious proportions of norovirus and F-RNA phage will vary in the same way across samples from a wide variety of sources.

6. Summary

- The recent development of norovirus cell culture methods using human intestinal enteroids has considerable potential for investigating inactivation methods of relevance to the food industry. However, at present these methods are not suitable for detection of viable norovirus in naturally contaminated food samples due to their complexity, low sensitivity, and variable performance dependent on norovirus strain. In addition, these methods are presently not considered quantitative.

- Indirect methods for determination of virus infectivity in food samples are more realistic in the near to medium term. These include:

    - Pre-treatments to increase the proportion of intact virus particles subjected to RT-qPCR (e.g., RNase protection, monoazide treatments)
    - Modified molecular methods to reduce detection of short RNA fragments (e.g., long-range RT-qPCR)
    - Use of culturable indicator viruses to estimate infectious norovirus (e.g., F-RNA phage)

- All these methods have limitations:

    - Individual methods may be effective only for certain types of viral inactivation
    - Incomplete removal of RNA from damaged particles using pre-treatments may lead to overestimation of infectious virus
    - Lower inherent sensitivity of long-range RT-qPCR may result in failure to detect low levels of infectious virus
    - Use of indicator viruses relies on the assumption that they have been subject, and have responded in the same way, to the inactivatory stresses experienced by the target virus.

- A lack of equivalent baseline data in naturally contaminated foodstuffs makes the interpretation and contextualisation of the results of indirect methods difficult.

- If carefully considered and validated, indirect methods can provide a more realistic demonstration of presence and levels of potentially infectious virus in food samples than RT-qPCR alone, can provide confirmation or otherwise of RT-qPCR results that can assist interpretation, and can be used to e.g., investigate inactivation methods of relevance to the food industry more simply and cheaply than target virus cell culture methods.

- The newly developed monoazide dye PMAxx appears the most successful and widely applied pre-treatment at present and is more easily photo-inducible in turbid samples such as bivalve homogenates compared to older monoazides such as EMA and PMA. Platinum compounds such as platinum chloride have also shown promising results but concerns over their toxicity may limit their routine use.

- Pre-treatments (e.g., PMAxx) that reduce signal from viruses with damaged capsids can in theory be combined with molecular detection methods (e.g., long-range RT-qPCR) that reduce signal from viruses with damaged genomes, to better monitor virus inactivation caused by a wide variety of mechanisms. This combination approach has shown some promise in laboratory investigations but to date has been applied in very few studies.

- To date, norovirus infectivity methods have been applied to naturally contaminated food samples (predominantly bivalve shellfish) in only a limited number of studies. In most cases levels detected using the infectivity method were lower than with RT-qPCR alone, suggesting that RT-qPCR alone overestimates levels of infectious virus in food samples. Most studies, however, found that a considerable proportion of RT-qPCR positive samples also contained potentially infectious virus as detected using the infectivity method. Given the low infectious dose of norovirus these results appear to indicate that it cannot be assumed that RT-qPCR positive samples are safe until proven otherwise using infectivity methods.

- The development of infectivity methods for foodborne viruses is a rapidly developing area and technologies that are not currently applied e.g., use of microfluidic separation of intact viruses from the test matrix, may be used in the future. The NRL will continue to monitor the relevant literature.

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