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Detection of foodborne viruses in berries – State of science and future considerations

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ABSTRACT

Enteric viruses are the leading cause of foodborne disease, with human norovirus (HuNoVs) the most prevalent, and hepatitis A virus (HAV) the more severe. Fresh and frozen berry fruits are a recognized vehicle for transmission, gaining increased international attention. The detection of these viruses is complicated because: (i) they cannot be cultivated routinely *in vitro*; (ii) their concentrations in foods are frequently low; (iii) and sample matrices are complex. ISO- standardized methods, released in the last decade, are widely used, but there remain complexities in their applications, interpretations, and risk-based decision making based on results. This paper describes deliberations of an International Expert Panel asked to address the following: (i) methods most often used to detect viruses in fresh and frozen berries; (ii) role of sampling in test reliability; (iii) means by which testing results are interpreted; (iv) typical uses of testing by various stakeholder sectors; (v) role/use of confirmatory testing; (vi) how testing results are used by various stakeholder sectors; and (vii) the overall value of testing. Critical unanswered questions are discussed, such as the relationship between RT-qPCR positive results and infection risk (virus infectivity) and the role of testing in risk management. Perhaps the most comprehensive work of its kind, this paper highlights the unique challenges posed by emerging molecular-based detection methods applied to non-cultivable foodborne pathogens and sets a stage for the questions that beg answers as these methods become more widely and routinely used.

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1. Introduction and context

In most of the world, foodborne viruses are recognized as an important, if not the leading cause of foodborne disease (Ahmed et al., 2014; Hall et al., 2016). Among foodborne viral outbreaks, human noroviruses (HuNoVs) are the most common cause, while hepatitis A virus (HAV) presents with the more serious illness. Both viruses are characterized by a low infectious dose, transmission via the fecal-oral route, environmental persistence, and resistance to many food safety control measures, all contributing to ease of spread by foodborne routes. Unlike bacterial foodborne contaminants, which can be readily grown in the lab, producing a live culture on which further confirmation and characterization can be done, routine cultivation of foodborne viruses is not possible. Significant effort has gone into developing alternative methods to detect viruses in foods, all of which rely on nucleic acid amplification methods, usually reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The current global gold standard virus testing method is the ISO 15216–2:2019 (ISO, 2019), but other protocols are used in different geographical regions, and equivalency of methods is often not established. This complicates food safety management efforts, as manifested in issues associated with sampling, test method choice, interpretation of results, and the association between positive detection findings and public health risk. To document the scope and significance of these issues, with a specific focus on the berry fruit supply chain, an International Expert Panel was convened in 2020, with deliberations delayed by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS CoV-2) pandemic. Specifically, and consistent with the approaches taken by bodies such as the U.S. National Academies of Sciences (NAS) and the National Advisory Committee on Microbiological Criteria for Foods (NACMCF), the Expert Panel was asked to deliberate on specific questions related to the topic. This paper summarizes those deliberations, providing both background and responses to the following seven questions:

- *Question #1: What methods are used to detect foodborne virus contamination in fresh and frozen berries and their products, and how do they compare to one another?*
- *Question #2: How is the information gained from foodborne virus detection in berries being used by various stakeholder sectors (academic, industry, government/regulatory)?*
- *Question #3: What is the sampling approach taken when instituting foodborne virus testing by the various stakeholder sectors (academic, industry, government/regulatory)?*
- *Question #4: How are the RT-qPCR testing data interpreted relative to sample status (positivity, negativity, or undetermined)?*
- *Question #5: Is additional laboratory testing being performed on RT-qPCR-positive samples to confirm and/or characterize genome sequences? If so, what methods are used and/or in the pipeline?*
- *Question #6: How are the data from the totality of the testing process (i.e., sampling, virus concentration and purification, and RT-qPCR) interpreted relative to lot or batch acceptability?*
- *Question #7 (Synthesis): Based on the totality of information known about the methods and their interpretation, what is the overall value of testing for foodborne virus contamination in fresh and frozen berries?*

1.1. Background

Fresh fruit and vegetable consumption has been increasing worldwide, particularly in many industrialized countries (Li et al., 2015), with berries of all types leading the consumer consumption trend (Kårlund et al., 2014). Human noroviruses and HAV were responsible for 72.3 % (68/94) of berry-associated foodborne outbreaks and more than 80 % of berry-related illnesses globally between 1983 and 2018 (Bozkurt et al., 2021). Because berries are often consumed raw or minimally processed, if virus-contaminated they can present an elevated public health risk; frozen berries also have an extended shelf life and are stored under conditions that preserve foodborne viruses (EFSA, 2014). Nevertheless, despite the increased importance of berry fruits as a vehicle for virus transmission, there is limited information concerning the prevalence and fate of viruses in the berry supply chain (Bozkurt et al., 2021).

Foods usually become contaminated with foodborne viruses by direct or indirect contact with human fecal matter (fecal-oral transmission) or occasionally, vomitus (de Wit et al., 2007), either from a single infected person or collectively, such as through untreated human sewage (de Wit et al., 2007; Mäde et al., 2013). The specific means of contamination varies by food commodity. For berries, potential sources of contamination include the following: virus-contaminated water/-wastewater/pesticides/fertilizer or planting in contaminated soil (pre--harvest); or manipulation of foods by infected food workers who have failed to practice appropriate personal hygiene measures (harvest or post-harvest) (Bozkurt et al., 2021; Derrick et al., 2021). The former tends to result in more widespread, diffuse contamination while the latter usually is more focal in nature, although comingling product can dilute but spread contamination. Often the exact details of how and where in the supply chain a frozen berry product becomes contaminated with foodborne viruses is difficult to ascertain, but it is more likely to occur prior to freezing.

1.2. Epidemiological significance of berries in foodborne virus outbreaks

Callejón et al. (2015) reported that HuNoVs were responsible for 55–60 % of the outbreaks associated with fresh fruits and vegetables in the U.S. and Europe during the period 2004–2012. Fresh berries were implicated in about 2 % (5/223) of the U.S. outbreaks but 51 % (55/108) of the EU outbreaks. Focusing on the U.S. alone, Bennett et al. (2018) reported that 54 % of outbreaks associated with fresh produce were caused by HuNoVs, 2 % by HAV. Specific to viruses, Chatziprodromidou et al. (2018) produced a systematic review of virus outbreaks linked to fresh and frozen produce reported in the literature through 2017, with global representation. These authors demonstrated the role of frozen over fresh berries as causative vehicles. Specifically, 55 % (84/152) of all identified outbreaks were linked to frozen berries, juxtaposed to <1 % (1/152) caused by fresh berries. Frozen strawberries, raspberries and berry blends were the major culprits. Relative to fresh produce, the most often implicated produce types in 29 % (70/245) of alert notifications pertaining to viruses (1998–2016) issued by the EU Rapid Alert System for Food and Feed (RASFF) were raspberries, blackberries, blueberries, strawberries, berry and fruit mixes, as well as dates and lettuce (ISBN: 978-1-905767-74-8, 2017).

Bozkurt et al. (2021) produced a very comprehensive review of foodborne viral outbreaks linked specifically to berries between 1983 and 2018, identifying a total of 68 outbreaks globally, resulting in 18, 851 illnesses. Human norovirus was the most common causative agent, implicated in 68 % (46/68) of the outbreaks with over 15,000 cases. The most frequently involved berry types were strawberries, raspberries, and berry mixes. For HuNoVs, China, Poland, and the Republic of Serbia were the most common countries of contaminated product origin, although HAV-contaminated product also came from Egypt, Morocco, Argentina, Chile, Mexico, and Turkey. The same authors noted that 50/68 (73.5 %) of these outbreaks were caused by frozen berries, of which 72 % (36/50) were HuNoVs and 28 % (14/50) were HAV. The HAV numbers accounted for more than 70 % of worldwide foodborne outbreaks associated with this virus. Again, frozen strawberries, raspberries and berry mixes caused most of these outbreaks. For example, frozen raspberries were responsible for more than 80 % of the HuNoV outbreaks while frozen berry mix (44 %) was linked to the majority of the HAV outbreaks. Collectively, the findings of these reviews illustrate the epidemiological significance of berries, particularly frozen products, in contributing to reported foodborne virus-associated outbreaks, an observation which has also been noted by others (Müller et al., 2015; Sarvikivi et al., 2012).

Unfortunately, the epidemiological significance of fresh and frozen berries to the overall viral foodborne disease burden, in comparison to other food commodities, is not well characterized. In an early U.S. study, Hall et al. (2012) used voluntary state reporting data on foodborne HuNoV outbreaks (2001–2008) to identify "fruits and nuts" as the second most prevalent food commodity responsible for outbreaks. Berries would be included in this group. For about 40 % of those outbreaks, a contamination source could not be found; for over 50 %, product was contaminated during preparation or service. There were very few outbreaks in which contamination was found to be associated with production or processing, although for fresh and frozen berries on the market, this would be the more likely contamination route. Bozkurt et al. (2021) reported that HuNoVs and HAV were responsible for 72.3 % (68/94) of berry-associated foodborne outbreaks and more than 80 % of berry-related illnesses globally between 1983 and 2018, suggesting that when berries are contaminated with foodborne pathogens, they are likely to be viruses. However, recognized berry-associated foodborne viral outbreaks occur infrequently relative to the overall high degree of consumption of these products. But that does not make them unimportant. In the recent (2023) FAO-WHO consultation on foodborne viruses, experts ranked HuNoVs and HAV in berries as among the leading viral foodborne disease risks (https://www.who.int/publications/m/item/jemra-of-viruses-in-foods-part1-food-attribution-analytical-methods-and-indicators). This suggests that the virus-berry pair is of substantial interest to the international food safety community.

1.3. Virus survival and persistence in freezing

Human noroviruses and HAV are environmentally stable under ambient conditions of temperature and relative humidity. They can survive adverse environments and are recalcitrant to extremes of pH and many commonly used food processes and sanitation practices, including most disinfectants (D'Souza et al., 2007). For this reason, the consensus is that they persist for long periods of time and are difficult to inactivate. Butot et al. (2009, 2008) demonstrated that freezing reduced concentrations of HAV, HuNoVs (GI and GII), and rotavirus in strawberries, raspberries, and blueberries, as evaluated using RT-qPCR, by less than one $\log_{10}$ over 90 days, and that HAV and rotavirus remained infectious over long-term frozen storage. Clearly, freezing has minimal effect on the infectivity of these non-enveloped viruses in berries, so if a pre-packaging contamination event occurs, the pathogens will continue to pose a risk to public health, likely through the duration of product shelf-life, unless subjected to an effective inactivation process prior to consumption.

1.4. Quantitative microbial risk assessment (QMRA) findings for contamination and human disease risk from viruses in berries

A review of the literature revealed a total of five published risk assessments addressing foodborne virus contamination and associated public health risk in berries. These focused on the two major contamination routes, i.e., infected pickers and/or packers, and virus-contaminated waters. In the first comprehensive work, Bouwknegt et al. (2015) developed a farm-to-fork QMRA model to explore the relative importance of various combinations of human handling (picking/packing), cross-contamination (conveyor belt), and water (drip irrigation) on HuNoV and HAV contamination of fresh berries (raspberries, strawberries). Overall, the simulations produced negligible risks, with neither virus predicted to be found in the berry supply chains, within the quantification capabilities of the model.

Jacxsens et al. (2017) produced a quantitative exposure model looking at HuNoV contamination along the Individual Quick Frozen (IQF) soft red raspberry supply chain, concluding that relative to water contaminated at documented frequencies, the combined effect of five or more infected pickers during harvesting was the major contributor to contamination (>95 %). For a one-day batch of 11 tons, the mean HuNoV concentration ranged from 0.5 to 36.2 particles/kg, depending upon the number of infected pickers. Proper hand hygiene reduced HuNoV concentrations in the raw product to ≤0.2 particles per kg. Sobolik et al. (2021) produced a QMRA to evaluate the effects of farm-based hand hygiene and worker furlough practices on the risk of HuNoV infection from raspberries. When an identified infected worker is on the premises, combinations of high handwashing compliance (100 %) and efficacy (>5 $\log_{10}$ removal) were necessary to produce infection risks below a threshold of <0.032 infections per consumption event. On the other hand, using community-based HuNoV prevalence estimates, single interventions like handwashing with 3 $\log_{10}$ virus removal or furlough of the worker reduced maximum infection risk to below that threshold.

Miranda and Schaffner (2018) published a QMRA model intended to replicate the condition(s) of the large (>11,000 cases) 2012 HuNoV outbreak associated with consumption of contaminated frozen strawberries in Germany. The model explicitly assumed that all contamination originated from surface water used for pesticide dilution during berry production. Examining the sensitivity of the model on a variety of input parameters [i.e., (i) HuNoV concentration in surface water; (ii) pesticide application; (iii) HuNoV prevalence in surface water; (iv) pesticide strength; (v) truck temperature; (vi) washing step; (vii) sanitizing step; and (viii) food service preparation (heat)], they found the model to be most sensitive to the concentration of HuNoVs in water (assuming 8 $\log_{10}$ genome copies/L), which resulted in up to 8000 illnesses. The assumption about the concentration of viruses in pesticide water was the only one capable of causing an outbreak of this magnitude and supported the conclusion that the 2012 outbreak resulted from the use of a highly contaminated water source applied to a large amount of product just prior to harvest.

The approach of Zhong et al. (2023) was like that of Miranda and Schaffner (2018) but with a focus on estimating the probability of infection with HAV or HuNoVs in a Chinese population consuming imported strawberries. These authors focused on three product types [fresh unprocessed, fresh processed, and frozen strawberries] imported from the U.S. and Australia. The main factors affecting the concentration of virus on consumed product were product type-specific but most largely associated with those variables impacting the two major contamination routes, i.e., human hands (i.e., prevalence, virus concentration, and transferability) and pesticide water (i.e., virus decay rates and time between last pesticide application and harvest). Likewise, the estimated mean concentration of virus in contaminated berries was product-specific, with fresh processed showing the lowest concentration [<0.001 PCR Detection Units (PDU)/berry) and frozen strawberries having the highest (~1.0 PDU/berry) When linked to dose-response models, the risk of illness showed the same trends by product type, with probability of illness never exceeding $\sim 5.0 \times 10^{-3}$ (95 % CI) and oftentimes in the 10^{-4} to 10^{-5} range.

While modeling exercises are not directly comparable for myriad reasons, there are a few overarching conclusions. Firstly, there have been more efforts taken to characterize HuNoV than HAV risks. Secondly, most of the effort has gone into exposure modeling. When extrapolated to human infection or disease risk, those are usually quite low, except for a major water event such as that modeled by Miranda and Schaffner (2018). It appears that contamination via water is more likely to result in diffuse virus distribution within a lot or batch, whereas human handling will be more focal in nature. Comingling product will distribute the contaminant more widely, but by how much, has not yet been modeled. In short, frozen berries are more associated with viral foodborne disease outbreaks than are fresh, likely due to myriad factors including long shelf lives, amenability to epidemiological investigation and attribution, and also because of supply chain practices such as more global sourcing and reliance of comingling product from many smaller farms.

1.5. Key conclusions

- Foods that are prone to exposure to human fecal material are at elevated risk of contamination with viruses.
- Epidemiologically speaking, HuNoVs and HAV are the two most significant viruses that cause produce-associated foodborne illness.
- Berries are epidemiologically linked to a larger proportion of produce-associated HuNoV and HAV outbreaks than is any other single produce commodity classification.
- Frozen berries, rather than fresh, have a greater likelihood of being attributed to foodborne viral disease outbreaks.
- Virus-contaminated berries originate more often from certain regions of the world (i.e., China, and parts of Eastern Europe, the Middle East, and Central and South America).
- If berries were to become contaminated with HuNoVs or HAV before freezing, the virus would be expected to remain infectious throughout the duration of product shelf-life.
- Based on QMRA, human viral disease risks associated with the consumption of (mostly) frozen berries are quite low, except in the case of a highly contaminated water source applied just prior to harvest, which results in diffuse contamination at high virus concentration. Contamination due to poor personal hygiene of infected pickers and packers results in focal "pockets" of viral contamination which yield overall lower public health risk (less cases of disease).

2. Question #1

What methods are used to detect foodborne virus contamination in fresh and frozen berries and their products, and how do they compare to one another?

2.1. Overview of virus detection in foods and environmental samples

Historically, the development of pathogen detection methods in foods and environmental samples has been driven by a number of important considerations: (i) an anticipated low concentration of the pathogen in naturally contaminated product; (ii) a high degree of heterogeneity in the distribution of the pathogen in the product; (iii) the need to screen fairly large samples sizes (≥25 g) and/or multiple samples; and (iv) the need to assure that the pathogen is viable or otherwise able to cause disease in a consumer. In classic food microbiology, these issues are dealt with in part using cultural enrichment, aimed to increase the concentration of the target pathogen, while suppressing the growth of competitors and diluting out matrix-associated inhibitors. The end-product of a successful positive test result is a viable culture that can be further screened to provide additional information on phenotype, genotype, serotype, virulence, and infectivity, among other pertinent features. Standard protocols that rely on this approach are available for important bacterial foodborne pathogens such as *Salmonella enterica* and *Listeria monocytogenes*.

Detecting viruses in food and environmental samples is much more complicated. This is driven by many factors, perhaps the most significant of which is the absence of *in vitro* cultivation methods that can be routinely used to propagate wild-type virus in naturally contaminated samples. This makes virus detection a poster child for what is now referred to as "non-culture-based detection methods." The general approach to detection of viruses in food and environmental samples consists of two major components: sample preparation (virus elution, concentration and purification from the sample matrix, including nucleic acid extraction); and detection of viral nucleic acid (usually done by molecular amplification, i.e., RT-qPCR). Various methods have been developed over the last three decades and have been comprehensively reviewed elsewhere (Bosch et al., 2011; Haramoto et al., 2018; Ikner et al., 2012). It is important to note that the endpoint for detection for all these methods is an amplified copy of a fragment of the viral genetic material, ***not*** an infectious virus culture.

The goal of the sample preparation steps is to separate, concentrate, and purify the viruses from the sample matrix, and to reduce the total sample volume while maximizing virus recovery. It is performed on a relevant sample size (e.g., 2 g digestive diverticula for molluscan shellfish; 10–50 g for solid foods; 100 or more ml for liquid samples) and is intended to achieve the following: (i) release of viruses from adherence to the sample matrix, leaving them in suspension (elution); (ii) removal of food substances from the resulting eluate (done by sedimentation, precipitation, centrifugation, among other methods); and (iii) virus concentration into a smaller volume (done by precipitation, centrifugation, flocculation, among other approaches). Historically, there are many protocols that have been reported in the literature for concentration and purification of viruses from foods (e.g., Bosch et al., 2011; Hrdy & Vasickova, 2022). Most capitalize on various characteristics of non-enveloped viruses, such as their ability to behave as proteins in suspension; their tenacious, charge-based attachment to matrix particulates; and their ability to withstand exposure to organic solvents and various enzymes without loss of capsid integrity. In almost all instances, two or more steps must be used sequentially, and the choice of those steps is matrix specific.

After virus concentration and purification, viral RNA is extracted, resulting in further sample volume reduction (generally to 50–100 μl) and additional purification. Many approaches have been used (Hrdy & Vasickova, 2022), but those relying on guanidinum isothiocyanate (GTC) for capsid disruption followed by one or more secondary nucleic acid clean-up steps are the most common. A good RNA extraction method should be able to recover viral RNA with high efficiency; remove most of the organic materials in the virus concentrate that can interfere with reliable downstream RT-qPCR detection; and produce an RNA concentrate of low volume. Ideally, the collective process of sample preparation should be simple, efficient, practical and robust. In reality sample preparation is often cumbersome, inefficient, and results in a concentrate in which matrix-associated inhibitors persist.

Downstream detection of viral RNA is done by nucleic acid amplification, specifically RT-qPCR. The specificity of the RT-qPCR reaction is largely determined by the selection of primers and probes. The assay sensitivity can be affected by many different factors, including but not limited to buffer composition, annealing temperature, cycling conditions, enzyme efficiency, and degree of matrix-associated inhibition. The ORF1-ORF2 junction is generally recognized as the most conserved region of the genome for HuNoVs and is usually targeted for genogroup-specific detection (Cannon et al., 2017; Kageyama et al., 2003; Lees, 2010; LeGuyader et al., 2009; Stals et al., 2012). The most recent protocol for detection of HuNoV RNA includes amplifying the 3′-end of ORF1 and 5′-end of ORF2 in a single genogroup-specific reaction with a sensitivity as low as five RNA copies per reaction for GI and 50 copies for GII HuNoVs (Chhabra et al., 2021). The most often used method for detecting HAV RNA targets the 5′ NCR for detection, and the VP1/2A region for strain typing (Costa-Mattioli et al., 2002; Costafreda et al., 2006; Sánchez et al., 2004). A recently developed HAV RT-qPCR protocol enables both sensitive detection and differentiation of IA, IB, and IIIA strains. It consists of two assays, a monoplex targeting the 5′NTR for detection, and a triplex assay for genotyping, although this assay has not yet been tested in food samples (Probert & Hacker, 2019).

Nucleic acid amplification assays can be designed to be qualitative (presence/absence) or quantitative. The latter is achieved by creating a standard curve, usually done by amplifying serial dilutions of a known quantity of virus, extracted viral RNA or transcript RNA, and plotting those concentrations (X-axis) vs. fluorescent signal value (Y-axis). Alternatively, cDNA can be used as the template for standard curves, ideally with some knowledge about the efficiency of the RT reaction. The nomenclature describing the fractional PCR cycle used for quantification is inconsistent, with cycle threshold (Ct), crossing point (Cp), and take-off point (TOP) all used in the literature. All three terms refer to the same value from the real-time instrument and correspond to the PCR cycle number at which the sample's reaction curve intersects the

threshold line. Recognizing that different laboratories use different nomenclatures, we will use the term quantification cycle (Cq), in accordance with the RDML (Real-Time PCR Data Markup Language) data standard (http://www.rdml.org). This is also consistent with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009). Extrapolation of the Cq value of an unknown sample to the concentration of target gives an approximation of the number of genome equivalent copies (GEC) in an unknown sample, which serves as a proxy for virus concentration. Standard curves are also an important component of calculating detection limits for qualitative assays.

Several controls and/or standards, designed to assure that each step in the process has proceeded appropriately and efficiently, are included in each assay. These are used to assure the absence of cross-contamination, the efficiency of the sample preparation steps, and the absence of residual matrix-associated inhibition. Up to four control reactions are included in the assay: (i) traditional negative control (target-free water or buffer as template); (ii) traditional positive control (usually reference strains extracted for RNA isolation or plasmids containing all or part of the viral genome); (iii) sample extraction or process control(s); and (iv) an internal or external amplification control. The extraction process control virus is usually a nonpathogenic cultivable virus [e.g., mengovirus MC0, murine norovirus (MNV), bacteriophage (MS2), or others] that is added in a known concentration to the sample being tested prior to virus concentration and purification. A fragment of the genome of this virus is amplified by RT-qPCR at the same time as is the target. For those assays that include an amplification control, this is often a non-target nucleic acid sequence added to the sample amplification. Both the extraction and amplification controls can be used to indicate if residual matrix-associated inhibition is impeding amplification of the target nucleic acid (reviewed by Hoorfar et al., 2004). The extraction process control virus also serves to monitor the efficiency of the upstream sample preparation steps. Failure to amplify either of these controls suggests residual matrix-associated inhibition (which calls for dilution of the concentrate with re-amplification) or, in the case of the latter, poor efficiency in the sample preparation steps (which invalidates the assay and necessitates starting again from the beginning). Depending upon the protocol, other controls may also be included.

If all controls perform adequately, the endpoint of a positive test will be the presence of a Cq value indicating successful amplification of the target viral RNA fragment. Also required is that the curve displays the typical sigmoidal shape. Specific criteria (such as exact Cq value range or replicate amplifications) for determining sample positivity are discussed elsewhere in this document. Although not technically specified in most protocols, a sample that tests positive by RT-qPCR is by some users, considered a positive test result with no need to proceed further. By others, an RT-qPCR positive result might be considered 'presumptive,' with further confirmatory steps recommended or even required. This, too, is discussed later.

2.2. Standardized methods for detection of foodborne viruses in berries

2.2.1. Berries: A special case

Berries are one of the more challenging food matrices for extraction and detection of HuNoV and HAV. This appears to be due to (i) the relatively poor efficiency of virus concentration and purification steps in the berry matrix; and (ii) the presence of residual inhibitors even after extensive sample preparation. Further variability in method performance may be impacted by product type, growing and processing conditions, and sample-to-sample variation (Zhou and Li, 2020). For frozen berries in particular, gel-like pellets often appear after polyethylene glycol (PEG) precipitation, a cornerstone step in many virus extraction methods. Zhou and Li (2020) hypothesized these pellets occur because of the release of pectin, which forms a complex with PEG that can entrap viruses, preventing them from being released into the retained aqueous layer. The pectin complexes are recalcitrant to current commercial pectinase products. The same authors also noted that residual phenolic compounds in berry RNA extracts can inhibit nucleic acid amplification.

In this paper, we are focused on the two widely used for detecting HuNoV and HAV in berries: the international standard ISO 15216 methods, and the US FDA Bacteriological Analytical Manual (BAM), Chapter 26 method. There are other methods that might be used on a per-country basis, and still others in development. Several studies have characterized virus recovery efficiency from berries, and residual matrix-associated inhibition. Such studies are usually in the context of comparing the international ISO 15216 standard to one or more experimental methods in development. In most cases, reported extraction efficiencies for HuNoV and/or HAV associated with the ISO 15216 method are low, <10 % and sometimes much lower (Bartsch et al., 2016; Fraisse et al., 2017; Plante et al., 2024; Raymond et al., 2021). Methods modifications in the form of added nucleic acid purification steps have been shown to improve virus yields and detection limits, and/or decrease inhibition (Fraisse et al., 2017; Park et al., 2021; Rajiuddin et al., 2020; Raymond et al., 2021; Trudel-Ferland et al., 2024). Use of digital RT-PCR in place of RT-qPCR has been shown to improve method performance (Fraisse et al., 2017) and there is some indication that this amplification method is less influenced by inhibitors (Sun et al., 2019). Collectively, the combination of relatively poor extraction efficiency and the need to dilute sample concentrates before subjecting them to RT-qPCR due to residual matrix-associated interference can result in an assay of reduced analytical sensitivity, with increased chances of false negative results.

2.2.2. The International Organization for Standardization (ISO) methods

In 2004, the European Committee for Standardization (CEN) Working Group on Microbiology of the Food Chain appointed a technical advisory group (CEN/TC275/WG6/TAG4) with the task of developing a European standard method for the detection of foodborne viruses (HuNoVs and HAV) in representative foods [i.e., bivalve shellfish; soft fruit (which includes berries); leaf, stem and bulb vegetables; bottled water; and food surfaces) using RT-qPCR. The result was eventually adopted as the joint International Organization for Standardization (ISO)/European technical specification, designated ISO/TS 15216 (ISO, 2013). The two parts of this document [Part 1 (−1) for Quantification and Part 2 (−2) for Detection) are broadly similar but differ in the number and type of controls applied. Validation data from a series of single and inter-laboratory studies conducted by an international group of scientists (2012–2014), including data for berries, were added to these documents when they were reissued as full standards in 2017 (ISO 15216–1:2017; method for quantification) and 2019 (ISO 15216–2:2019; method for detection), respectively (Lowther et al., 2019). The ISO 15216–2:2019 for detection is currently the most widely used method for foodborne virus elution, followed by concentration, purification, and detection in berries, and has been embraced by most of the world, including regulatory agencies, industry, testing labs, and academic laboratories.

Over many years, data have been amassed for the ISO 15216 methods, the vast majority of which have focused on molluscan shellfish (Bigoraj et al., 2024; Dirks et al., 2021; Mangeri et al., 2024). The ISO 15216 protocols for soft fruits differ from that for shellfish, mostly because of differences in the matrices and in the localization of viral contamination. Molluscs are filter feeders that concentrate viruses in their digestive diverticula during production, and those tissues are exclusively processed for virus detection. On the other hand, viral contamination in soft fruits localizes predominantly on the product surface, necessitating virus elution before further sample processing steps are undertaken. Based on comparisons between monitoring studies done on molluscan shellfish vs. fresh and frozen berries, the overall frequency of virus contamination is higher in shellfish and the proportion of samples with high levels/lower Cq values is larger for shellfish (Li et al., 2023; Romalde et al., 2018; Bozkurt et al., 2021; see also Table 3).

The ISO 15216–2:2019 method for soft fruits is diagramed in Fig. 1

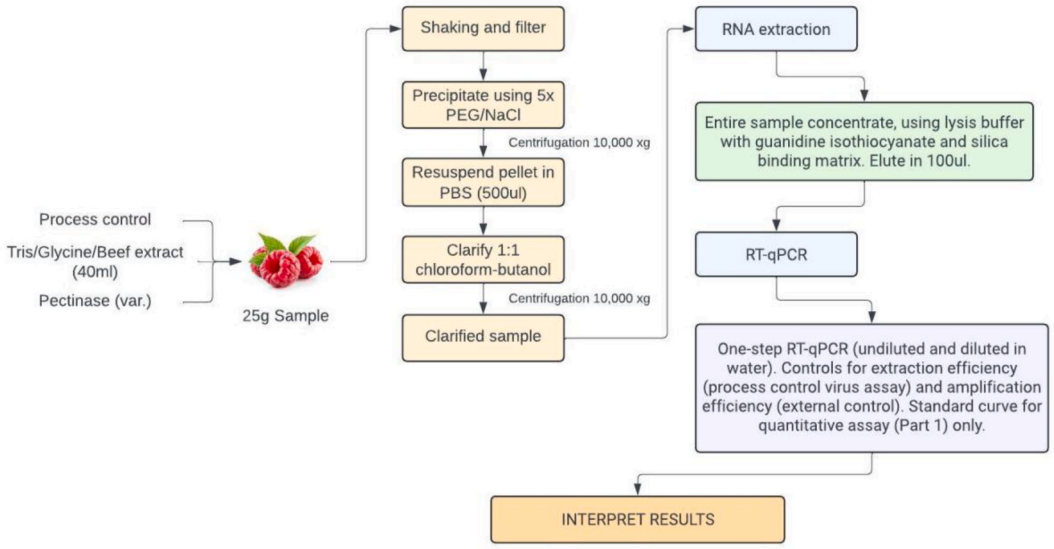

Figure Q1.A. Schematic diagram of the ISO 15216 method for foodborne virus extraction and detection from soft fruits.

and described below. Beginning with a 25 g berry sample, the viruses are eluted (released from attachment to the sample matrix) using a buffer solution of high ionic strength with addition of pectinase, an enzyme that degrades pectin, a well-documented RT-qPCR reaction inhibitor (Suther & Moore, 2019). Once elution and digestion are complete, the decanted eluate is centrifuged for clarification, with recovery of the supernatant. After pH adjustment, virus concentration is performed by adding polyethylene glycol (PEG) supplemented with NaCl and subsequent mixing, followed by centrifugation to facilitate precipitation of viruses. The resulting pellet is resuspended in a small volume (500 μl) of phosphate-buffered saline (PBS) and further clarified (purified) by chloroform/butanol extraction followed by centrifugation and recovery of the aqueous phase. This entire sample concentrate (approximately 500 μl) is then subjected to RNA extraction using an appropriate guanidine thiocyanate (GITC) method designed to disrupt the viral capsid and release the nucleic acids. This is followed by further purification of viral RNA by adsorption to silica particles or magnetic beads. The purified RNA is eluted into a volume of 100 μl and retained for RT-qPCR. The annexes of ISO 15216–2:2019 provide recommended TaqMan assays with specified primer and probe sequences, but these are not mandatory, although the oligos used must be in the specified region, peer-reviewed, and in-house optimized. For detection of HAV, the amplification region is in the 5' NCR and produces an amplicon of 157–188 bp (Costafreda et al., 2006). Recommended HuNoV GI and GII RT-qPCR assays amplify regions of the capsid protein, with the GI assay producing an amplicon size of 86 bp (da Silva et al., 2007; Hoehne & Schreier, 2006; Svraka et al., 2007) and the GII assay an amplicon of 89 bp (Costafreda et al., 2006; Kageyama et al., 2003; Loisy et al., 2005).

Although several different commercial reagents were used in the development of the ISO 15216 methods, use of commercial reagents is not mandated in the main body of the standards, in accordance with the ISO policy that disallows commercial endorsements. Hence, the main body details the mandatory minimum requirements of a method element to be compliant with the ISO. The best example of this is the virus extraction method, which details exactly the reagents, centrifugation steps and incubation conditions that must be done to perform a valid extraction. Other aspects of the method provide more flexibility, and often, protocols for these aspects are provided in the annexes of the standard and can be adopted (or not) by the end-user at their discretion. This includes assay components such as (i) choice of process control virus; (ii) RNA extraction method and reagents; (iii) RT-qPCR reagents, platforms, and cycling parameters; and (iv) primer and probe sequences. Taken together, the ISO 15216–2:2019 method permits considerable flexibility, and it is possible for two laboratory protocols to differ while still both being compliant with the requirements of the standardized method.

Data from the ISO 15216 assays can be interpreted quantitatively (Part 1) or qualitatively (Part 2) at the discretion of the user, but qualitative (presence/absence) determination is usually done for testing of berries. The ISO 15216–2:2019 RT-qPCR method for soft fruit requires simultaneous testing of two 5 μl aliquots of undiluted and 10-fold diluted (10^{-1}) sample RNA and also includes a range of different controls. Negative controls (water only and the negative extraction process control virus) should always give negative results, while the external amplification control (EC RNA) also functions as a positive control for the RT-qPCR and should always provide positive results within an expected range of values. A process control virus amplification is also included in the assay. If any of these controls give a discordant result, samples may require retesting.

RT-qPCR inhibition is assessed on a sample-by-sample basis using the EC RNA control. This is a fragment of synthetic RNA including the PCR target sequence that is added to test reactions containing water only, and undiluted and 10^{-1} diluted sample RNA (in separate reactions from the main test reactions). The assessment of inhibition for undiluted sample RNA is carried out by comparing the results of the EC RNA + undiluted sample RNA reaction with the EC RNA + water reaction as a reference. Where the difference between the reactions is $<$ 2.0 Cq (corresponding to less than 75 % RT-qPCR inhibition), the inhibition for the undiluted sample RNA is considered acceptable and results generated using the undiluted RNA are used for that sample. Where the difference is $\geq$ 2.0 Cq, the undiluted RNA is considered inhibitory, and the assessment is repeated for the 10^{-1} diluted sample RNA. If the difference between the EC RNA $+10^{-1}$ diluted sample RNA and EC RNA + water reactions is $<$ 2.0 Cq, the inhibition for the 10^{-1} diluted sample RNA is considered acceptable and results generated using the 10^{-1} diluted RNA are used for that sample. Where the difference is $\geq$ 2.0 Cq, the sample is considered inhibitory and the test would normally be considered invalid, and the sample would need to be retested.

Extraction efficiency is determined on a sample-by-sample basis by comparing the Cq value for the process control virus (added to the sample at the start of the virus extraction) in the sample RNA (undiluted or 10^{-1} diluted depending on the results of the inhibition assessment) with a standard curve generated using RNA from a reference extraction (water plus process control virus). When the extraction efficiency for the

sample is ≥ 1 %, the assay is considered valid; <1 % extraction efficiency is considered unacceptable, and the sample would normally need to be retested.

An exception to these control interpretation guidelines is allowed if an otherwise valid RT-qPCR positive result is obtained for a sample, even if it showed unacceptable RT-qPCR inhibition or extraction efficiency. In this case, results may still be expressed as positive.

2.2.3. *The U.S Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) method*

Besides the ISO 15216 methods, other national standards have been developed, published, and some validated, most of which are quite similar to the ISO methods. One alternative method was developed by the U.S. Food and Drug Administration (FDA) and described in the FDA Bacteriological Analytical Manual (BAM), Chapter 26 (U.S. Food and Drug Administration, 2022). The BAM contains laboratory protocols for microbiological analyses of foods and cosmetics, as governed by guidelines described in FDA's Methods Development, Validation, and Implementation Program (MDVIP) Standard Operating Procedures (U.S. Food and Drug Administration, 2021). Method validation was done by FDA internal laboratories and is described in the appendices to FDA-BAM Chapter 26, including for berries. Sample matrices for use of this method include molluscan shellfish; green onions and leafy greens; berries and soft fruit; and scallops and finfish. The FDA-BAM method for soft fruits is diagrammed in Figure Q1B. A 50 g berry sample is processed, and the viral particles are eluted using a high pH alkaline buffer containing pectinase and beef extract, with periodic pH adjustment to 7.5. The eluate is subsequently purified using conventional centrifugation and the virus-containing supernatant further concentrated using ultracentrifugation (45 min at 170,000×*g* at 4 °C). The resulting pellet is resuspended in 600 μl of PBS, clarified by chloroform extraction, and the final suspension aliquoted into three subsamples. One of these subsamples is subjected to RNA extraction, the other two are retained. RNA isolation is done using guanidine thiocyanate-mediated lysis, followed by further purification by adsorption to silica particles. Additional RNA extract purification is done as a sequential three-step process with kits specified in the formal procedure. Two multiplex TaqMan assays [including specified primers and probes for target(s) and internal amplification control] are used for RT-qPCR detection of HAV or HuNoVs (GI and GII). More specifically, the HAV assay is designed with primers and probe targeting the conserved 5′ untranslated region and detects all HAV genotypes (Gardner et al., 2003). The RT-qPCR protocol for GI and GII HuNoVs amplifies the ORF1-ORF2 junction, the most conserved region of the genome, using primers derived by Kageyama et al. (2003), with minor modifications.

Several controls are used in the assay. Process control virus [MNV or mengovirus, commercially available through American Type Culture Collection (ATCC), Mannasas, VA], which is added to the sample right before sample preparation, is used to evaluate extraction efficiency. Heterologous internal amplification controls (IAC) (available from BioGX, Birmingham, AL; 750-0001), which are a fragment of reference RNA coamplified with target RNA, are used in process control and test sample RT-qPCR reactions. Positive controls consist of synthetic HuNoV RNA (fragments from the RNA-dependent RNA polymerase and VP1 (ORF1-ORF2 junction) regions for HuNoV GI and GII [available from ATCC; VR-3234SD (GI); VR 3232SD (GII)] and RNA extracted from cell culture-propagated strain HAV HM-175-18f (also available from ATCC; VR1402). A total volume of 3 μl of concentrate is amplified per reaction, with triplicate reactions per sample. A sample is considered valid under the following criteria: no viral RNA is detected in the uninoculated sample; RT-qPCR negative control is negative; RT-qPCR positive control is positive; and the IAC is positive for process control and test samples. When the average of the Cq values corresponding to the IAC in the process control or unknown sample exceeds 4.0 Cq values of the IAC amplified in a negative control tube, the sample is deemed to have significant matrix-associated inhibition, and the RT-qPCR assay must be repeated (using 1 μl of RNA rather than 3 μl). If the repeated RT-qPCR indicates significant inhibition yet again, then a new extraction is to be performed using the retained berry sample. If, however, the sample tests positive for HAV or HuNoVs, with the classic sigmoidal curve shape, but the IAC reaction(s) exceeds the 4.0 Cq difference, the sample is considered positive without having to repeat the assay. Unlike ISO 15216, the FDA-BAM method does not appear to describe performance criteria for the process control virus relative to the test samples. For the HAV test only, a single control exclusion amplification (CEA) is also included. If the CEA RT-qPCR is negative and all controls are satisfactory, the virus detected in the HAV RT-qPCR is considered to be wild-type, not the laboratory strain. If positive, the sample is submitted to further confirmatory steps.

The FDA-BAM assay is interpreted qualitatively. Unlike the ISO 15216–2:2019 method, the FDA method is highly prescriptive and there is no opportunity for the user to modify protocols, reagents, primer and probe sequences, equipment and settings, cycling parameters, and interpretations.

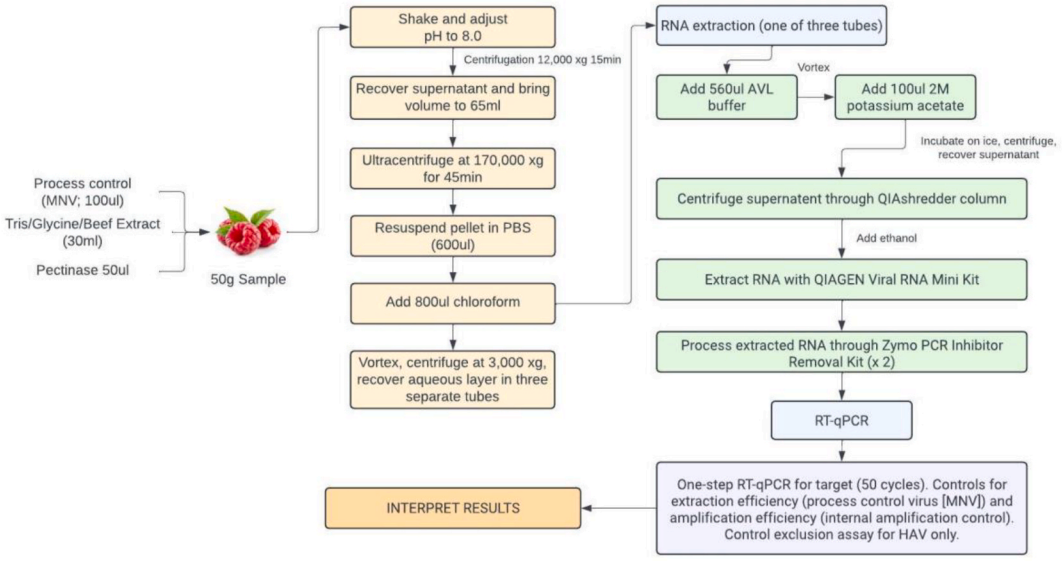

Figure Q1.B. Schematic diagram of the U.S. FDA Bacteriological Analytical Manual (BAM) method, Chapter 26, for virus extraction and detection from soft fruits.

2.2.4. Comparison of ISO 15216–2:2019 and U.S. FDA-BAM chapter 26 methods

Both the ISO 15216–2:2019 and the U.S. FDA-BAM methods share common principles, but they also diverge in important ways. Table 1 provides an overview of the major differences between the two. Note that, at the time of this writing, the two methods have not been subjected to a head-to-head performance assessment to establish equivalency. Consequently, comparisons should be made cautiously and some of the issues discussed here might be considered academic, although they are grounded in scientific experience. Starting with the sample size, testing a larger quantity of berries (50 g in FDA-BAM versus 25 g in ISO 15216:2–2019) may be advantageous, as foodborne viruses are usually in relatively low numbers and not homogeneously dispersed in berries. However, larger sample sizes could potentially result in higher concentrations of PCR inhibitory substances, although this has not been confirmed in this case.

An important distinction between the two methods is the concentration protocol. Precipitation with PEG is an inexpensive and simple technique that has been extensively used with a variety of non-enveloped viruses in a diversity of food and environmental samples. However, there have been concerns that it may lead to co-precipitation of PCR inhibitors (Schrader et al., 2012). On the other hand, ultracentrifugation can be faster than PEG precipitation and, in some situations, it has been suggested that it provides improved recoveries of HuNoVs from certain produce items (Hida et al., 2018). However, ultracentrifugation requires expensive and specialized equipment and higher reagent costs, making it difficult to implement in many laboratories, particularly in middle to lower income countries. In addition, it may not be suitable for all types of matrices and can result in rather large pellets (Rutjes et al., 2006; Rzeżutka et al., 2006). More minor differences between the methodological concentration protocols include elution volume, exact composition of elution buffer (i.e., use of beef extract), elution conditions (pH, time), and concentration of pectinase.

The RNA purification protocols differ somewhat from one another, with the FDA-BAM method having the mandatory inclusion of multiple sequential RNA purification steps, while this is an option for ISO 15216–2:2019. The FDA method amplifies from a sample concentrate volume of ~200 μl (representing one-third of the final 600 μl sample concentrate, equivalent to ~16.7 g of the original berry sample), while the ISO 15216 amplifies from the entire volume of the sample concentrate in RNA extraction. Both incorporate isothiocyanate lysis with silica-based purification, but the FDA method specifies sequential purification steps that include homogenization (QIAshredder) followed by lysis and two sequential silica-based steps (QIAmp and Zymo One Step RT-PCR inhibitor remover column, twice). The Expert Panel members pointed out that many ISO-compliant labs incorporate one or more additional clean-up steps in their RNA extraction protocols, although this is not required in the formal ISO 15216–2:2019 protocols. It is recognized that the nucleic acid purification step(s) is very important in reducing residual matrix-associated inhibition associated with the berry matrix (Zhou and Li, 2020) and this is being deliberated upon in consideration of the upcoming ISO 15216 revisions.

There are also differences in the detection (RT-qPCR) protocols and associated controls when comparing ISO 15216–2:2019 to FDA-BAM Chapter 26 method (Table 2). These include the volumes of RNA to be amplified (3 μl for FDA-BAM, 5 μl for ISO 15216–2:2019); the need for RNA dilution before amplification (undiluted for FDA-BAM method; undiluted and 10-fold diluted for ISO method); the number of replicate amplifications (two for ISO 15216–2:2019, three for FDA-BAM); the sequences of the oligonucleotide primers and probes; and the number of cycles for the PCR amplification. The FDA-BAM method specifies the exact oligonucleotide primer and probe sequences, the brand of RT-qPCR kits, thermocyclers, and threshold/baseline settings, while the ISO 15216–2:2019 provides mandatory criteria for selection of primers and probes but not specific sequences. Most protocols specify 45 PCR amplification cycles, although the FDA-BAM method uses 50 for detection of HAV RNA.

In terms of controls, the FDA-BAM method mandates the use of MNV or mengovirus as the process control, while ISO 15216–2:2019 does not specify the exact virus, but rather provides the criteria for selecting such

Table 1
Major differences between ISO 15216–2:2019 and U.S. FDA-BAM Chapter 26 sample preparation steps for detection of foodborne viruses in soft fruits.

Protocol Step	ISO 15216 Method	FDA-BAM Method
Sample size	25 ± 0.3 g	50 ± 5 g (with 1/3 of sample concentrate screened by RT-qPCR)
Virus concentration and purification method	PEG/NaCl solution	Ultracentrifugation
RNA extraction method	Guanidine thiocyanate virus particle lysis and silica-based RNA binding, brands unspecified Additional purification steps are often conducted at the discretion of the testing lab	Guanidine thiocyanate virus particle lysis and silica-based column RNA binding using QIAshredder homogenization, QIAmp column purification and Zymo column purification, hence brands specified
Process control virus	Culturable non-enveloped positive sense ssRNA virus of a similar size to the target viruses (e.g., Mengo virus but others are acceptable)	Murine norovirus type 1 strain CW1
Performance of process control virus	Recovery >1 % is considered acceptable	Not specified
Endpoint	Quantitative (15216-1) OR Qualitative (15216-2)	Qualitative only

Table 2
Major differences with respect to the detection step, and the interpretation of results, when comparing the ISO 15216-2-2-19 to the U.S. FDA-BAM Chapter 26 methods for detection of foodborne viruses in soft fruits.

Parameter	ISO 15216 Method	FDA-BAM Method
Volume of RNA per reaction	5 μl	3 μl
Total volume of reaction	25 μl	25 μl
Number of reactions	2 each of undiluted and 10^{-1} diluted RNA extract per single sample; total of one sample per test	3 each of undiluted RNA extract per single sample; total of 3 samples per test
Controls	Positive, negative, EC RNA, process control virus	Positive, negative, IAC, process control virus
Performance of Internal Amplification Control	>2.0 Cq value difference between EC RNA amplification in sample vs. control (water) indicates unacceptable degree of inhibition—qualitative method only	When average Ct value corresponding to the IAC >4.0 what is expected, sample has unacceptable degree of inhibition
If inhibition, then ….	Use results from 1:10 in the event that RT-PCR inhibition is >75 % for undiluted sample RNA	Repeat amplifications with 1 μl of sample RNA
Cycles of run	At least 45	45 (HuNoVs) or 50 (HAV)
HAV probe	FAM-CCT GAA CCT GCA GGA ATT AA- MGBNFQ (recommended)	Cy5-AGA CAA AAA CCA TTC AAC GCC GGA GG-IB-RQ
HuNoV probes	NVGG1p (GI): FAM-TGG ACA GGA GAY CGC RAT CT-TAMRA TM9 (GI): FAM-TGG ACA GGA GAT CGC-MGBNFQ QNIFs (GII): FAM 5′-AGC ACG TGG GAG GGC GAT CG 3′; TAMRA (recommended)	COGP (GI): Cy5-(TAO) AGA TYG CGA TCY CCT GTC CA-IB-RQ COG P1 (GI): Cy5-(TAO) AGA TCG CGG TCT CCT GTC CA-IB-RQ COG P2 (GII): Cy3-TGG GAG GGC GAT CGC AAT CT-IB-RQ

a virus and establishing its performance (recovery ≥1 % is considered acceptable). The FDA-BAM method does not establish criteria for performance of the MNV process control. To monitor residual matrix-associated inhibition, both use amplification controls but they differ in nature and interpretation. The FDA-BAM method includes an IAC, while the ISO 15216–2:2019 uses an EC RNA, both discussed above. The amplification control approach for the FDA-BAM method involves multiplexing.

Whenever a molecular amplification method is used, care must be taken to prevent cross-contamination between positive controls and samples, or between contaminated and non-contaminated samples. Good PCR practice dictates that positive controls and samples are handled in different laboratory areas with specific controls to avoid contamination of samples, and these procedures are described elsewhere (ISO, 2024). Negative control assays are included in all tests done using ISO 15216–2:2019 and FDA-BAM Chapter 26 methods and provide batch level assurance that gross cross-contamination is not occurring. In terms of positive controls, although not mandatory, some ISO-compliant labs use positive controls that contain genetic modifications (generally substitutions) which provide a means by which to identify cross-contamination by sequencing or restriction digestion, if so desired (ISO, 2017). The FDA-BAM method uses positive controls that are commercially available through the American Type Culture Collection (ATCC). These do not have inserts, making it difficult to discriminate between an amplicon sequence corresponding to a wild-type strain vs. the control sequence. The FDA-BAM method attempts to identify cross-contamination with a positive control for HAV using the control exclusion assay (CEA), but this is not well described in the documentation. No CEA is used for HuNoV detection in the FDA-BAM method.

2.2.5. Other methods

The reader is referred to the recent FAO-WHO (2024) report which details methods used in various global locations (https://www.who.int/publications/m/item/jemra-of-viruses-in-foods-part1-food-attribution-analytical-methods-and-indicators). As stated above, ISO 15216–2:2019 is the most widely used method worldwide for foodborne virus concentration, purification, and detection in soft fruits, by competent authorities, academic researchers, and commercial testing labs. Some entities in some countries use modifications of this method which make them not entirely ISO-compliant. For example, the Canadian Food Inspection Laboratory published a new method, with virus capture being accomplished using magnetic silica, replacing the PEG precipitation step in the ISO 15216 methods (Raymond et al., 2021). This method was applied to raspberries artificially contaminated with HuNoVs with method performance similar to the ISO 15216–1:2017 standard (ISO, 2017); it was also used in a recent national surveillance study (Steele et al., 2022). The U.S. is the only country which uses its own method to the exclusion of the ISO 15216 protocols. Other methods periodically appear in the literature but are beyond the scope of this document.

2.2.6. The infectivity dilemma

A critically important point is that these RT-qPCR tests are not designed to detect whole, intact virus particles, rather viral nucleic acid. The nucleic acid being detected could be derived from a non-infectious virus particle, defective in capsid or full-length nucleic acid or both; or could be a remnant of viral nucleic acid which may persist much longer in environmental samples than initially suspected (Julian and Schwab, 2012; Trudel-Ferland et al. (2021). Hence, a positive RT-qPCR signal does not equate to the presence of whole, intact, and infectious virus. And the method does not produce an infectious 'culture' which can be subjected to further characterization. In short, a positive RT-qPCR test does not assure that an infectious virus has been detected, making it difficult to correlate test results to risk of infection.

This infectivity dilemma is simply illustrated using data in the U.S. FDA-BAM Chapter 26, Appendix F, Figure F2 and Table F6 (available at: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-26-and-appendices-concentration-extraction-and-detection-enteric-viruses-food; Williams-Woods et al., 2022). The calibration curve presented here is derived from amplification of the RNA extracted from a cell culture lysate of the cultivable HAV HM-175 18f strain, presented as PFU (infectious virus, X-axis) vs. Cq (Y-axis). In this graph, Cq values exceeding about 35 correspond to a fraction of an infectious virus. For instance, at a concentration of 0.1 PFU, 10 of 10 amplifications were positive with a mean Cq value of 41.13 ± 0.55. This could be interpreted to mean that at a Cq of about 41, there is a 100 % likelihood of detection by RT-qPCR, with a one in 10 chance that what is being detected is infectious. Although the curve loses log linearity beyond this point, the authors extrapolate a limit of detection (degree of confidence/statistical significance not specified) of 0.001 PFU at a Cq value of 43.48 + 1.04, with eight of 10 replicate samples yielding positive results.

Although the Expert Panel identified flaws to this approach, primarily the erroneous use of PFU to calibrate an assay based on the detection of viral RNA, the results are illustrative of the fact that there can be relatively reliable detection of "fractions" of an infectious unit at higher Cq values. The FDA-BAM HAV calibration graph was produced using cell culture lysate from a cultivable strain. This may be considered a special case, perhaps with lower infectious:non-infectious virus ratios (Deng et al., 1994; Jansen et al., 1988). Klasse (2015) discusses the molecular determinants of inert to infectious virus ratios, and notes that for most viruses, these ratios range between 1:1 to 1:1000, sometimes higher. Unfortunately, there is limited information about the typical infectious:non-infectious ratios for environmentally present HAV and HuNoVs, and how these viruses and/or their RNA might present or persist, as detectable and/or infectious, in the environment or in foods. While one PFU may represent an aggregate of many particles, with some infectious and some not, it is likely that each one of these particles would contain amplifiable RNA and a single "infectious unit" could be interpreted as many. Taken together, this figure represents the difficulties associated with interpreting RT-qPCR results from the perspective of virus infectivity.

Recent publications have suggested caution in interpretations. For example, using virome capture sequencing, Tan et al. (2021) compared the results from a subset of market oyster samples testing positive for HuNoVs to positive control samples spiked with serially diluted HuNoV GII fecal specimens. They found that the naturally contaminated samples generated much lower read counts (>7-$\log_2$ cumulative sum scaling difference) and genome coverage (406 nt. vs 3715 nt) than did the spiked samples. These investigators concluded that, based on the methodology used, the RT-qPCR positive signals detected from market shellfish samples in this study could have corresponded to degraded RNA derived from inactive virus particles that otherwise persist in the environment. Trudel-Ferland et al. (2021) demonstrated that inactivated HAV on frozen (−20 °C) blueberries remained fully detectable for up to 90 days. Interestingly, in their human challenge study, Eshaghi, Tan, Zhao, and Li (2021) demonstrated the absence of signs of infection in individuals consuming berries testing positive for HuNoVs below the assay limit of quantification (<120 genome copies/g), calling into question the public health risk of low genome copy numbers. Collectively these data demonstrate that a clear relationship between detection of viral RNA, virus infectivity, and risk of infection cannot be determined and may even be sample or situation dependent. In risk terms, the argument could be made that a positive test result suggests a risk of exposure to virus but does not clearly equate to a defined risk of infection or illness associated with that exposure.

2.3. Key conclusions

- The ISO 15216–2:2019 method is considered the international standard for qualitative detection of foodborne viruses in berries. While the FDA-BAM Chapter 26 method is used in the U.S., the ISO

15216–2:2019 remains the method of choice for most competent authorities and commercial testing labs.

- The ISO 15216 methods (Part 1, quantitative; Part 2, qualitative) consist of the major steps of virus elution with pectinase treatment, PEG precipitation, chloroform-butanol extraction, RNA extraction, and RT-qPCR. They have been internationally validated, and those studies have been published in the peer-reviewed literature.
- The FDA-BAM Chapter 26 method consists of the major steps of virus elution with pectinase treatment, ultracentrifugation, chloroform extraction, RNA extraction and purification, and RT-qPCR. It has been validated within the Agency and is published in the BAM. At the time of this writing, data on external validation of this method, as applied to soft fruits, has not been published in the peer-reviewed literature.
- The major differences between these two methods are as follows: total sample size (25 g for ISO 15216, 50 g for FDA-BAM, although the latter only subjects one-third of the sample concentrate to RNA extraction and RT-qPCR); virus concentration by PEG (ISO 15216) vs. ultracentrifugation (FDA-BAM); and the complexity of the RNA extraction protocol [(guanidinium isothiocyanate with silica beads (ISO 15216) vs. the same but with further downstream purification (FDA-BAM)], although many ISO-compliant labs also perform additional RNA purification steps.
- The FDA-BAM method is highly prescriptive while the ISO 15216 methods provide more methodological flexibility, although there are some required steps of any protocol that claims to be ISO-compliant. Notable here is that both qualitative and quantitative ISO methods specify the exact protocol for virus extraction from soft fruit, including reagents, centrifugation steps and incubation conditions. They are less prescriptive about the RNA extraction and detection protocols/kits.
- At the time of writing, there were no published head-to-head comparison studies establishing equivalency the ISO 15216 and U.S. FDA-BAM methods.
- A positive RT-qPCR test does not assure that infectious virus has been detected, particularly at higher Cq values. This makes it difficult to correlate test results to human disease risk. Perhaps it is safe to say that positive results indicate exposure risk, but not necessarily infection or disease risk.

3. Question #2

How is the information gained from foodborne virus detection in berries being used by various stakeholder sectors (academic, industry, government/regulatory)?

3.1. Uses of data from foodborne virus testing of berries

Efforts to develop standardized methods to detect foodborne virus contamination in berries began two decades ago with the expressed purpose of contributing to our ability to effectively carry out outbreak investigation; understand the natural prevalence of virus contamination in fresh and frozen berries; perform root cause analysis in the case of contamination events; and otherwise protect public health by preventing contaminated product from entering the marketplace. Testing also has utility in providing data for risk assessment, advising regulatory decision-making, and aiding risk management approaches along the supply chain. The detection methods described above, and modifications thereof, have been used by academic and government researchers, regulators, and industry alike, oftentimes for different purposes.

3.1.1. Monitoring data obtained by academic and government laboratories

Publicly available results of representative monitoring and surveillance studies on foodborne virus contamination of berries, mostly collected from the retail market and led by academic or government researchers, are summarized in Table 4. In most surveys, some evidence of contamination was detected. In their recent meta-analysis, Miotti et al. (2024) reported a combined global frequency of HuNoVs and HAV in berries at 2.1 % (95 % CI 1.7–2.6 %). Collectively, positivity rates for the presence of foodborne viral RNA in studies such as these range from no detection (0 %) to as high of 33 %. Two large and recent academic laboratory studies are illustrative of the high degree of variability in results. On the one hand, Li et al. (2018) collaborated with Nestle and PROFEL (the European Association of Fruit and Vegetable Processors) to screen a range of (mostly frozen) berries for contamination with HAV and HuNoVs (2009–2016). Testing was done using the ISO/TS 15216-2 (ISO, 2013) method by several independent laboratories. Seven of a total of 2015 samples analyzed (0.4 %) showed evidence of viral RNA; 0.2 % (3/2015) positive for GI HuNoVs; 0.1 % (2/2015) positive each for GII HuNoVs and HAV. The berries were sourced from all over Europe as well as the U.S., Russia, and Turkey. On the other hand, Gao et al. (2019) surveyed 1800 retail berry samples (2016–2017) from Heilongjiang Province, China, finding the frequency of HuNoV RNA to be 9.0 % (81/900) and 12.1 % (109/900) for frozen and fresh berries, respectively. Of the positive samples, 35.8 % (29/81) and 29.4 % (32/109) were GI; 54.3 % (44/81) and 60.6 % (66/109) were GII; and 9.9 % (8/81) and 10.1 % (11/109) showed evidence of viral RNA associated with of both genogroups. HAV was not screened in this study. It does not appear that there were any illnesses associated with products which tested positive in any of the studies listed in Table 3.

The Expert Panel was able to identify several large berry surveillance/monitoring studies performed by government entities, or organizations sponsored by such entities. A synopsis of the results of these studies is provided below. Bolded, italicized text provides results related to Cq values and sample positivity criteria which will be discussed in greater detail later in the report.

- The UK Food Standards Agency screened close to 500 fresh and frozen raspberry samples obtained from the commercial market (2015–2016) for HuNoV contamination using the ISO 15216-2 (2013) method (Cook et al., 2019). Of the fresh berries, 2.3 % (7/310) were positive for HuNoVs; the positivity rate was 3.6 % (10/274) for the frozen raspberries. Most of the positive fresh samples were imported from Morrocco and Spain, but it was difficult to determine country of origin for the positive frozen samples. The authors acknowledged difficulties in discriminating between natural virus contamination and cross-contamination with positive control, and although amplicon sequencing was attempted, it was not successful. ***Cq values for positive samples were not reported.***
- In the first Canadian monitoring/surveillance study (2014–2016), pre-packaged, ready-to-eat (RTE) fresh and frozen fruits were screened for HAV and HuNoV contamination using the Canadian Food Inspection Agency (CFIA) internally validated methods [(CFIA -VAD-02 for HAV and CFIA-CRNVA-0 RT-PCR for HuNoV GI and GII). A total of 1991 samples were analyzed. No viral RNA was detected in almost all (99.6 %) of the samples tested; only HuNoV (GII) RNA was detected in 0.4 % (7/1991) samples (6 berry samples, one other fruit). ***Cq values did not exceed 40 for all seven of the positive samples*** (Government of Canada, 2018).
- In a follow-on study, CFIA analyzed 926 fresh berry samples, and 3292 samples of frozen berries, including pomegranate aril samples, collected from retail chains and groceries across the country from 2016 to 2021 using the method of Raymond et al. (2021) (Steele et al., 2022). None of the samples were positive for HAV RNA. Positivity for HuNoVs was 0.2 % (2/926) for fresh and 0.4 % (13/3292) for frozen berries. Subdividing the HuNoV data for frozen berries, 0.1 % (3/3292) positivity was found for GI, and 0.3 % (10/3292) for GII strains. ***Cq values of positive samples ranged from 33.9 to 42.2.***
- In an Eastern European study not yet published (Branko Velebit, personal communication), the Institute of Meat Hygiene and Technology (INMES, Belgrade, Serbia) screened fresh and IQF raspberries and blackberries for HuNoV contamination (2017–2020) using the

Table 3
Summary of surveys of foodborne virus detection in retail fresh and frozen produce, by study.

Study	Study Venue	Year(s)	Location(s)	Method(s)	Berry Items	# samples positive/total # samples tested (% Positivity)	Confirmation Yes/No
Baert et al. (2011)	Academic	2009–2010	Belgium, Canada, France	PEG precipitation Real-time PCR [No ISO standard specified]	Raspberries and Strawberries	Belgium raspberries and strawberries: HuNoV: 10/29 (34.5 %) France raspberries and strawberries: HuNoV: 10/150 (6.7 %)	Yes Subset only; amplification of different region, sequencing
Stals et al. (2011)	Academic	2010	Belgium	PEG precipitation RT-qPCR [No ISO standard specified]	Raspberries Strawberries	Raspberries: HuNoV: 4/10 (40.0 %) Strawberries: HuNoV: 6/20 (30.0 %)	No Attempted reamplification and sequencing; unsuccessful
De Keuckelaere, Li, Deliens, Stals, & Uyttendaele, 2025	Academic	2011–2012	Belgium	PEG precipitation RT-qPCR [No ISO standard specified]	Frozen mixed/ minced Raspberries	Frozen mixed/ minced Raspberries: HuNoV: 6/70 (8.6 %)	Yes Reamplification with different primers and sequencing
Loutreul et al. (2014)	Academic	N/A	Various	PEG precipitation RT-qPCR [No ISO standard specified]	Raspberries Strawberries Blackberries Mixed berries	Raspberries: HuNoV GI: 27/162 (16.8 %) HuNoV GII: 0/162 (0.0 %) Strawberries: HuNoV GI: 3/32 (9.4 %) HuNoV GII: 1/32 (3.1 %) Blackberries: HuNoV GI: 1/2 (50.0 %) HuNoV GII: 0/2 (0.0 %) Mixed berries: HuNoV GI: 0/4 (0.0 %) HuNoV GII: 0/4 (0.0 %)	No
Parada-Fabián et al. (2016)	Academic	2016	Mexico	PEG precipitation RT-PCR [No ISO standard specified]	Frozen Strawberries	Frozen strawberries: HAV and HuNoV: 0/20 (0.0 %) Rotavirus: 4/20 (20.0 %)	No
Li et al. (2018)	Academic	2009–2016	Various Germany, Bulgaria, France, Poland, Switzerland, Czech Republic, USA, Spain, Russia, and Turkey	ISO/TS 15216-2: 2013	Strawberries Blueberries Red currants Mixed berries	Strawberries: HAV:1/918 (0.1 %) HuNoV GII: 1/918 (0.1 %) Blueberries: HAV: 0/126 (0.0 %) HuNoV GI: 2/126 (1.6 %) Red currants: HAV: 1/39 (2.6 %) HuNoV GII: 1/39 (2.6 %) Mixed berries: HAV: 0/122 (0.0 %) HuNoV: 1/122 (0.8 %)	No
Gao et al. (2019)	Academic	2016–2017	China	ISO 15216-1: 2013	Strawberry Raspberry Blackcurrant Blueberry Cranberry Blackberry	Export: HuNoV: 0 for all items (0.0 %) Domestic: HuNoV Frozen: 81/900 (9.0 %)	No

(*continued on next page*)

Table 3 (*continued*)

Study	Study Venue	Year(s)	Location(s)	Method(s)	Berry Items	# samples positive/total # samples tested (% Positivity)	Confirmation Yes/No
					Export & Domestic Retailed *For export, included only frozen; For domestic, included both frozen and fresh	HuNoV GI: 29 HuNoV GII: 44 HuNoV GI and GII: 8 HuNoV Fresh: 109/900 (12.1 %) HuNoV GI: 32 HuNoV GII: 66 HuNoV GI and GII: 11	
Shin et al. (2019)	Academic	2016–2017	South Korea	ISO/TS 15216–1:2013	Strawberries	Strawberries: HuNoV GI: 0/120 (0.0 %) HuNoV GII: 0/120 (0.0 %) HAV: 1/120 (0.8 %)	No
Pavoni et al. (2022)	Academic	2014–2019	Italy	ISO/TS 15216–2:2013	Frozen Berries	All samples: 5/2749 (0.2 %) HAV: 2/2749 (0.1 %) HuNoV: 3/2749 (0.1 %), with: HuNoV GI: 1 HuNoV GII: 2	No
Bennett et al. (2023)	Academic	2018	Ireland	ISO 15216–1:2017	Fresh strawberries Fresh raspberries Frozen strawberries Frozen raspberries	Fresh strawberries: HAV: 2/63 (3.2 %) Fresh raspberries: HAV: 2/60 (3.3 %) Frozen strawberries: HAV: 1/57 (1.8 %) HuNoV GII: 2/57 (3.5 %) Frozen raspberries: HuNoV GII: 1/60 (1.7 %)	No
Chatonnat et al. (2023)	Academic	2021	Canada	ISO 15216–1:2017	Fresh cranberries Fresh blueberries	Fresh cranberries: NoV GI: 3/234 (1.3 %) HAV: 0/234 (0.0 %) Fresh blueberries: HEV: 0/150 (0.0 %)	Yes Reamplification; nucleic acid intercalating agent; Sanger sequencing
Canadian Food Safety Authority (2017)	Government	2014–2016	Canada	CFIA-VAD-02 [HAV] CFIA-CRNVA-05 RT-PCR [HuNoV]	Fresh berries (blackberry, blueberry, strawberry) Frozen berries (blackberry, blueberry, strawberry) Frozen fruits	Fresh berries: HAV: 0/930 (0.0 %) HuNoV GI: 0/930 (0.0 %) HuNoV GII: 4/930 (0.4 %) [Blackberry, Blueberry(2), Strawberry] Frozen berries: HAV: 0/656 (0.0 %) HuNoV GI: 0/656 (0.0 %) HuNoV GII: 2/656 (0.3 %) [Blueberry, Strawberry] Frozen fruits: HAV: 0/405 (0.0 %) HuNoV GI: 0/405 (0.0 %) HuNoV GII: 1/405 (0.3 %) [Peach]	No
Cook et al. (2019)	Government	2015–2016	UK	ISO 15216–2:2013	Fresh raspberries Frozen raspberries	Fresh raspberries: HuNoV: 7/310 (2.3 %),	Yes Replicate amplifications only

(*continued on next page*)

Table 3 (*continued*)

Study	Study Venue	Year(s)	Location(s)	Method(s)	Berry Items	# samples positive/total # samples tested (% Positivity)	Confirmation Yes/No
						specifically: HuNoV GI: 3 HuNoV GII: 5 HuNoV GI & GII: 1 Frozen raspberries: HuNoV: 10/274 (3.7 %), specifically: HuNoV GI: 8 HuNoV GII: 5 HuNoV GI & GII: 3	
Steele et al. (2022)	Government	2016–2021	Canada	ISO/TS 15216-2 (2013) ISO 15216-2 (2019)	Fresh blackberries Fresh blueberries Fresh raspberries Fresh strawberries Frozen blackberries Frozen blueberries Frozen raspberries Frozen strawberries Frozen pomegranate arils Frozen mixed berries	NA NA Fresh raspberries: HuNoV GI: 1/120 (0.8 %) Fresh strawberries: HuNoV GII: 1/368 (0.3 %) Fresh blackberries: HuNoV GI: 1/204 (0.5 %) Frozen blueberries: HuNoV GII: 1/316 (0.3 %) Frozen raspberries: HuNoV GI: 1/808 (0.1 %); HuNoV GII: 3/808 (0.4 %) Frozen strawberries: HuNoV GI: 1/1056 (0.1 %); HuNoV GII: 4/1056 (0.4 %) Frozen pomegranate arils: HuNoV GII: 1/192 (0.5 %) Frozen mixed berries HuNoV GII: 1/716 (0.1 %)	Yes Cloning and Sanger sequencing
Oteiza et al. (2022)	Government	2016–2017 and 2020	Argentina	ISO 15216–2:2019	Strawberries Blueberries Raspberries Blackberries	Strawberries: HuNoV: 0/75 HAV: 0/75 Blueberries: HuNoV: 0/68 HAV: 0/68 Raspberries: HuNoV GII: 1/10 (10.0 %) HAV: 0/10 Blackberries: HuNoV: 0 HAV: 0	Yes Heminested re-amplification and sequencing
U.S. Food and Drug Administration, 2025	Government	2018–2024, (pause during 2020–2022 due to SARS CoV-2	United States	Method consistent with US FDA-BAM Chapter 26	All frozen product, Domestic and imported Strawberries Raspberries Blackberries	HAV (total): 8/1558 (0.5 %) Domestic: 3/538 (0.6 %), 2/3 confirmed Imported: 5/1020 (0.5 %), 5/5 confirmed HuNoV (total): 10/1558 (0.6 %) Domestic: 4/538 (0.7 %), 3/4 confirmed Imported: 6/1020 (0.6 %), 3/6 confirmed	Yes Nested PCR and Sanger sequencing

Table 4
Summary of berry-associated foodborne disease outbreaks, not likely contaminated by food handlers, having positive viral RNA detection results in implicated product.

Outbreak Date	Berry Product	Date/Location and Info about Outbreak	Methods Used	Overall Results	Context	Citation (Year of study)
2012	Frozen strawberries	September/October 2012 Germany	Virus Extraction: 1) PEG Precipitation method 2) Ultrafiltration method Real-time RT-PCR	HuNoV: 7/16 for precipitation HuNoV: 3/16 for ultrafiltration	While using outbreak-derived samples, this study also compared two different methods of virus extraction.	Mäde et al. (2013)
2013	Frozen mixed berries	2013 Italy	RT-PCR Next-generation sequencing	Only 2 mixed berries samples were examined: HAV: 1/2 (50 %)	This study focused on examining the presence of HAV in the previously collected berry sample from an outbreak.	Chiapponi et al. (2014)
2014	Frozen mixed berries	November 2013–June 2014 Norway	ISO/TS 15216-2 RT-qPCR Sequencing	Norway: HAV: 1/5 (20 %) berry samples from an implicated mixed cake tested positive	Virological investigation of European HAV outbreak from which positive detection was obtained for implicated product	Guzman-Herrador et al. (2015)
2016	Frozen raspberries (Imported from China)	August 2016 Minnesota, US Frozen raspberries used for making raspberry chocolate chip ice cream	GCSL WI September 2016 duplex real-time RT-PCR	Chinese raspberries: 100 % for HuNoV GII.17 (P17)	Outbreak report that included features of the epidemiological investigation and lab-based detection of HuNoV in implicated product	Saupe et al. (2021)
2018	Frozen strawberries (imported from Poland)	June–September 2018 Sweden, Austria	Real-time RT-PCR with sequence analysis confirming outbreak strain	Sweden: HAV: 100 % detected (No specific sample numbers are given for food sampling)	European outbreak investigation in which implicated product was tested	Enkirch et al. (2018)
2019	Frozen raspberries (imported from China)	July–October 2019 Europe and US (Cruise ship) Smoothie made from frozen fruits and berries	RT-PCR (no further information provided)	Frozen fruit and berries: HuNoV: 3/16 (18.75 %) Specifically: Raspberries: HuNoV GII Tropical fruit cocktail & berry mix: HuNoV GI	Investigation of multiple cruise ship HuNoV outbreaks from which Chinese frozen raspberries were inplicated	Rispens et al. (2020)
2019	Frozen bilberries	January–February 2019 Finland	Environmental sampling: ISO 15216-2 Real-time RT-PCR	Recall batch: HuNoV GII: 2/2 (100 %) from outbreak 1 HuNoV GII.17: 1/2 (50 %)	This paper focused on a series of HuNoV outbreaks linked to frozen bilberries in Finland.	Summa et al. (2024)

ISO 15216-2 (2013) method. ***The laboratory used certain pre-established criteria for sample positivity: a pre-determined Cq value cut-off calculated as 2 successful duplicate amplifications lower than the Cq value of the LoD_{95} + 1 Cq; ranged from 39-40); curves of the typical sigmoidal shape; and positivity in duplicate amplifications.*** The Cq cut-off and sample retesting approaches used in this study were significant modifications compared with what is written in ISO 15216. Of the 2244 samples tested, 1.9 % (43/2244) of samples were positive for HuNoV GI RNA; 6.1 % (137/2244) were positive for GII HuNoVs; and 0.2 % (4/2244) for HAV.

- In another unpublished European study (Branko Velebit, personal communication), 10 % of imported frozen raspberry batches presented at the Serbian – Hungarian border (N = 1444 samples) was screened for HuNoV contamination by the Border Control Authority (2016–2020). The ISO 15216-2 (2013) method was used. Positivity rates were 0.1 % (1/1444) for HuNoV GI RNA, and 0.3 % (4/1444) for GII RNA. ***The Cq values ranged between 34.2 and 39.3.***
- In late 2018, the U.S. FDA began sampling and testing commercial packages of frozen single-component berries for HAV and HuNoVs using the FDA-BAM method, which was temporarily halted and restarted during the SARS CoV-2 pandemic. The FDA collected and tested 1558 domestic and import samples of three type of frozen berry commodities (585 strawberries, 528 raspberries, and 445 blackberries) (https://www.fda.gov/media/185087/download). A frequency of 0.5 % (8/1558 samples) was reported for HAV RNA, and 0.6 % (10/1558) for HuNoV RNA. Of domestically produced products, 3/538 (0.6 %) were positive for HAV RNA (2/3 were confirmed by Sanger sequencing) and 0.7 % (4/538) for HuNoVs (3/4 were confirmed by Sanger sequencing). In imported product, 0.5 % (5/1020) of samples were positive for HAV RNA (5/5 confirmed by Sanger sequencing) and 0.6 % (6/1020) for HuNoVs (3/6 sequence-confirmed) (U.S. Food and Drug Administration, 2025). ***For all positive lots (n = 10) to which the Expert Panel had data access, Cq values exceeded 40 (range, 40.75–49.98). In all but two instances, only one of three subsamples tested was positive, and in those cases, only one of nine total amplifications produced a Cq value. In the two instances in which two subsamples tested positive, two of nine amplifications produced Cq values.***

It should be noted that it is difficult to compare studies to one another. Lack of harmonization of detection methods and positivity criteria are important reasons. Sample numbers per study vary widely, from fewer than 50 to greater than 1000. One might expect contamination frequency differences by product country of origin, but studies specifically designed to identify these differences have not been done, nor have studies to systematically compare virus contamination frequency by berry type, fresh or frozen status, and/or product intended for domestic or export markets. Some of this information can be derived from existing studies but more data are necessary, preferably performed regularly, globally, and with some degree of harmonization amongst one another.

3.1.2. Outbreak investigation

The purpose of testing implicated berry products associated with an outbreak is to attempt to confirm the presence of the virus (via its nucleic acid) as together with strong epidemiological data, this provides

a more direct link to causality. The U.S. FDA-BAM or ISO 15216 methods have been used to detect contamination in outbreak investigations in which fresh and frozen berries have been implicated (summarized in Table 4). Investigators sometimes deviate from the standard methods in outbreak investigation and will often apply additional or alternative laboratory steps that further facilitate virus concentration, sample purification, and/or greater analytical sensitivity, such as using multiple/sequential RNA extraction protocols or nested amplifications.

3.1.3. Testing for regulatory purposes

Evidence of routine monitoring for foodborne virus contamination in fresh or frozen berries in the EU did not appear to be publicly available, although member states have undertaken time-limited studies in association with concerns presented by outbreaks. Several European Commission Implementing Regulations introduced amendments to Regulation 669/2009 (no longer in force and repealed by Regulation 2019/1793 in December 2019) on official controls on imports of certain feed and food of non-animal origin (available at Regulation - 669/2009 - EN - EUR-Lex) detailing legal requirements to test strawberries imported into the EU from China (for HuNoVs and HAV), and raspberries imported into the EU from Serbia (for HuNoVs) for limited time periods (2012–2014 and 2015–2019, respectively). Instructions to EU Member States regarding what to do in the event of a positive test result, e.g. destruction, diversion for further processing or redispatch, were contained in the generic European Commission Regulation 882/2004 on Official Controls Performed to Ensure the Verification of Compliance with Feed and Food Law [no longer in force and repealed by Regulation 2017/625 in December 2019 (available at Regulation - 882/2004 - EN - EUR-Lex), however the extent to which official testing was carried out does not appear to be publicly available.

More specific to the Serbian situation, in 2013 the EU Directorate-General (DG SANTE) audited the system of controls for raspberries exported to the EU, producing recommendations for improvement. In response, Serbian Competent Authorities imposed a national monitoring program and produced a rulebook on hygiene of fresh and frozen berry fruit. From 2016 to 2018, 410 official samples were tested, of which nine (2.2 %) were non-compliant. In 2017, the non-compliance rate jumped to 7.7 % (raspberry and blackberry samples). In 2019, DG SANTE conducted a second audit (DG SANTE, 2019-6698) that produced the following recommendations to Serbian competent authorities: (i) extend the risk-based control system and increase to include the entire production chain; (ii) institute a sampling and analysis program on frozen product intended for EU export; and (iii) ensure the effective functioning of the system for transmission of Rapid Alert System for Food and Feed (RASFF) notifications from Serbia to the EU. Following this audit, EU and Serbian authorities actively enhanced the food safety system, with a focus on human waste management and utilization of fresh water in raspberry growing, resulting in a lift on the export controls on Serbian frozen raspberries in 2020 (Commission Implementing Regulation (EU) 2020/625).

Consideration of regulatory action has been associated with both large frozen berry monitoring/surveillance efforts undertaken in North America (U.S. and Canada) over the last decade. In the U.S. FDA sampling assignment (2018–2023), any samples testing positive by RT-qPCR in accordance with the criteria specified above for the FDA-BAM method were considered adulterated and the remaining product on the market was subject to Class I (HAV) or Class II (HuNoVs) recalls [Note: In the US, a Class I recall (the most serious type) is issued if there is reasonable probability that the food will cause serious adverse health consequences, including death. A Class II recall is reserved for foods whose consumption may cause temporary or reversible adverse health consequences, with remote probability that these would be serious]. There was no epidemiological evidence of illness associated with these products. The FDA-BAM Chapter 26 method does not require sequence confirmation, although the agency incorporated Sanger sequencing of RT-qPCR positives as a confirmatory step before regulatory action was taken. No information was publicly shared or published by the agency regarding the sequencing protocol(s) or criteria for sequence quality/analysis.

The Canadian Food Inspection Agency (CFIA) operates a National Microbiological Monitoring Program (NMMP) and a targeted surveys program. Under both programs, a wide variety of domestic and imported products are randomly selected and tested for select microbes. As part of these programs, CFIA undertook HAV and HuNoV surveillance of fresh and frozen berries, and pomegranate arils (2016–2021). The results were recently published (Steele et al., 2022) and are described above. The CFIA follow-up activities on viral RNA-positive samples might comprise a variety of activities, including but not limited to reviewing the facilities' food safety program; inspecting facilities to verify food safety practices; assuring adequate traceability for the lot(s) in question; assuring the absence of related epidemiological evidence of human illness; and conducting or seeking risk assessment. CFIA follows a risk-based approach to take any risk mitigation action as appropriate. According to Canadian Expert Panel members, no regulatory action was taken on any of the samples testing positive during these surveillance activities, nor were any illnesses detected.

In 2023, the Taiwan Food and Drug Administration (FDA) started testing for HAV in imported frozen berries (products originated from U.S., Chile, and Mexico), presumably in response to the 2023 frozen organic strawberry outbreak (available at: https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-hepatitis-virus-infections-frozen-strawberries-february-2023) in the U.S. Samples testing positive were subjected to sequencing and the products for which sequence could be obtained were considered violative, resulting in removal of the imported product from the market. Additionally, the Taiwan FDA instituted a moratorium on imports from the U.S. supplier, which was still in effect at the time of this writing. No information is available on the sampling schemes used by the Taiwan FDA; review of protocols indicates the agency used a "double" (dual amplification of the same PCR product) traditional RT-PCR method with gel electrophoresis, rather than a single RT-qPCR reaction. This protocol deviation increases the risk for cross-contamination and makes the method non-compliant with the ISO 15216 standard. The sequencing data were of poor quality and the approach taken limits the ability to make valid conclusions as to the HAV strain to which those sequences map, and if that strain corresponds to wild-type or control HAV.

3.1.4. Testing by commercial laboratories

There are several commercial laboratories that offer virus testing of foods and environmental samples, with all of them currently using the ISO 15216–2:2019 method, and a few U.S. labs offering both the ISO 15216–2:2019 and FDA-BAM Chapter 26 methods. Particularly large and multi-national testing companies usually limit their virus testing services to one or two laboratory locations. It appears that when producers and processors do choose to test, it is almost always done because of specifications issued by their customers, with results included in Certificates of Analysis (COAs). There can be variability in the quality of commercial testing, and there is limited effort to harmonize or validate methods across labs using spiked samples, although some labs participate in commercial proficiency testing as part of accreditation. Exclusively, results are currently reported qualitatively. Conversations between the Expert Panel and several commercial testing labs indicate that positive test results are extremely rare if not entirely absent (L. Jaykus and S. Gummalla, personal communication).

An ongoing outbreak or recall associated with HuNoVs or HAV in berries generally heightens emphasis on testing across the supply chain. This was evident during the 2023 HAV outbreak linked to frozen organic strawberries (https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-hepatitis-virus-infections-frozen-strawberries-february-2023), which caused brand owners, mostly in response to legal concerns, to stipulate requirements that led to a cascade of product testing upstream of the frozen berry supply chain. This has resulted in greater use of commercial testing services, driven by customer

requirements within the supply chain. While most of this testing is done using the ISO 15216–2:2019 method, there is no clear guidance for determining lot positivity based on single sample results, or further confirmation of RT-qPCR-positives, which can complicate decision-making relative to lot disposition.

An unexplored area is the use of testing during production or processing, not just on finished product. For instance, testing theoretically could be incorporated as a means of verifying Good Agricultural Practices or Critical Control Points. This has not been done to-date. There are two significant impediments here, i.e., (i) there are no standard methods for screening production/processing waters or the hands of food workers for HAV or HuNoVs; and (ii) high testing cost and lengthy time-to-result limit its practicality. While analytical virus monitoring programs may be useful tools to obtain baseline data and to increase awareness about viral contamination among the different actors in the berry supply chain (Li et al., 2018), an effective preventive approach is what will ultimately reduce contamination risk and improve the safety of these products.

3.2. Key conclusions

- Data from testing is of interest to industry, governments, and academic sectors.
- Data from foodborne virus testing of berries can be used for many purposes. These data have historically been used to determine baseline contamination frequency; in outbreak investigation; for supply chain management by private industry; and for public health protection/regulatory purposes.
- Collectively, positivity frequencies for the presence of foodborne viral RNA in berries obtained from surveillance/monitoring studies range from no detection (0 %) to a high of 33 % (summarized in Table 2Q1 and 2Q.2). It is very difficult to compare frequencies between studies due to differences in study design, sample numbers, methodology, and region.
- As is the case for some recent reports, it would be helpful if these studies included detailed data on Cq values or genome copy number, positivity criteria/replicates, and confirmation, if undertaken.
- Detection of foodborne viral RNA in berry samples associated with identified disease outbreaks has been possible in some cases but certainly not all cases.
- Virus testing of berries done by commercial laboratories is almost always in response to supplier needs to provide certificates of analysis to their downstream customers.

4. Question #3

What is the sampling approach taken when instituting foodborne virus testing by the various sectors (academic, industry, government/regulatory)?

4.1. General sampling considerations

Decisions about if, when, and how to test pathogens in foods are driven by the value of the test relative to whether it is appropriate for determining if the pathogen is present or absent. This is affected by myriad properties of the test itself, but also by the quality and quantity of the sample(s) collected and tested. Sampling plans for detection of bacterial foodborne pathogens have been extensively described and used, with an eye to statistical validity of the resulting data (Cowell & Morisetti, 1969; DOD, 1963; Kilsby & Baird-Parker, 1983). It must be recognized that these plans are implemented under the assumption that cultural enrichment will be applied before detection, usually with zero tolerance (detection limits of 1 CFU/sample) expectations. For illustrative purposes, take a standard *Salmonella* assay applied to a 25 g sample. The enrichment process itself results in an increase in the pathogen concentration, usually 10^3-10^6- fold (Wang et al., 2015). When followed by a PCR detection method, a small aliquot of enrichment broth (say 1 ml of 250 ml or 0.4 % of the enrichment) would still contain 40+ viable *Salmonella* cells. The nucleic acid from these would be further 'enriched' a million-fold or more by PCR, yielding plenty of template to achieve a positive signal. Also, one would be assured that what was detected was a viable pathogen.

That same 25g sample, when subjected to virus testing using the ISO15216 methods, might be concentrated to a volume of 1 ml, the entirety of which is extracted for RNA isolation yielding a final sample volume of 100 μl, essentially a 250-fold concentration factor. Of this, the RT-qPCR test accommodates a 5 μl volume of RNA, meaning that only 10 % of the sample is being tested (in the ISO 15216 method, amplifications are done in duplicate). When performed on a 10^{-1} dilution of RNA (which is frequently necessary for berries), in duplicate, 1 % of the sample is being tested. Assuming 100 % efficiency in both concentration and extraction (which rarely if ever happens), and an RT-qPCR method with a limit of detection of 1 template copy (which is difficult on these sample types), the starting concentration of virus in the 25g sample would need to be at least 10 viruses, 100 if RNA dilution is required. A "perfect" test would have an overall detection limit of 10–100 viruses per 25g sample, as juxtaposed to 1 CFU per sample when using bacterial enrichment. And what is being detected is viral RNA, not necessarily infectious virus. These concepts will be elaborated upon later.

A major consideration when designing sampling plans is the distribution of a pathogen in a contaminated lot or batch of berries. Although gross contamination of berries (e.g., from contact with untreated sewage or wastewater) could result in relatively high concentrations of viruses distributed uniformly through the lot or batch, in real life, this is rather rare. More likely is a 'focal' contamination event (e.g., contact with the hands of an infected farm worker, or the comingling of contaminated with pristine product) in which the spatial distribution of virions in a contaminated lot or batch is not homogenous but rather "clustered" or "localized" (Butot et al., 2014). The overall virus concentration in that lot or batch may be quite small, although there will be pockets of contaminated products with higher concentrations of virus. So, a single test may miss a contaminated region of the lot or batch. Systematic sampling of many small sample units would increase the probability of virus detection, but the methods are cumbersome and expensive. Increasing sample numbers may not be practical or even feasible.

The rigor of testing can also be improved by increasing sample size. This is commonly done when testing for Shiga toxigenic *Escherichia coli* (STEC), for instance, in ground beef and is especially useful when compositing multiple samples. However, the sample size is clearly designated in both the ISO 15216 (25 g) and FDA-BAM (50 g) methods and even if deviation to a larger sample size were desired, it is not feasible as it would have significant trickle-down effects on virus concentration and purification parameters like reagent concentrations and reconstitution volumes.

4.2. Sampling approaches to foodborne virus testing in berries

4.2.1. Sampling approaches for outbreak investigation

When possible, the entities involved in outbreak investigation will obtain clinical and food samples, sometimes also performing an environmental survey or root cause analysis. Often, they will obtain as many samples as possible from implicated products [i.e., by code or production date, implicated ingredient(s), or even what can be easily pulled off retail shelves or from a consumer's home]. While standardized detection protocols like the ISO 15216 or FDA-BAM are likely to be applied to test these samples, laboratorians engaged in outbreak investigation often take great efforts to detect viral RNA in outbreak-related samples, meaning protocol deviations or sometimes, even completely different methods, may be used. And often, many samples are tested.

4.2.2. Sampling approaches for regulatory surveillance or monitoring purposes – U.S. And EU

For large, formalized sampling plans, such as those performed by

regulatory agencies in the U.S. and EU, multiple samples from one batch are often screened. For instance, for the FDA Frozen Berry Sampling Program, three bags of frozen product (each bag constituting a single sample) were collected from distribution. A 50 g sample was taken from each bag and processed separately through the RT-qPCR detection step. From a volume perspective, three 50 g samples (150 g total) would be considered representative of a single lot or batch of product.

The EU mandated testing from 2012 through 2019 on imported products originating from countries with a history of contamination events and linkage to illness incidents in the EU. At the time, the EU legislation stipulated only the percent of imports to be tested, but nothing about sampling plan or replicate samples. The Center for Environment, Fisheries, and Aquaculture Science (CEFAS) proposed a sampling plan that relied on food safety criteria set forth in Regulation (EC) No. 2073/2005 for similar food commodities (i.e., pre-cut fruit and vegetables) and a faecally-derived pathogen (i.e., *Salmonella*), as well as Codex Alimentarius CAC/GL 50–2004 General Guidelines on Sampling (Codex Alimentarius, 2004). The CEFAS plan required at least 5 samples to be taken when testing a batch and was used as an example the in mathematical modeling summarized in Table 7 below. However, in the absence of agreed upon regulation for virus contamination of foods, this sampling approach has not yet made it into actual EU legislation.

4.2.3. *Sampling approaches for academic laboratories and private testing for industry*

For most surveillance or monitoring studies led by academic researchers (Gao et al., 2019; Li et al., 2018; Loutreul et al., 2014; Parada-Fabián et al., 2016; Purpari et al., 2019; Shin et al., 2019; Stals et al., 2011), a single berry sample is collected and processed for virus extraction and detection. Similarly, in Expert Panel discussions about sampling strategies with representatives from some of the larger berry processing companies, it appears that when testing is done on frozen berries, the producers/processors almost always collect and send their own berry samples to an outside testing lab, one single sample at a time (typically several hundred grams each), theoretically representing a single lot. These are submitted to a single test (where the test unit is 25–50 g). Note that the weight of a single berry may range from 1 to 10+ g, so a 25–50 g sample consists of only a few individual berries. Even when multiple samples are sent, they are usually composited into a single test unit. Many commercial testing labs offer services to help companies develop targeted sampling plans, but testing for viruses is up to 10-fold higher in cost than bacterial pathogen screening, sometimes with an extended time-to-result, both barriers to screening multiple samples from a single lot or batch of product. As stated above, when processors do choose to test, it is almost always done because of the requirements of their customers.

While virtually all berry processors use commercial labs when they test, the Expert Panel had the opportunity to speak with one very progressive berry processor who has since established their own virus testing lab. This processor moved from a grab sampling approach to a modified sampling protocol in 2022 based on a square root model and informed by their risk assessment parameters such as type of berry, country of origin, previous history, supplier/grower audits, lot size, number of cases, etc. Their approach and findings are described below and the criteria detailed in Table 5.

- A "container" was defined as equivalent to 600–1000 cases of 10–15 kg each. A container may comprise multiple lots received from the same supplier or origination country. Multiple containers may be comprised of products with the same lot number as well.
- From one to up to 80 farms may be associated with a single container; some containers can represent upwards of thousands of kilograms of product that have been comingled and may or may not be part of a single lot.
- When a container represents the same lot/batch, 36 random samples of 50 g each are collected.
- When a container represents multiple lots/batches, 36 random samples of 50 g each are collected but the company ensures that each lot is represented in at least one of the 36 samples.
- In both cases, all 36 samples are pooled, leaving a composite sample of 1800 g.
- One 25 g subsample is taken from the composite and tested using the ISO 15216–2:2019 protocol
- Before and until 2021, the above sampling approach was used, testing a single 25 g subsample from the composite sample of 1800 g.
- In 2022 and thereafter, this baseline plan was further modified to apply an intensified sampling program determined by a risk level ascribed by the company. The company assesses the risk level of a 'container' based on several risk factors such as the specific commodity (berry type), country of origin, supplier history, use of inactivation interventions and wash cycles, etc.
- Based on these factors, an intensified sampling program comprising four possible sampling and compositing plans (A – D) was implemented. In general, depending on the risk assessment, each container was subject to sampling from a greater number of cases; setting a maximum number of cases for compositing; and increasing the number of composites (see table).
- By setting the maximum number of cases for compositing, more testing was achieved in plans A and B versus the baseline plan.
- In the same way, in plans C and D, further increasing the number of composites resulted in a more rigorous sampling of the container.

Table 5
Criteria for sampling based on modified square root-based approach instituted by industry stakeholder (IQF processor/packer) to inform risk-based composite sampling plan.

	Sampling and testing plan utilized before and until 2021 (Baseline sampling program)	Sampling and testing plan utilized in 2022 and beyond (intensified sampling program)[a]			
		Plan A	Plan B	Plan C	Plan D
Number of cases in a container from which samples (25g) are collected	12–36 cases	12–36 cases	12–36 cases	15–45 cases	15–90 cases
Number of cases from which collected samples were composited	Samples collected from all cases are composited	Samples collected from a maximum of 18 cases are composited	Samples collected from a maximum of 9 cases are composited	Samples collected from a maximum of 9 cases are composited	Samples collected from a maximum of 9 cases are composited
Number of composites from each container for testing	1	1–2	1–4	1–5	1–10

A 25 g test unit is collected from each composite that is subsequently subject to viral extraction and concentration, RNA extraction and purification, and RT-qPCR.

[a] The intensified sampling program comprised four possible sampling and compositing plans (A – D) which differed by the (i) number of cases per lot from which samples were collected (ranging from 12 to 90); (ii) number of cases from which samples were composited (ranging from 9 to 18); and (iii) number of composite samples tested (ranging from one to 10). All intensified sampling plans were more rigorous than the pre-2021 baseline plan. Collectively, the rigor of testing using the intensified sampling plan increased from Plan A (least rigorous) to Plan D (most rigorous).

The processor used a Cq cut off ≤43 (a value provided by the test-kit manufacturer) and for lots which tested positive, the extracted RNA was retested by RT-qPCR as a means of confirming the positive; all samples were confirmed by repeat amplification. Over the two-year course of the more intensified sampling program, not a single sample tested positive for the presence of HAV RNA. As detailed in Table 6, a few HuNoV positives were identified, leading to a two-year contamination frequency of 0.2–0.3 %. Data from the company did not delineate whether the GI or GII genotype were detected. Cq values ranged between 34.7 and 38.2. Products that tested positive were sourced from Chile, Mexico, and Peru. All cases of positively tested product were returned to the supplier.

Table 6 also provides a comparative summary of testing results obtained from the laboratory of industry stakeholders (IQF processor/packer) using random sample-grab (traditional) vs. square root-based sampling (intensified) approaches. A few interesting trends emerge. While the positivity rate was quite low, application of the intensified sampling program in the years 2022 and 2023 resulted in more positive hits than did the traditional sampling program used prior to and in 2021, during which there was not a single positive sample. In addition, in the years in which the strategic sampling model was used, the volume of product represented by testing was about two-thirds lower, meaning that more positive results were observed with less volume of overall product screened. At three to four positive samples per year, each representing about nine million kilograms of product, this equates to one positive sample for every 2.25–3.0 million kilograms of product screened.

This company is of the strong opinion that sampling should be linked to traceability, largely because the likelihood of contamination varies by berry ingredient production location, with certain countries producing products at greater risk than others. In many instances, the riskier production areas are dominated by very small farmers who batch their product to create large lots that may represent many, many individual growers. The company is continuing to test in this manner using a combination of third-party laboratory services and running their own microbiological laboratory at a total cost approaching hundreds of thousands of dollars. While commendable, this might be cost or resource-prohibitive for most frozen berry processors and illustrates the inadequacy of singular reliance on sampling and testing for management of virus contamination in berries intended for the frozen market.

It is generally recognized by industry that testing alone may convey a false sense of security, particularly in cases where the hazard presents at low concentrations and/or low and variable contamination frequency within the lot (i.e., non-uniform pathogen distribution), as is the common case for foodborne viruses in berries (EFSA, 2014). Indeed, the absence of positive samples is not a guarantee for the absence of foodborne viruses in the batch of product and thus the overall 'safety' of the lot (Dahms, 2003; 2004). Nonetheless, despite their limitations, monitoring programs may be powerful tools to obtain baseline data and to increase awareness of food safety relative to foodborne viruses among the different actors in the berry supply chain (Li et al., 2018). Hence, they can effectively contribute to the "assessment-education-continuous improvement" process (Julien-Javaux et al., 2019).

Table 6
Comparative summary of testing results obtained from the laboratory of industry stakeholder (IQF processor/packer) using random sample-grab (baseline) vs. square root-based (intensified) sampling approaches.

	Baseline sampling program (Year 1, 2021)	Intensified sampling program (Year 2, 2022)	Intensified sampling program (Year 3, 2023)
Representative volume of product (kg)	Approx. 14 million	Approx. 9 million	Approx. 9 million
Number of composite samples and RT-qPCR tests for HAV/HuNoVs	1570	1388	1598
# of positive hits hNoV	0	4	3
# of positive hits HAV	0	0	0
% positivity	0	0.3 %	0.2 %

4.3. Statistical evaluation of frozen berry sampling for foodborne viruses – an example

To demonstrate the potential impact of sampling on the likelihood of achieving a positive test result in berries contaminated by a variety of routes, an abbreviated modeling exercise was undertaken. A spreadsheet has been provided as supplemental material which describes the model and calculations used in the analysis below. In this case, five different contamination scenarios, using two different test methods and two different sampling approaches, were evaluated. The four combinations modeled were: (i) single 25 g sample, ISO 15216-2 method; (ii) five 25 g samples, ISO 15216-2 method (as per CEFAS surveillance/monitoring proposal described above; see also Table 7); (iii) single 50 g sample, FDA-BAM method; and (iv) three 50 g samples, FDA-BAM method (FDA surveillance/monitoring approach).

4.3.1. Key assumptions

- A container was defined as a single shipping unit consisting of 800 bulk cases of 13.6 kg (30 lbs) each, for a total product weight of 10,886 kg (24,000 lbs)
- Each container consists of from one to four lots of products
- The contents of any one lot may represent as few as one farm to as many as 80 different farms
- For purposes of this analysis, one virus particle was considered equivalent to one amplifiable genome copy
- Both testing methods were assumed to be 100 % specific and 100 % sensitive (i.e., no false positives and no false negatives; a "perfect" test)

4.3.2. Five potential contamination scenarios

Scenario #1: This scenario represents an event in which irrigation or wash water (assume this occurs at the pre-harvest phase) might have contaminated product at a very low virus concentration, such as would occur with an unknown septic tank leak into groundwater. This scenario is most likely to occur in an integrated business operating out of a middle to high income country. Quantitatively, it was assumed that a container consists of 800 bulk cases (10,886 kg), composed of one lot from one single supplier farm. The virus is present at a very low concentration (0.01 virus particles per g of product, i.e., one virus particle per 100 g) in a uniform manner across the product. This same concentration is present in each of the 800 cases.

Scenario #2: This scenario represents an event occurring at a single farm (pre-harvest contamination) in conjunction with poor personal hygiene of an infected picker in the field. This scenario could also happen in any location in which there was an infected processing line worker who did not wash their hands after using the restroom (post-harvest contamination). Quantitatively, it was assumed that a container consists of 800 bulk cases (10,886 kg) composed of one lot from four supplier farms. The virus is present in only one of the 800 bulk cases (13.6 kg product), and its concentration ranges from 10 to 10,000 virus particles per g in that bulk case.

Scenario #3: This scenario represents an event in which raw sewage is used to irrigate the product on a very small farm (pre-harvest contamination), most likely to occur in product imported from a lower income country and used as an ingredient or directly repacked in a product line processed elsewhere. Quantitatively, it was assumed that a container consists of 800 bulk cases (10,886 kg), composed of four lots. Each lot is 200 bulk cases (2722 kg each) with product from 20 farms (80 farms represented by the container). One farm has contributed eight bulk cases (109 kg), and the virus is present at 10 particles per g finished product. The berries from this farm have been uniformly mixed with the

Table 7
Proposal on a sampling plan and analytical methods from the EU Reference Laboratory in 2012 (CEFAS, Weymouth, U.K.) in view of the enhanced monitoring for HuNoVs and HAV in strawberries from China in accordance with Art. 15(5) of Regulation (EC) No. 882/2004. Note: this proposal was never officially mandated into legislation.

Food Category	Etiological Agent(s)	Sampling Plan [a]		Limits		Analytical Reference Methods	Stage At Which Criterion Applies
		n	C	m	M		
Strawberries from China	Norovirus genogroup I (GI) and genogroup II (GII); and hepatitis A virus	5	0	Not detected in 25 g [b]		Qualitative detection of norovirus and hepatitis A virus in soft fruit [c]	Products placed on the market during their shelf life

[a] Sample units should be distributed throughout the batch to test as much of the consignment as possible.
[b] Theoretical limit of detection is 20 genome copies of GI or GII norovirus, or hepatitis A virus, per 25 g.
[c] EURL generic protocol - Qualitative detection of norovirus and hepatitis A virus in soft fruit.

other berries in that lot (2722 kg; 200 bulk cases; one-quarter of the container). One-quarter of the cases in the container are therefore contaminated at 0.4 viral particles per g.

Scenario #4: This scenario represents a gross, focal contamination event in which an infected child defecates directly on product in the field (pre-harvest contamination). This scenario is most likely to occur in products imported from a lower income country and used as an ingredient or directly repacked in a product line processed elsewhere. Quantitatively, it was assumed that a container consists of 800 bulk cases (10,886 kg), composed of four lots. Each lot is 200 bulk cases (2722 kg each) with products from 20 farms (80 farms total). One farm has contributed 109 kg (8 bulk cases) with a very high concentration (100 virus particles/g) in only two of the bulk cases. Thus 2/800 bulk cases in the container are contaminated.

Scenario #5: This scenario is similar to Scenario #4, but with a lower magnitude of contamination. It represents a vomiting incident that occurs in an IQF processing plant that results in indirect contamination of packed product. This scenario is most likely to occur in a domestic repacking operation and illustrates post-harvest contamination. Quantitatively, it was assumed that a container consists of 800 bulk cases (10,886 kg) being processed in a single shift. Although lots are irrelevant as contamination is happening at the post-process phase, the scenario assumes the entire container is one lot for sampling purposes. A low level of contamination (0.1 virus particles/g) is present in 3/800 bulk cases.

4.3.3. Methods for initial analysis

The Monte Carlo simulation Excel add-in XLRisk Version 1.00 (https://github.com/pyscripter/XLRisk) was used for calculations. Calculations were checked using R, and similar results were obtained. In instances in which the concentration of the virus was less than one particle per g, the expected concentration was multiplied by the expected contamination frequency (where the term "frequency" is used to describe the proportion of the lot in which virus would be present) and the sample size to give the probability of a positive sample. For example, if the frequency was 25 % at 0.1 virus particles/g, and the sample size was 0.5g, the assumed frequency would become:

0.25 * 0.1 virus particles/g * 0.5g = 0.0125 or 1.3 %

The ISO 15216 methodology uses a single sample of 25 g. Each RT-qPCR reaction (5 μl of total RNA concentrate of 100 μl volume) is representative of the entire sample but constitutes the equivalent of 1.25 g of berries per amplification, and two RT-qPCR assays are performed per sample. A single positive amplification of either sample is considered a positive test result. For modeling purposes each of the two amplifications were evaluated separately for the probability of containing the virus. For extended sampling plans as proposed by CEFAS, results are multiplied by five.

The US FDA BAM methodology uses a single sample of 50 g. One-third of the sample concentrate is processed for RNA extraction, and 3 μl is used in each RT-qPCR reaction, done in triplicate. This is considered representative of the entire sample but constitutes the equivalent of 1.5 g of berries. A single positive amplification of any of the three samples is considered a positive test result. For modeling purposes each amplification is evaluated separately. For extended sampling plans as used in the FDA surveillance study, results are multiplied by three (details of analysis are provided in supplementary materials).

4.3.4. Results

The results of the modeling are shown in Table 8. By rank order (highest to lowest), it was much more likely that contaminated product would be picked up by testing for contamination Scenario #3. There was moderate likelihood for Scenario #1, then #4, especially when multiple samples were tested. The likelihood for positive test results for Scenarios #2 and #5 were remote, irrespective of sample number.

There was little relationship between $\log_{10}$ virus concentration per container and likelihood of detection. There was a positive correlation between lot contamination frequency (i.e., the proportion of the lot containing viral contamination) and the likelihood of detection. This suggests that the distribution of the virus in the lot or batch is a key driver of probability of detection by testing. Secondary to this was an elevated concentration of virus. Not unexpectedly, increasing sample number usually resulted in higher likelihood of detection.

The only scenario showing a high likelihood of detection was Scenario #3, with detection probabilities ranging from 48.8 to 86.6 % (FDA-BAM method, one vs. 5 samples) and 75.0–99.9 % (ISO 15216 method, one vs. 5 samples). This scenario represents an event in which raw sewage was used to irrigate the product on a very small farm (gross contamination event), with that product being comingled with non-contaminated product, yielding an estimate that one-quarter of the cases in the container were contaminated at a concentration of 0.4 viral particles per g.

The relationship between methods and sampling schemes was somewhat consistent across the scenarios, where the probability of detection was highest for the ISO 15216 method combined with sampling done in monitoring programs, followed by the FDA method and surveillance sampling, although both methods performed somewhat similarly. As expected, likelihood of detection always increased with increasing number of samples tested.

For three of the scenarios (#1, #3, and #5) the ISO 15216 method applied to a single sample outperformed the FDA-BAM single sample method. For the two scenarios in which the virus concentration per gram of contaminated product was elevated (#2 and #4), relative to the other scenarios, the FDA-BAM single sample method outperformed the ISO 15216 single sample method, probably because the FDA-BAM method amplifies the RNA from three subsamples while the ISO 15216 method amplifies only two RNA aliquots, and perhaps due to the larger samples size for the FDA method.

Taken together, some overarching conclusions can be reached. Not unexpectedly, the more samples processed and tested, the greater the likelihood of detection (single sample vs. sampling associated with

Table 8
Results of mathematical modeling to compare likelihood of detection (expressed as percentage) using two different sampling strategies and the ISO 15216–2:2019 or U. S. FDA-BAM detection methods.

Scenario	Contamination frequency per lot (% of lot with contaminant)	Concentration (# virus particles per gram contaminated)	Log_{10} virus per container	Likelihood of Detection (%)			
				ISO		FDA-BAM	
				Single sample (n = 1)	Surveillance/ Monitoring (n = 5)	Single sample (n = 1)	Surveillance/ Monitoring (n = 3)
1	100.0 %	0.01	5.04	2.5 %	11.8 %	1.5 %	4.4 %
2	0.1 %	~5000	7.83	0.5 %	1.2 %	0.4 %	1.1 %
3	100.0 %	0.4	6.04	75.0 %	99.9 %	48.8 %	86.6 %
4	1.0 %	100	7.04	2.0 %	9.6 %	3.0 %	8.6 %
5	0.4 %	0.1	3.61	0.1 %	0.5 %	0.1 %	0.2 %

monitoring programs). While the likelihood of detection for the two methods differed by contamination scenario when only one sample was analyzed, when the sampling criteria associated with surveillance/monitoring were considered, the ISO 15216 method always outperformed the FDA-BAM method, likely because of the larger number of samples (5 vs. 3) for the ISO method. Pathogen distribution in a given product lot is an important driver in the likelihood of detection. Finally, the likelihood of detection is actually quite high (>~50 %) when there is a gross focal contamination event (such as using sewage for irrigation) after which contaminated product is comingled; less so but somewhat reliable (~2–12 %) when there is a smaller gross, focal contamination event (such a child defecating in a field with subsequent product comingling) or a diffuse event with low virus concentration (such as a leaking septic tank contaminating irrigation water); and quite low (<~1 %) for an isolated focal event (such as contamination by a single infected food handler or a vomiting incident during processing).

4.4. Key conclusions

- In most contaminated lots or batches, virus particles are heterogeneously distributed, so while the occasional sample may have elevated concentrations of virus, most of the product is virus-free. This presents challenges for developing effective sampling plans.
- Private testing, usually performed by commercial laboratories under contract with berry producers and/or processors, is mostly based on the ISO 15216–2:2019 method applied to a single 25 g sample. This approach likely has limited value as a means of assuring that end-product is virus-free and preventing positive batch release. Nonetheless, many commercial entities see it as an important aspect of due diligence and supply chain requirements.
- It is possible to make the sampling phase of monitoring programs more rigorous, but this will require more complicated sampling protocols and likely, additional tests. The time and cost of these programs may be prohibitive, particularly when incorporated on a routine basis for monitoring the supply chain.
- Both standardized methods (U.S. FDA-BAM and ISO 15216–2:2019) do not explicitly specify a carefully designed sampling plan. For the U.S. FDA, the general approach for surveillance has been collection of three grab samples per lot or batch, processing 50 g from each sample, with each sample concentrate tested thrice (9 total) by RT-qPCR. For the ISO 15216–2:2019, most surveillance/monitoring testing has been done on a single sample with duplicate RT-qPCR amplifications, although on occasion five grab samples have been tested per lot or batch.
- Mathematical modeling of the proposed contamination scenarios illustrates that reliable detection of viral RNA (defined as a likelihood >50 %) occurs only when there is a gross focal contamination event, in this case, with comingling. In other words, the combination of high amounts of virus that are, or become, more uniformly distributed amongst large proportions of the batch or lot significantly drives the likelihood of a positive test result. This is the likely case for and representative of the very large 2012 German HuNoV outbreak (Mäde et al., 2013).

5. Question #4

How are the RT-qPCR testing data interpreted relative to sample status (positivity, negativity, or undetermined)?

Interpretation of RT-qPCR data serves as the basis for determining sample status, i.e., whether a given sample is designated as positive, negative, or undetermined. To call a test valid, all controls must produce the expected results, including meeting the specified performance criteria for amplification and process controls. In general, samples are designated as positive using a combination of Cq value and sigmoidal shape of the amplification curve. The criterion for sample positivity using the FDA-BAM method is a single Cq positive from 3 subsamples, each amplified in triplicate (i.e., even if one of nine amplification reactions is positive) and proper shape to the amplification curve. Criteria for sample positivity is not explicitly set out in the ISO 15216–1:2017 (quantification), however the approach for quantification of a sample giving a single positive amplification reaction out of a total of two is described (the negative replicate is given a zero concentration then the average of the concentrations for the two replicates is used for quantification), implying that a sample with one out of two positive replicates is treated as positive. In the ISO 15216–2:2019 (Part II, qualitative), it is explicitly stated that a sample with one out of two positive replicates is treated as an overall positive for detection. As is the case for the FDA-BAM method, all amplification plots are checked to confirm sigmoidal amplification and rule out false positive results caused by high or uneven background signal. Neither method uses a Cq cut-off value.

Virus monitoring studies provide a snapshot on how testing data are interpreted in the field. To better understand how data interpretation is approached when the test is applied in the field, the Expert Panel was able to secure relevant data from several large national monitoring studies of foodborne virus detection in berries. These data are described in the answer to Question 2 (pertinent information bolded and italicized) and summarized below. For comparative purposes, the data from extensive surveillance/monitoring of enteric virus contamination of bivalve molluscan shellfish is included.

- For the first Canadian monitoring study (2014–2016), Cq values did not exceed 40 for all seven of the positive samples (Government of Canada, 2018). For the second Canadian study (Steele et al., 2022), Cq values of positive samples ranged from 33.9 to 42.2. In the first two Eastern European monitoring studies (2017–2020), the laboratory used pre-established criteria for sample positivity which included a Cq cutoff (between 39 and 40) and duplicate positive amplifications. So, all positive samples would have presented with Cq values < 40. In the second study (2016–2020), Cq values for positive samples ranged between 34.2 and 39.3. In the case of the FDA surveillance study, for all of the positive lots (n = 10) to which the Expert Panel had data access, Cq values exceeded 40 (range,

40.75–49.98), and in all but two instances, only one of three subsamples (9 amplifications) tested positive.

- The ISO 15216–2:2019 method (with minor modifications including use of 3 rather than 2 RT-qPCR replicates for each genogroup) has been used in several surveys on HuNoVs in UK oysters, revealing high positivity rates of 76.2 % in harvesting area samples during 2009–2011 (Lowther, Gustar, Hartnell, & Lees, 2012) and 68.7 % in retail samples during 2015–2016 (Lowther et al., 2018). These two datasets, comprising 1474 samples in total, were combined and further analyzed for the distribution of Cq values (unpublished data, personal communication, J. Lowther). The criterion for sample positivity for each genogroup was one single RT-qPCR amplification (out of a total of three) presenting a positive Cq value, and appropriate amplification curve shape. Results showed that 66.0 % (1326/2009) of the positive RT-qPCR reactions for HuNoV GI, and 92.8 % (1709/1842) of the positive GII reactions produced Cq values < 40. It can be extrapolated that 34.0 % and 7.2 % of the GI and GII amplifications, respectively, presented with Cq values exceeding 40. For the subsets of 923 GI positive and 802 GII positive samples, the following breakdowns relative to positive replicates were obtained: GI: 1/3, 30.7 % of the time; 2/3, 20.9 % of the time; and 3/3, 48.4 % of the time. For GII, 25.3 %, 19.5 %, 55.2 % of the time 1/3, 2/3, and 3/3 replicate amplifications were positive, respectively. A total of 190 samples (20.6 % of GI positives) were positive based on a single positive replicate with Cq of $\geq$40; 47 samples (5.9 % of GII positives) were positive on the basis of a single positive replicate with Cq of $\geq$40. In total, 118 samples (11.0 % of overall positives) were positive for HuNoVs (GI or GII) on the basis of a single positive replicate with Cq of $\geq$40. Viewed collectively, a much higher frequency of HuNoV contamination is observed for oysters compared with berries, and in general, the Cq values are lower and the proportion of positive replicate amplifications higher. This makes it difficult to directly compare shellfish data to those for berries.

Considering only the berry data, several trends emerge: (i) interpretation criteria differ somewhat by method and lab; (ii) the frequency of a positive RT-qPCR signal for soft fruits is often quite low (<1 %); (iii) Cq values for soft fruits are usually high; and (iv) the absence of replicate positives is common. This suggests very low concentrations of detectable viral RNA and likely non-homogeneous distribution of a target in a tested product lot.

5.1. Expert Panel discussion on interpretation of RT-qPCR results

In most instances, elevated Cq values (>38, with particular attention to >40) are the rule, rather than the exception in berry samples testing positive for foodborne viral RNA. High Cq values present unique challenges from a data interpretation standpoint. This is related to both assay detection limit and statistical considerations. Bustin et al. (2009), the authors of the MIQE guidelines, advocated the need for PCR calibration curves, even for qualitative assays, stating that obtaining an accurate yes/no answer requires information about the low-end sensitivity of the assay. MIQE defines the limit of detection (LoD_{95}) as *"…within a group of replicates containing the target at concentrations at the LoD_{95}, no more than 5 % failed reactions should occur."* They state that, in general, limits of detection of <3 copies per PCR reaction, which almost always occur in the Cq range of 37–40, are not possible. It is also important to note that once outside the assay limit of detection, interpretation of results must be done based on Poisson statistics, and the Poisson assumption only holds true for about a range of 3–4 Cq values. Beyond that, (approx. Cq values > 45), the nature of sample positivity becomes stochastic, or random; it cannot be predicted statistically, resulting in uncertainty over the validity of the results (Burns & Valdivia, 2008). In instances in which positive RT-qPCR reactions for viruses in berry products present with Cq values > 40, in no more than one or two of nine replicate amplifications, the assay results do not meet the criteria of <5 % failed reactions and could be considered below the LoD_{95}. This is not to say that there is absence of target; simply, that we are interpreting results below the assay detection limit. This is not the case for the vast majority of molluscan shellfish testing results. In berries, the template may certainly be present, but it is important to be cautious in interpretation.

In the absence of template, one possible explanation for false positive results is the occurrence of PCR artifacts. Artifacts are produced due to unintended non-specific amplification (Ruiz-Villalba et al., 2017). This phenomenon is more likely when using intercalating dyes for amplicon detection rather than probes, which is not the case for the ISO 15216 or FDA-BAM methods. However, what is happening in the latter cycles of PCR is not well understood, although hypotheses have emerged from recent SARS CoV-2 testing. For example, a 2020 publication suggests that certain physicochemical phenomena, including instability of probes and quenchers after many rounds of amplification, can drive the production of high Cq values independent of template amplification (Public Health Ontario, 2020). The likelihood of non-specific amplification increases with the RT-qPCR cycle number, meaning that false positives occur more frequently at high Cq ($\geq$40) (Ruiz-Villalba et al., 2017). Late non-specific amplification has also been reported, due to dimerization events, for certain SARS CoV-2 targets (Jaeger et al., 2021). Although it is unknown if non-specific amplification and hybridization are occurring in RT-qPCR assays applied to food and environmental samples tested for viral RNA, there is a need to study this further. This is particularly important because the RNA extracts derived from food and environmental samples usually contain some residual sample matrix components which could increase the likelihood for non-specific primer-probe binding and/or spurious amplification, resulting in high Cq values (Ruiz-Villalba et al., 2017).

Another cause of false positive results is cross-contamination between samples with the positive control materials (e.g., viral RNA, cDNA); or from reagents or a laboratory environment having low levels of amplicon contamination. There are international guidelines for control of PCR contamination in diagnostic labs (ISO 22174:2024). Careful adherence to these guidelines, including separation of pre- and post-PCR work areas, and handling of sample and positive control material in different work areas, should ensure that contamination of samples or PCR reactions with positive control materials or amplicons occur very rarely. However, some labs have tighter adherence to such standards than others, and the use of positive controls means that there is always at least a minute opportunity for cross-contamination during a test run. The ISO 15216 method recommends positive controls that contain genetic modifications (for instance, to introduce a novel restriction site) which provides a molecular means by which to identify cross-contamination (ISO, 2017). This method also uses external amplification control RNA standards (ECs) that produce signals which can be distinguished from wild-type targets by restriction enzyme analysis or sequencing (D'Agostino & Cook, 2018). Such downstream analyses can rule out cross-contamination but are not always feasible (as discussed below).

The FDA-BAM method uses positive controls that are commercially available through the ATCC and are described as synthetic GI and GII HuNoV RNA, and HAV RNA. For the HAV RT-qPCR reaction, the FDA method employs the 'control exclusion assay' (CEA) to rule out potential cross-contamination. This protocol is described only briefly in the FDA-BAM Chapter 26 but appears to be a single amplification specific to the positive control that is done in parallel with the test sample. From a statistical standpoint, the number of CEA amplifications used for any one test should be chosen based on a combination of the assay limit of detection and sample positivity criteria. So, if the sample positivity criterion is 1/9 amplifications positive (at any Cq) and the goal is to be 95 % confident that a true positive is occurring (LoD_{95}), not just one, but many (20–30) CEA amplifications would need to be done to rule out cross-contamination. The inability to make clear distinctions between positive controls and naturally occurring virus puts the method at

greater risk of producing false positive results in the event of a cross-contamination incident.

Taylor et al. (2019) pointed out that when target DNA concentration is very low (the authors used a benchmark of <10 copies per reaction), primers may not land on all the template molecules in the first cycle, a particular problem for samples with residual matrix-associated debris. This means that different fractions of the original starting template are amplified in subsequent cycles before complete amplification of all the template has been initiated, resulting in poor repeatability of the assay. While single amplifications presenting at high Cq can represent the presence of viral RNA at very low levels, in the field, some laboratories view weak signals or highly sporadic replicate sample positivity as suspicious and will perform additional amplifications or even repeat parts of the overall procedure, to obtain more data to aid in decision-making. This approach is not specified in the ISO 15216 standard and would be done at the discretion of the individual laboratory.

It is also important to note that Cq values are highly dependent on the specific thermocycler used, corresponding software, fluorescence measurements and applied algorithms, quality of chemical reagents, brands, pipetting and weighting operations, among other factors. There is also considerable variability between or within matrix categories, from run-to-run, or among laboratory staff. Harmonization studies between laboratories can help to manage such differences (Lowther et al., 2019). An alternative would be to establish a fixed cut-off value for determination of sample positivity/negativity. This option would require that all these factors be highly conserved from lab to lab, which for the ISO 15216–2:2019 method is nearly impossible. This is not to say that a single laboratory could not define cut-off values for their own validation/verification plans, and use them in routine work, but it would be difficult to expand these to more universal use. This may not necessarily be the case for the FDA-BAM method, as it is highly prescriptive and mostly used within a tightly controlled regulatory laboratory network. There is also precedent for Cq cut-off values for another important non-cultivable pathogen, *Cyclospora cayetanensis* (FDA-BAM, Chapter 19b; available at: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-19b-molecular-detection-cyclospora-cayetanensis-fresh-produce-using-real-time-pcr). Also of note is that two of the major kit manufacturers use Cq cut-offs in interpretation of HAV testing results. Specifically, the BioMerieux CEERAMTOOLS HAV test kit package insert indicates a Cq < 40 is considered positive (https://www.biomerieux.com/us/en/our-offer/industry-products/ceeramtools.html), while the Eurofins kit uses a Cq < 43 (https://www.goldstandarddiagnostics.com/virseek-food-hepatitis-a-virus-real-time-rt-pcr.html).

Collectively, the Expert Panel was not in favor of establishing a universal Cq cut-off value for virus testing in berries. They agreed that Cq cut-off values may be appropriate for an individual lab but would need to be carefully established for a well-validated assay. They pointed out the need to acknowledge that imposition of a Cq value cut-off, while it may reduce some false positives, will result in a higher false negative rate as some genuinely contaminated samples will be treated as negatives. Accordingly, any use of a Cq value cut-off is a question of balancing priorities, sometimes called producer-consumer risk. Nonetheless, Cq cutoffs do appear in the literature, but usually applied to academic laboratory studies (e.g., Bustin et al., 2009; Stals et al., 2012).

In the absence of international standards, the Expert Panel did, however, agree to certain general guidelines for interpretation of Cq values for berry samples processed by standardized methods and yielding sigmoidal RT-qPCR curves. Specifically, the laboratory should establish a limit of detection based on a validated (reference) method that has been verified in that laboratory. Note that, at the time of this writing, the only reference method that is fully validated internationally is the ISO 15216–2:2019. The laboratory should also be accredited by an appropriate body. If the sample test result demonstrates a sigmoidal curve shape and assuming cross-contamination is ruled out, samples testing with a Cq which signifies that detected virus is at or below the assay limit of detection are interpreted as positive. However, in cases such as these, it was also suggested that the lab follow up with further evaluation that could include additional testing (e.g., consider repeat analyses, or additional lot sampling and testing); facility visits with assurance of traceability; risk assessment; nucleic acid sequencing; and/or other measures discussed below that constitute further characterization. In some cases, an inevitable consequence of further characterization is a second negative test that could misclassify a lot or batch that is indeed contaminated, albeit at a likely low level of contamination. When a sample presents with an elevated Cq value but in the absence of a sigmoidal curve, it is sometimes interpreted as negative.

5.2. Key conclusions

- The ISO 15216:2–2019 and U.S. FDA-BAM methods have defined criteria for sample positivity, with the commonality that one of the replicate amplifications present with a positive Cq value and display the typical sigmoidal amplification curve shape.
- In most instances, elevated Cq values are the rule, rather than the exception, for berry samples testing positive for foodborne viral RNA. High Cq values present unique challenges from a data interpretation standpoint.
- Two major factors can lead to false-positive results: spurious or non-specific amplification and cross-contamination. Both will often present high Cq values and must be ruled out when positive results with high Cq values are obtained.
- In some instances, it may not be as simple as calling the test “positive;” a term like “presumptive positive” may be more appropriate. Certainly, this should be the case in instances for which cross-contamination with a target-identical positive control cannot be ruled out. Some laboratories also view samples with very high Cq values that lack replicate amplifications as suspicious and these may be subjected to further testing, be it repeat analysis or use of a reliable confirmation method.
- The Expert Panel did not recommend the establishment of a universal Cq cut-off, although recognized its value for use by a single laboratory if carefully validated. The Expert Panel did agree that more standardized guidelines for interpretation of Cq values, and the requirement for replicate amplifications, are useful and merit further scientific discussion.

6. Question #5

Is additional laboratory testing being performed on RT-qPCR-positive samples to confirm and/or characterize genome sequences? If so, what methods are used and/or in the pipeline?

The need for additional laboratory testing following an RT-qPCR positive test result (this process is sometimes referred to as ‘confirmation’) has been debated. Confirmatory testing is often attempted in the case of berry products associated with a recognized foodborne illness outbreak. It is sometimes performed in routine foodborne virus sampling and monitoring programs [e.g., by U.S. FDA and recently by the Canadian Food Safety Authority (Steele et al., 2022),]. Commercial testing labs rarely obtain positive results for virus detection in berries and often do not have the capabilities to confirm such complex samples using sequencing or metagenomics (S. Gummalla, personal communication), although out-sourced Sanger sequencing of the real time product to discriminate wild type vs. positive control may be an option.

Historically, the method of choice for further characterization of samples testing positive for viral RNA using RT-qPCR has been Sanger sequencing. In the case of clinical samples, sequencing is often done on RT-PCR amplicons from two or more select virus genome regions. Ideally, in outbreak investigations, the same amplification regions as used in clinical diagnosis would be used for sequencing viral nucleic acid derived from berry samples. In this case, identical sequences would serve as strong evidence that the tested product was the most likely cause of the outbreak. This is rarely done for non-culturable pathogens,

though. In the case of surveillance or monitoring, additional testing may be done to (i) rule out potential cross-contamination with a positive control or co-processed samples; and/or (ii) characterize virus genotype (s) or strains associated with natural contamination of the berry product. Some ISO 15216-compliant laboratories use positive control sequences with inserts, making it possible to rule out cross-contamination by restriction digestion. However, neither this control design nor restriction digestions to exclude cross-contamination are required of the ISO 15216 method. The FDA-BAM method uses commercially available positive control sequences for both HAV and HuNoV testing, supplementing this with the CEA for HAV (see also response to Question 4). For the FDA-BAM method, Sanger sequencing is currently used to confirm that the target amplicon detected from the sample was not the result of non-specific amplification or cross-contamination.

6.1. Sanger sequencing

6.1.1. Hepatitis A virus

Both ISO 15216 methods require the amplification of a fragment from the highly conserved 5′NCR (non-coding region) of the genome in the soft fruit (berry) method. This RNA region has extensive secondary structure and contains the IRES (Internal Ribosome Entry Site), a non-coding but functional structure that recruits the ribosome and directs CAP-independent translation (Brown et al., 1991). This intrinsic structure is required for proper functionality. Therefore, it is a region highly conserved between the different strains and ideal for the design of robust RT-qPCR assays. Sequencing this region would be appropriate and there is enough sequence divergence to distinguish wild-type from cross-contaminating positive control. Were a second region to be necessary, using the same criteria, it would be logical to target another highly conserved and structured region in the HAV genome. A potential optimal region would be the CRE (cis-active RNA element) located near the 5′ end of the polymerase coding region (Yang et al., 2008, https://doi.org/10.1128/jvi.00787-08). Similarly, the 3′NCR is another amplification region candidate (Rohll et al., 1995; https://doi.org/10.1128/2Fjvi.69.12.7835-7844.1995). Both RNA regions fold in secondary structures, which are involved in the process of genome replication; hence, they are highly conserved among the different HAV strains. The HAVnet typing protocol [sponsored by Dutch National Institute for Public Health and the Environment (RIVM); available at: https://www.rivm.nl/en/havnet] is widely used for typing of clinical and food samples, and sequences are deposited in the HAVnet database for comparison purposes.

6.1.2. Human norovirus

The ISO 15216 methods require genogroup (GI and GII)-specific RT-qPCR amplification of the ORF1-ORF2 junction region as it is the most conserved region across the genetically diverse HuNoV genotypes. However, traditional HuNoV genotyping is based on the sequence of a small 5′-region of the capsid gene (ORF2). Since recombination around the ORF1-ORF2 junction region can occur, the sequence-based typing system for HuNoVs has now been updated to include P-types, which are based on sequence diversity of the RNA-dependent RNA polymerase (RdRp) gene, located at the 3′-end of ORF1 (Chhabra et al., 2021). Dual typing of HuNoVs not only provides the necessary resolution for source tracking and outbreak investigation but also allows for tracking of recombinant strains (Green, 2018). Sequences can be typed using the norovirus typing tool [https://www.rivm.nl/mpf/typingtool/norovirus/] or the human calicivirus typing tool [https://calicivirustypingtool.cdc.gov/bctyping.html]. Both typing tools use the same reference sequences.

The standard methods used for detecting HAV and HuNoVs in berries and their products does not necessarily match what is being done for clinical samples. Further the use of Sanger sequencing of RT-PCR amplicons derived from berry samples is much more difficult than it is for clinical samples. This is driven by two major factors: (i) the low concentrations of viral RNA anticipated in these products; and (ii) the effects of residual matrix-associated inhibitors. Although sequence has been obtained from foodborne outbreak samples presenting with Cq values in the 40's (Woods et al., 2016), the Expert Panel agreed that the higher the Cq value, the less likely it will be possible to obtain high quality sequence using the Sanger method. This can sometimes be overcome using nested amplifications.

The Expert Panel discussed potential guidelines for recommending further analysis of samples testing positive for foodborne viral RNA using standardized RT-qPCR methods and yielding sigmoidal curves as:

- Cq values $\leq$ 35: clearly positive
- Cq values of 36–39: additional testing/confirmation is recommended
- Cq values $\geq$ 40: additional testing/confirmation is required

From previous discussion, it appears that most positive samples are going to fall in the latter two categories. These are exactly the categories where sequencing becomes less reliable. Further, even if sequence can be obtained, its quality may not be the best. Unfortunately, there are no standardized quality specifications for sequences derived from food and environmental samples, nor are there clear bioinformatics guidelines for alignment and interpretation of such sequences. The Expert Panel recommended a few "best practices" that include the following, and ideally, would be harmonized within the scientific community:

- Use of a proof-reading polymerase to ensure sequence quality
- Reading sequences from both directions
- Establishing minimum quality scores for sequence data
- Determining minimum number of amplicons and/or sequence length required for reliable interpretation
- Establishing criteria for clearly determining a wild-type sequence

For identified berry outbreaks, sequencing has been successfully applied in a few instances (summarized in Table 4). There are many more cases in which investigators were unable to obtain quality sequence. Most of these are not detailed in the scientific literature. In many, if not most of the cases in which sequence has been obtained from berry samples corresponding to outbreaks, nested PCR was necessary to sufficiently enrich the amplicon(s) to facilitate sequencing. Inclusion of nested amplifications increases the likelihood of cross-contamination, meaning that best laboratory practices are even more important, and the analyst must be very sure that the sequence obtained for the sample is not that of the positive control. Cloning is sometimes used in place of nesting (Raymond et al., 2022).

6.2. Whole genome and next generation sequencing (WGS and NGS)

Whole genome sequencing (WGS) has been proposed for foodborne virus surveillance and outbreak investigation, with promises and challenges in food virology detailed elsewhere (Desdouits et al., 2020). Different WGS methods, including the metagenomics approach and the genotype-specific approach, use different next-generation sequencing (NGS) platforms such as Illumina MiSeq, Oxford Nanopore, and Ion Torrent. They have been successfully employed on HuNoV clinical samples for surveillance purposes as well as to delineate linked cases (Fischer et al., 2019; Kundu et al., 2013; Nasheri et al., 2019). Metagenomic techniques rely on *de novo* assembly of virus-specific reads without using any virus-specific primers for enrichment purposes. The success of this approach requires high virus titers. On the other hand, genotype-specific approaches use primers that allow for enrichment of virus-specific sequences and are more amenable to low titers (Cotten et al., 2014; Kundu et al., 2013). However, the rapidly evolving nature of HuNoVs means that primers must be periodically updated. Parra et al. (2017) employed a technique that would render full-genome amplicons for all HuNoV genotypes within each genogroup that would be ideal for analysis by high-throughput NGS platforms.

Because viral sequences are often difficult to obtain and tend to be of

poorer quality when RNA template concentrations are low, it is likely that enrichment approaches will be necessary when testing food and environmental samples. Enrichment strategies are often based on sequence-specific capture probes such as SureSelect (Brown et al., 2016), or a poly(A) capturing technique (Fonager et al., 2017). While these approaches overcome the problem of primer design in amplicon sequencing, they have not been found to be more efficient when compared to conventional RNA-Seq methods for samples with low viral loads (Thomson et al., 2016). The third-generation sequencing platforms, which can produce long sequencing reads (>1 kb), including Pacbio Single-Molecule Real-Time (SMRT) and Oxford Nanopore (MinIon), have been recently applied for genomic characterization of HuNoVs from clinical samples (Thomson et al., 2016) and for HuNoV outbreak tracking (Silva et al., 2021). In addition, the use of NGS platforms for "metabarcoding" (simultaneous characterization of multiple viral strains following amplification of viral genome fragments using conventional RT-PCR) has been successfully applied to norovirus in bivalve shellfish (Ollivier et al., 2022). WGS and NGS have limited use for HAV, mostly applied to clinical samples (Batista et al., 2020; Cleary et al., 2023; Lee et al., 2022; Zufan et al., 2023; Yang et al., 2018; Sabrià et al., 2019).

The application of WGS methods to confirm viral contamination in berries is in its infancy. The first reported success was that of Chen et al. (2019), who obtained near-complete genome sequence of HAV subgenotype Ib from a frozen raspberry sample obtained from a 2013 infection "event." Using a transitional RNA sequencing metagenomics approach, that sequence is deposited in GenBank but upon alignment, appears to be similar to ones reported for the cultivable HM-175 strain. Using metagenomic approaches, near-full genome sequences have been obtained from samples with viral titres higher than 10^6 genome copies (Nasheri et al., 2017; Petronella et al., 2018; Sabrià et al., 2018), but this is too high to be of utility for the 10^1 to 10^3 viral genome copies found in naturally contaminated foods. For example, exhaustive data mining of 29 million sequence reads obtained from RNA-Seq analysis of naturally contaminated frozen strawberries resulted in recovery of only two short reads, with a length of 146bp that showed homology to the HuNoV genome (Bartsch et al., 2018). More recently, Flint and colleagues used a different approach to perform HuNoV WGS using both Illumina MiSeq and Oxford Nanopore platforms on samples with an RNA copy number <200, although this was only applied to clinical samples (Flint et al., 2021).

Several recent studies have systematically evaluated these next generation technologies, specifically for HuNoVs and HAV in berries, often on spiked samples. Buytaers et al. (2022) performed a comparative study using a spiked raspberry matrix and a variety of nucleic acid preparation and sequencing technologies. Even with target enrichment, it was difficult to obtain sufficient quality sequence at spike levels $<10^5$ genome copies (as lenticules) per 25 g of sample. Yang et al. (2024) performed a similar spike study on blackberries, specifically evaluating non-target pre-amplification methods. In general, the pre-amplification methods coupled with WGS facilitated a sufficient number of reads for confirmation and genotyping from samples producing initial RT-qPCR Cq values of 35 or below. For those samples in an intermediate group ($35 \leq Cq \leq 40$), or for the single naturally contaminated blackberry product tested ($Cq \geq 40$), the approach was successful in producing some useful sequences, but not consistently. Raymond et al. (2022) demonstrated successful sequencing of a 2.4-kb cDNA fragment corresponding to the HuNoV ORF 2 and ORF3 capsid genes amplified using a multiplex long-range two-step RT-qPCR method. As applied to frozen raspberry samples associated with several 2017 outbreaks, they were able to use NGS for confirmation and demonstrated the presence of multiple genotypes. Most of these samples initially tested positive at Cq values ranging from 34 to 39. Most investigators conclude that, when Cq values are very high, only a few viral reads, short sequences, and poorer quality data are obtained using NGS/WGS. Efforts continue to make these techniques more amenable to food and environmental samples.

6.3. Alternatives to sequencing

The Expert Panel pointed out that NGS is labor intensive, time-consuming, and expensive, and that Sanger sequencing often requires multiplexing. Many laboratories do not have the equipment, time, or expertise to routinely confirm samples testing positive by RT-qPCR. In addition to ruling out cross-contamination, ideally sequence analysis would also be used for genotyping or strain designation. Given the complexity of sequence analysis, this begs the question 'is sequencing the best way to confirm?' The following alternatives to sequencing were discussed by the Expert Panel.

6.3.1. Microarrays

This technology was advocated for many years and developed for the detection and potential genotyping of HuNoVs and HAV (Quiñones et al., 2017; Yu et al., 2016), with proof-of-concept in some produce commodities, but not berries (Yu et al., 2020). In produce, detection limits were still in the range of 10^3-10^5 genome equivalents and genotyping resolution was poor. These research efforts appear to be dwindling, although commercial microarray detection devices are still in development (Kosai et al., 2021).

6.3.2. Multiple amplification targets on the same viral genome

The wider use of multiple amplification target PCR diagnostics emerged during the SARS CoV-2 pandemic, as was the introduction of wastewater-based epidemiological surveillance (Arena et al., 2021; Hamouda et al., 2021). While the nature of targets used is the subject of debate, different viral genome target regions amplified by RT-qPCR assays should ideally be longer, equally sensitive and, preferably, distantly located on the viral genome, providing added assurance that the full viral genome is present. This is particularly challenging for HuNoVs, for which there is substantial genetic diversity (particularly in the capsid region), necessitating the use of degenerate primers to accommodate assay inclusivity (Oh et al., 2023). It does not appear that the multiple amplification target method has been thoroughly investigated for confirmation of either HAV or HuNoVs.

6.3.3. Repeat testing

Some of the Expert Panel members cited repeat testing to confirm positive results, particularly when sample positivity is not present among all replicate samples, or when Cq values are very high. Repeat testing can take the form of rerunning an additional aliquot of the sample through the procedure in its entirety; performing a new RNA extraction on a retained sample aliquot; and/or performing additional amplifications on retained RNA extracts. A few laboratories have established criteria to identify when repeat testing might be recommended, the protocols to be used, and how the data might be interpreted. However, the use of repeat testing varies by individual laboratory and there are no standards for when and how to use it, and no Expert Panel consensus on whether repeat testing is even appropriate as a form of confirmation.

Depending on the nature of decision-making, strategic resampling and testing might aid in determination of lot disposition, but again, there are no standards to advise such a policy. Running split samples in another laboratory or reliance on multiple labs could also be done to assure results are repeatable. Another form of repeat testing might be the use of alternative platforms such as digital PCR. While fundamentally not more sensitive or robust than the standard real-time format is, digital PCR is not susceptible to distortion of quantitative results due to higher Cq values caused by inhibition (Coudray-Meunier et al., 2015; Fraisse et al., 2018). Of course, repeat testing can be expensive and time-consuming, especially if quick decisions are necessary. Its routine or mandatory use would require significant changes and/or additions to the standardized tests and might take years to validate and accept. Statistical analysis of the value of repeat testing might be an appropriate approach to justifying its use as a confirmation method.

6.3.4. Addressing the infectivity dilemma

Keeping in mind that routine laboratory cultivation of wild-type HAV and HuNoVs is not possible, several alternative approaches have been proposed to deal with the infectivity dilemma. Given that berry Cq values are higher, this might prove difficult for this commodity. Greater reliance on fecal indicators has been proposed, although there remain conflicting data on the strength of the association between foodborne viruses and indicators (Victor et al., 2021). Some methodological alterations or add-ons, like proteinase K and/or RNase A pre-treatments, integrated RT-qPCR, nucleic acid intercalating dyes, and binding assays (e.g., preceding RT-qPCR with ligand-based virus capture, using for instance porcine gastric mucin or antibodies), have been studied and are discussed in greater detail elsewhere (Escudero-Abarca et al., 2014; Fraisse et al., 2018; Knight et al., 2012; Raymond et al., 2023; Stals et al., 2013). At the time of this writing, none of these have been fully validated for widespread use.

6.4. Key conclusions

- Additional testing as a means of confirming a positive RT-qPCR result may be called for in certain circumstances. The two most important reasons to confirm are to (i) rule out cross-contamination; and (ii) provide additional information on virus genotype and/or strain.
- To date, the most common confirmatory method is Sanger sequencing. While successfully used in some contexts, its utility is complicated by low viral RNA concentration and residual matrix-associated inhibition, both of which can result in failure of sequencing or poor sequence quality. This can sometimes be overcome using nested amplifications.
- There is a need for harmonized best practices for interpretation of amplicon sequences from samples testing positive, including but not limited to (i) reading sequences in both directions; (ii) establishing minimum quality scores for sequence data; (iii) determining minimum number or amplicons and/or sequence length required for reliable interpretation; and (v) establishing criteria for clearly determining what constitutes a wild-type sequence.
- Whole genome and next generation sequencing have several benefits, including the ability to resolve sequences from samples contaminated with multiple virus strains. However, they remain in their infancy and cannot yet reliably accommodate samples having low virus titer or those derived from berries.
- Alternatives to confirmation by sequencing (e.g., multiple amplification targets, repeat testing, digital PCR) are possible but have not been fully investigated.
- Regardless of method, confirmation will increase cost and time to result and may not be practical for many laboratories.
- If confirmation were to become mandatory, the question remains, what to do when it fails or is not possible? Required confirmation would inherently decrease the likelihood of false positive but increase the likelihood of false negative results. The implications of this policy have not yet been investigated on a global scale but deserve careful consideration.

7. Question #6

How is the data from the totality of the testing process (i.e., sampling, virus concentration and purification, and RT-qPCR) interpreted relative to lot or batch acceptability?

7.1. Test accuracy

Accuracy is a test feature that describes the ability of the test to correctly identify positive and negative samples. Accuracy is expressed as a percentage, i.e., 100 % accuracy means that the test *always* produces negative results when the sample does not contain virus and *always* produces positive results for samples with virus. In other words, it *never* misclassifies a sample. Since knowledge of the contamination status of the sample is necessary to provide accurate values for sensitivity and specificity, most test development studies use relevant food samples seeded with pre-determined quantities of the pathogen to evaluate test accuracy. Accuracy is described by two test parameters, sensitivity, and specificity. Sensitivity and specificity can be further subdivided into analytical aspects and predictive (sometimes called diagnostic) aspects. Analytical aspects are features of the test itself; predictive aspects are features of the performance of the test in real life applications. For greater detail, consult Saah and Hoover (1998).

7.1.1. Analytical sensitivity, specificity, and limits of detection

Analytical specificity is defined as the ability of the assay to detect a particular organism to the exclusion of others, in a given sample type. Analytical sensitivity also refers to the lowest quantity of the target organism that can be detected, with a sufficient degree of confidence that the interpretation of the result is correct. The latter is equivalent to the assay limit of detection. For example, an LoD_{95} would be interpreted as the lowest concentration (corresponding to higher Cq values) of target virus that can be consistently detected in 95 % of samples tested under routine laboratory conditions. In this case, one would be highly confident that a positive test result truly reflects a positive sample. The LoD_{50} corresponds to the lowest virus concentration that can consistently be detected in 50 % of the samples, so inherently means a greater likelihood that the test will misclassify a positive sample as negative.

The test signal (in the case of RT-qPCR, the Cq value) corresponding to the chosen degree of confidence in analytical sensitivity is called the cut-off value. A change in cut-off value will increase or decrease analytical sensitivity, with the corresponding opposite effect on analytical specificity. However, standard methods for detection of foodborne virus contamination in berries do not rely on cut-off values, and for most laboratories, the presence of a Cq value (given the typical amplification curve shape) is considered as evidence of viral contamination, while the absence of a signal qualifies as evidence of no detectable viral RNA. In the absence of assay cut-off values, when a result presents too far below the limit of detection, results become more stochastic in nature. and consequently, drawing results from a single sample or single amplification can be tricky.

7.1.2. Analytical sensitivity and specificity values for foodborne virus testing in berries

Since knowledge of the contamination status of the sample is necessary to provide the inputs upon which analytical sensitivity and specificity values are calculated, most test development studies use food samples seeded with pre-determined quantities of the pathogen of interest. This means that limits of detection are calculated based on data for the relevant matrix using the entirety of the assay (including sample preparation, RNA extraction, and RT-qPCR); and by reference to a standard curve of Cq value as a function of template copy number. In the case of RT-qPCR, RNA copy number is often used as a proxy for numbers of viruses (without consideration of the infectivity dilemma), and the Cq value is translated to correspond to RNA copies per g or sample of berries. In a comprehensive study, Lowther et al. (2019) reported on validation of the ISO 15216–2:2019 method, reporting LoD_{95} data for soft fruit, using raspberries as the representative commodity. Based on a standard curve generated from a dilution series of dsDNA containing the target sequence, they reported LoD_{95} values of 3.97, 0.65, and 0.79 genome copies/g for HAV, HuNoV GI, and GII, respectively. Limit of quantification data were also provided in this paper, but are not relevant to this discussion.

7.1.3. Predictive (diagnostic) sensitivity and specificity

Predictive (diagnostic) sensitivity is the ability of the test to correctly identify samples having the presence of virus within a population of similar samples. Predictive sensitivity is influenced by assay limit of detection. When the ISO 15216–2:2019 method was applied to

raspberries (Lowther et al., 2019), the predictive sensitivity per contamination level was e.g. 100 % when samples were seeded with over HAV 76 genome copies per gram, but 87 % at the lowest concentration (12 copies/gram). In this case, predictive sensitivity was calculated by contaminant concentration, with values of 100 % when HAV concentration exceeded 7.6×10^1 copies/g, and 89.5 % at the low contamination level tested, which was 1.2×10^1 copies/g. For HuNoVs, predictive sensitivity was 100 % at concentrations exceeding $1.1–1.5 \times 10^1$ genomes copies/g. At the lower contaminant concentrations tested, predictive sensitivity was 73.7 % at $2.1 \times 10^{\circ}$ copies/g (for GI); and 61.1 % at $1.5 \times 10^{\circ}$ copies/g (for GII) (ISO, 2019). Predictive sensitivity values are not reported for the FDA-BAM Chapter 26 method. It should be noted here that as the limit of detection is approached, predictive sensitivity begins to drop. In other words, a very analytically sensitive method does not perform as well near or below the assay limit of detection.

$$\text{Positive Predictive Value (PPV)} = \frac{(\text{sensitivity x prevalence})}{(\text{sensitivity x prevalence}) + [(1 - \text{specificity}) \text{ x } (1 - \text{prevalence})]}$$

$$\text{Negative Predictive Value (PPV)} = \frac{[\text{specificity x } (1 - \text{prevalence})]}{(\text{sensitivity x } 1 - \text{prevalence}) + [(1 - \text{sensitivity}) \text{ x prevalence})]}$$

Predictive specificity is the ability of the sample to correctly identify samples that do not contain virus, from within a population of similar samples. In the case of virus testing in berries, this value is largely mediated by the quality of the primers and probes, and in most cases, the reported specificity for the standard methods is quite high. For instance, for the raspberry matrix, the specificity of the ISO 15216–2:2019 is 100 % each for HAV and HuNoV GII, and 94.4 % for HuNoV GI (ISO, 2019). Predictive specificity values are not provided for the FDA-BAM Chapter 26 method.

Besides the exclusivity of the RT-qPCR assay, specificity can also be impacted by non-specific amplification or cross-contamination with positive samples or controls. Given the assumption that all labs will adhere to standard protocols and use appropriate practices, these latter possibilities are generally not considered in calculating specificity. Predictive sensitivity is influenced by the degree of statistical confidence chosen for the limit of detection calculation; matrix-associated RT-qPCR inhibition; and/or sample dilution. These factors are sometimes, but not always, considered in predictive sensitivity calculations. Given the considerable flexibility of the ISO 15216 methods, the Expert Panel recommended that individual labs determine the predictive sensitivity of the assay as applied at their location.

7.2. Predictive value of the test

While it is important for a test to accurately classify samples with virus as positive, and those without virus as negative, what the user is most interested in is how likely it is that the contaminant is truly present in a sample testing positive or absent in one testing negative. In screening assays, this phenomenon is described by the term predictive value, a foundational test statistic described in greater detail by Monaghan et al. (2021). Positive predictive value (PPV) describes the probability of contaminant presence for a sample testing positive, while negative predictive value (NPV) reflects the probability of contaminant absence for a sample testing negative. While predictive value is driven in part by assay sensitivity and specificity, it is also highly dependent upon the inherent likelihood or prevalence of contamination. Positive predictive value and NPV are calculated using sensitivity, specificity, and prevalence based on the following formulae, derived from Bayes equation:

By way of illustrative example, juxtapose a test with 99 % specificity and 90 % sensitivity applied to berries having HuNoV contamination prevalence (frequency) of 0.1 % vs. 10 %. For the 0.1 % prevalence, PPV would be 8.3 % and NPV would be virtually 100 %. This means that only 8.3 % of samples testing positive are "true positives." For the 10 % prevalence, PPV would be 90.9 % and NPV would be 98.9 %. One would be much more assured that a positive is a true positive. Note very little impact of prevalence on NPV, but PPV changes dramatically with prevalence; the higher the prevalence, the better the test is at predicting the presence of virus. Most monitoring studies suggest that virus prevalence in berries is quite low, usually <1 %. Under these same test parameters described above, but with a 1 % virus prevalence, PPV is predicted to be 47.6 %. In other words, about half of the positive test results would be true positives, while the other half would be false positives. It is, however, important to note that as specificity rises ever closer to 100 %, PPV improves, and the likelihood of false positives disappears. It is for reasons of predictive value that inclusion of prevalence/frequency of contamination is used in the mathematical modeling exercise described below.

7.3. Integrating the concepts

There are several takeaways from this discussion. The first is that the confidence chosen for limit of detection calculations is relevant: the greater the confidence (e.g., 95 %), the less likely that false classifications will occur. Secondly, when assay results move at or below the limit of detection, predictive sensitivity is reduced and there is an increased chance for false negative results. Based on standard curves reviewed during Expert Panel deliberations, when samples present with Cq less

than the mid-30's, one can be reasonably confident that viral RNA has been detected. As Cq approaches 40 or higher, that confidence is reduced, and users should be cautious in their interpretations. Below assay limits of detection, results begin to show stochasticity and replicate samples and/or amplifications can be valuable. Thirdly, specificity is a key driver in being assured that positive test results are truly positive, so cross-contamination control is extremely important. In instances in which many samples are being tested, or there is a concern for cross-contamination, the inclusion of more controls is better. Fourthly, sampling is important, particularly when trying to determine lot or batch disposition based solely on testing results. The interplay of test accuracy (limit of detection, sensitivity, and specificity), natural prevalence of contamination, virus concentration and distribution, and sampling, complicates interpretation. Issues such as poor extraction efficiency and the potential for residual matrix-associated inhibition (necessitating dilution) might make one conclude that any positive test result must be reflective of the presence of the contaminant (assuming cross-contamination has been ruled out), since the initial sample would likely to have started with higher virus numbers. On the other hand, the difficulty in interpreting RT-qPCR results at or near assay limit of detection, along with naturally low likelihood of contamination, puts one into that PPV territory in which an elevated proportion of positive results may actually be false positives. We are dealing with an interpretive paradox: is the test too sensitive, or not sensitive enough? The answer is both. It depends on the context.

7.4. The combined impact of assay sensitivity, specificity, virus prevalence and concentration, and sampling on likelihood of detection

The above discussion does not include issues of sampling, which adds a further layer of complexity. In this regard, a mathematical modeling exercise was undertaken to characterize the impacts of assay accuracy; virus contamination frequency (prevalence), concentration, and distribution; and sampling on the overall likelihood of producing reliable testing results under different berry contamination conditions. The same contamination and sampling scenarios as described in Response to Question 3 were used in this exercise. Only ISO 15216–2:2019 test results were modeled, as the FDA-BAM did not evaluate all the necessary assay performance measures. Representative values for sensitivity, specificity, and LoD_{50} were taken from the ISO 15216–2:2019 (ISO, 2019). Since sensitivity and specificity are different for HuNoV GI and GII, the assumption was that 20 % of all scenarios would involve GI and 80 % would involve GII (details of analysis are provided in supplementary materials).

7.4.1. Analytical specificity

The first part of the analysis focused on the impact of predictive specificity within the context of the different contamination scenarios. Recall that in most cases, predictive specificity is quite high, close to 100 %. As a worst-case scenario, the specificity value for the HuNoV GI assay was used (94.4 %), yielding a false positive rate of 5.6 % for all five contamination scenarios. Increasing the number of samples to five per batch or lot (representative of sampling for surveillance/monitoring purposes) and under the assumption that all five tests are independent, the false positive rate jumps to about 25 % (data not shown) for all scenarios except Scenario 3, for which the overwhelming number of samples are true positives, so false positives are rare. Overall, the predictive specificity analysis suggests that when the probability of the virus being present is low, even a fairly high specificity (95 %), will result in a significant number of false positives. However, as stated above, as specificity increases, this false positivity rate drops. Two important issues are revealed here. The first is that specificity calculations are best done with a high number of samples, allowing for more precision in the estimates. Second, since cross-contamination can result in loss in specificity, tight control to prevent this phenomenon will present as very high specificity values.

7.4.2. Analytical sensitivity as a function of LoD_{50}

Table 9 shows the LoD_{50} values presented in the ISO 15216–2:2019 (Appendix I) document (ISO, 2019), including lower and upper confidence bounds. Recall that LoD_{50} is defined as the concentration in genome equivalent copies (GEC)/g for which the probability of detecting virus in a truly positive sample is 50 %.

The calculated LoD_{50}'s for each virus were then applied to the five scenarios, assuming that the collected sample is indeed contaminated in the same ways and given the parameters described in the response to Question 3. Results are shown in Table 10. The two scenarios associated with higher virus concentrations (Scenarios #2 and #4) produced test results in a range of 100 % analytical sensitivity, i.e., the test will always produce positive results if applied to a sample having the specified virus concentration. In Scenario #1, the virus concentration is below the lower bound of the LoD_{50} for all three viruses. It is unclear how to simulate the effectiveness of testing below the lower bound for LoD_{50}, but a reasonable assumption would be that the test would fail to detect any virus most of the time. This assumption would also hold true for HAV in Scenarios #3 and #5. In the remaining four scenario simulations, the virus concentration is either within LoD_{50} upper and lower bounds, or above LoD_{50} upper bound, but below the lowest concentration tested. Again, the value of testing is inconclusive, but the likelihood of detecting the virus if it is present in the sample would be approximately 50 %, but no greater than about 75 % (explicitly 73.7 % for HuNoV GI, Scenario #3).

In the next phase of this exercise, the scenario simulation results were updated for HAV and HuNoVs with consideration of each sampling strategy using the revised analytical sensitivity calculations in Table 10. Comparative results are summarized in Table 11. For Scenarios #2 and #4, the likelihood of HuNoV or HAV detection was unaffected by use of the revised analytical sensitivity data. For Scenario #1, use of the adjusted analytical sensitivity values resulted in failure to detect either virus, regardless of sampling strategy, because the virus concentrations were now below assay LoD_{50}. The simulations for Scenarios #3 and #5 are more nuanced, and since the HAV assay is less sensitive than the HuNoV test (Table 9), the combined impacts of sampling and assay performance were more dramatic for HAV. For example, for Scenario #5, the ability to detect HAV, regardless of sampling plan, dropped to 0 % using the revised analytical sensitivity values, while for HuNoVs, this

Table 9
LoD_{50} data for raspberries from the ISO 15216–2:2019 (Appendix I).

Virus		Test attribute		LoD_{50}		Contamination Level	
		Lower bound	Value	Upper bound	Low	Medium	High
HAV	GEC/g	0.41	0.92	2.04	12	76	370
	Sensitivity (%)	–	–		89.5	100.0	100.0
Norovirus GI	GEC/g	0.06	0.15	0.35	2.1	11	45
	Sensitivity (%)	–	–		73.7	100.0	100.0
Norovirus GII	GEC/g	0.07	0.18	0.45	1.5	15	48
	Sensitivity (%)	–	–		61.1	89.5	100.0

Table 10
Mathematical modeling of likelihood of HuNoV or HAV detection using the ISO 15216-2 method (as expressed by relationship to assay LoD_{50}) as applied to the scenarios described in the Response to Question 3.

Scenario[1-5]	Prevalence (contamination frequency) per lot	Concentration (virus particles per gram)	HAV	HuNoV GI	HuNoV GII
1	100.0%	0.01	Below LoD_{50} lower bound	Below LoD_{50} lower bound	Below LoD_{50} lower bound
2	0.1%	~5000	100% anal. sensitivity	100% anal. sensitivity	100% anal. Sensitivity
3	100.0%	0.4	Below LoD_{50} lower bound	Above LoD_{50} upper bound, but below the lowest concentration tested.	Within LoD_{50} upper and lower bounds
4	1.0%	100	100% anal. sensitivity	100% anal. sensitivity	100% anal. Sensitivity
5	0.4%	0.1	Below LoD_{50} lower bound	Within LoD_{50} upper and lower bounds	Within LoD_{50} upper and lower bounds

Note: Uncolored boxes, results correspond to 100 % analytical sensitivity; Light gray shading corresponds to results within upper and lower LoD_{50} bounds; Dark gray shading corresponds to results below the LoD_{50} lower bounds.
[1] Scenario 1 is representative of an irrigation water contamination event.
[2] Scenario 2 is representative of contamination due to poor personal hygiene of an infected picker.
[3] Scenario 3 is representative of a pre-harvest raw sewage contamination event.
[4] Scenario 4 is representative of a gross, focal contamination event such as field defecation.
[5] Scenario 5 is representative of a vomiting incident in an IQF processing plant.

Table 11
Mathematical modeling of the likelihood of HuNoV or HAV detection based on analytical sensitivities modified by the results presented in Table 10, as applied to the scenarios described in the response to Question 3, using single sample as well as surveillance/monitoring sampling designs and the ISO 15216–2:2019 method.

Scenario	Prevalence (contamination frequency) per lot	Concentration (virus particles per gram)	Log_{10} virus per container	ISO sample results for HuNoVs and HAV			
				One sample (100 % sensitivity)	One sample (modified sensitivity) [a]	Surveillance/ Monitoring (5 samples, 100 % sensitivity)	Surveillance/Monitoring (5 samples, modified sensitivity) [a]
1	100.0 %	0.01	5.04	2.5 %	0 %	11.8 %	0 %
2	0.3 %	~5000	7.83	0.3 %	0.3 %	1.2 %	1.2 %
3	100.0 %	0.4	6.04	75.0 %	37.5 % (0 %)	99.9 %	90.5 % (0 %)
4	1.0 %	100	7.04	2.0 %	2.0 %	9.6 %	9.6 %
5	0.4 %	0.1	3.61	0.1 %	0 % (0.05 %)	0.5 %	0 % (0.2 %)

[a] Non-parenthetical numbers correspond to the likelihood of HuNoV or HAV detection using corrected analytical sensitivity values: parenthetical values refer to the likelihood of HAV detection when that differed from the HuNoV detection percentages.

probability was reduced by approximately one-half. The most dramatic effect was observed for Scenario #3, in which case the likelihood of detecting HAV was reduced to 0 % (as compared to 75–99.9 % using original estimates), irrespective of sampling plan. The probability of detecting HuNoVs in Scenario #3 using five-sample testing reduced to about 90 % (as juxtaposed to almost 99.9 % using unadjusted analytical sensitivity values).

This analysis supports a variety of conclusions that are consistent with what is generally known about sampling. Any sampling scheme will not be very effective if what is being sampled for is present at low prevalence (or sporadically within a lot) or at low concentration. Analytical sensitivity, assay limit of detection, and specificity have profound effects on the results of sampling. For instance, in the case where the number of true positive samples is low, if the specificity is even marginally less than 100 % (e.g., 95 %), then some detection events will be false positives. Based on the published sensitivity values for the ISO 15216 method, there are several scenarios where the method will fail to detect low concentrations of virus, unless that virus is distributed evenly through the sample lot and present at a concentration at or above the assay LoD_{50}. Taken together, the value of testing is driven by many parameters associated with both the test itself and the sampling approach, but one would expect that unless both are highly optimized, and quite efficient, the test will often fail to detect virus if it is present in low concentration and/or focally distributed within the tested lot or batch of product.

7.5. *Balancing risk when evaluating test results*

In the establishment of test interpretation recommendations, it is necessary to consider two categories of risk. ‘Consumer risk’ relates to public health and refers to the likelihood of false negative results, translating to the probability of missing a contaminated sample. The second category, ‘producer risk,’ refers to the likelihood of false positive results, or the probability of falsely classifying a non-contaminated (and hence safe) product. Accepting higher producer risk goes hand-in-hand with accepting lower consumer risk, and vice versa. Clearly, both producers and competent authorities have the responsibility to not place products on the market that pose a public health risk. Under those circumstances, a positive test result, for which cross-contamination has been ruled out, would indicate the presence of viral RNA which may signal the *potential* for public health risk.

However, “acceptable” consumer and producer risk have not been determined for the non-cultivable pathogens for which we do not know the public health significance of an amplified piece of nucleic acid.

Based on Expert Panel discussion, some competent authorities have argued that any positive test for viral nucleic acid (even at Cq values in the high 40's) is evidence that the product has been contaminated with human fecal material and hence is adulterated and must be withdrawn from the market. Proponents of this stance support it by pointing out that standard methods often result in large virus losses before the detection phase, so a positive result is likely to represent a true public health risk. This essentially equates to use of a PCR test, without prior cultural enrichment or enumeration, as a microbiological indicator for product safety. However, targeted studies to determine the validity of nucleic acid amplification as a microbiological indicator have yet to be done. The opposite argument revolves around the absence of a defined means by which to correlate the detection of a short fragment of viral RNA with the presence of an infectious virus, and the fact that withdrawal or diversion of product presenting a public health risk has tangible economic, brand, and sustainability implications. This may be particularly relevant given recent studies demonstrating the long-term environmental persistence of viral RNA fragments in blueberries (Trudel-Ferland et al., 2021) and molluscan shellfish (Tan et al., 2021), and the absence of reported illness in individuals consuming berry fruits (Eshaghi et al., 2021) and oysters (Lowther et al. 2010, 2012) testing positive for HuNoVs at high Cq values. These questions directly relate to the need for developing science-based, risk-based policies, for which there is an urgent need for risk assessments that have applicability to interpret public health risk in the context of positive test results. Subsequent risk management decisions should involve all key stakeholders. Until that time, a pragmatic approach that entails integrating testing results with specific elements of food safety management systems, including production, harvest, and processing assessments, and records of traceability, can effectively support decisions about the disposition of lots or batches with positive RT-qPCR test results, particularly those presenting high Cq values (>40).

7.6. Key conclusions

- Test accuracy is influenced by specificity, sensitivity, and limit of detection. The specificity for the ISO 15216–2:2019 standard for berries is generally quite high, meaning that false positive results are rare as long as the laboratory is adhering to best PCR practices. Mathematical modeling shows that even small reductions in specificity can result in elevated likelihood of false positive results.
- The sensitivity of the ISO 15216–2:2019 standard for berries, as determined by inter-laboratory validation, ranges from 61 to 100 %, with values < 100 % occurring only as the LoD_{50} is approached. This means that at low virus concentrations, typical for naturally contaminated berries, there is an elevated risk of false negative test results.
- Positive predictive value (PPV) of a test is highly dependent upon prevalence of contamination, the lower the prevalence, the higher the likelihood of false positive results. The results of most surveillance/monitoring studies suggest that the prevalence of foodborne viral contamination in berries is low, usually <1 %. Positive predictive value is also impacted by analytical sensitivity (assay limit of detection); the lower the sensitivity, the poorer the PPV. Cq values for most berry samples testing positive fall close to assay limits of detection. For these reasons, interpretation of high Cq values in berries testing positive for viruses should be approached cautiously.
- Mathematical modeling shows that the value of testing is driven by a complex interplay between viral load and distribution, test accuracy, and the sampling approach. The test will often fail to detect virus if it is present in low concentration and/or focally distributed within the tested lot or batch of product.
- Making management decisions in response to positive testing results requires balancing of consumer and producer risk. Quantitative risk assessment and open dialogue among stakeholders is critical to making policy that keeps dangerous product off the market without needlessly excluding the sale of safe product.

8. Question #7

8.1. Synthesis

Based on the totality of information known about the methods and their interpretation, what is the overall value of testing for foodborne virus contamination in fresh and frozen berries?

The Expert Panel deliberations resulted in some overarching conclusions about foodborne virus contamination in the berry supply chain. ***Contamination is more likely to occur during production and harvest than during processing. Virus-contaminated berries originate more often from certain regions of the world, driven by virus endemicity and riskier growing, irrigation and harvesting practices that are more prevalent in these regions. Outbreaks occur more often in association with frozen rather than fresh products, likely due to regional origin of berries, the comingling of products harvested from multiple small farms, the persistence of viruses under frozen conditions, and extended shelf-life for frozen products.*** This implies that greater concern for product safety, and targeted controls, should focus on these particular risk factors for contamination in frozen berries.

Microbiological testing has long been relied upon as a key component in 'ensuring' the safety and quality of food products. Its use in pathogen detection is believed, by most consumers, to be a means by which to keep contaminated products off the market. However, food microbiologists understand that even a perfect test (with 100 % accuracy) is dependent upon the prevalence of contamination, the distribution of the pathogen within a lot or batch of food, as well as myriad issues associated with sampling such as sample size and number. ***While certificates of analysis may claim a lot or batch is "pathogen-free," the real interpretation is that only the tested sample is pathogen-free. The only way to assure complete freedom of pathogens is to test the entire lot or batch. One can never test into safety.***

Tests are never perfect. Even in bacterial pathogen testing in which cultural enrichment is possible and widely used, there remain concerns about enrichment bias influenced by factors such as residual matrix inhibition; the behavior of sub-lethally injured cells; and competition by the rest of the microbial community. Routine testing is often based on a single sample, so sample representation is also an issue. The impact of these and other factors will remain unknown because these phenomena cannot yet be monitored or accurately quantified. The case is more complicated for non-cultivable pathogens. ***The absence of cultural enrichment means that a live culture cannot be obtained. The exclusive reliance on molecular amplification for detection means one cannot know for certain what the presence of nucleic acid represents. Due to low efficiency of extraction and purification steps, a positive test result could represent many more copies of nucleic acid, but if those copies derive from infectious or non-infectious viruses cannot be determined. At high Cq values, it can even be doubtful if the product poses any health risk at all.***

The Expert Panel viewed that the availability of standardized foodborne virus test methods for foods, including berries, as an important advancement in science that should not be undermined. ***They concluded that test method validation and report in the peer-reviewed scientific literature are critical for transparency, providing*** support ***for the credibility of, and confidence in, test methods. The ISO 15216 fulfills these requisites and is the method most often used globally for foodborne virus testing of berries. In the case of other methods, demonstration of equivalency in performance to the ISO 15216 is necessary for their widespread adoption. Such equivalency studies are currently lacking.***

The major purposes for testing are outbreak investigation; routine surveillance or monitoring to understand frequency of contamination and inform risk assessments; and routine industry monitoring. ***The***

utility of testing in outbreak investigation is well established if relevant samples are available, although there are instances in which viral RNA simply cannot be detected from foods epidemiologically implicated in the outbreak. Many surveillance/monitoring studies done by both academicians and regulatory agencies have been completed in the last 15 years and used the ISO 15216 methods. The data from only some of these are publicly available. The baseline frequency of foodborne viral RNA contamination in berries varies by study, project, and region, but it is usually quite low (<1 % of collected samples test positive for viral RNA). There are no international standards on how to extrapolate sample positivity to lot or batch disposition, which presents difficulties for the industry and in international trade. Apart from the U.S., regulatory actions have not been taken, in the form of market restriction or diversion, in response to positive findings during routine surveillance and monitoring studies.

A significant but often overlooked aspect of testing is the role of sampling. While two RT-qPCR amplifications per sample are performed when using the ISO 15216–2:2019 standard, and three subsamples followed by three RT-qPCR amplifications each (9 total) are screened using the FDA-BAM Chapter 26 method, neither method describes systematic sampling strategies. ***Mathematical modeling associated with the Expert Panel efforts revealed that sampling has a significant impact on the ability to detect viruses in a contaminated lot or batch of berries. In general, testing is most reliable when virus is present in high concentration and distributed uniformly through the lot or batch of berry product. There are few contamination scenarios for which this occurs. No matter how it occurs, the likelihood of detecting foodborne viral RNA in contaminated product increases with increasing number of samples tested.*** Systematic and increased sampling, however, requires statistical considerations and results in increased cost and perhaps time-to-result.

Interpretive guidance provided in the ISO 15216–2:2019 and FDA-BAM Chapter 26 both state that one single RT-qPCR replicate presenting with a detectable Cq and the typical sigmoidal amplification curve constitutes a positive test. Data from surveillance and monitoring studies suggests that when berry samples test positive for viral RNA, they often present with high Cq values, often approaching assay detection limits. The Expert Panel noted that the same exquisite sensitivity that we value in PCR assays means that the test is sensitive to cross-contamination if laboratories do not strictly adhere to best practices. ***The Expert Panel cautioned that care should be taken in interpretation of data presenting as elevated Cq values (corresponding to low genome copy number). Although they did not recommend establishment of a universal Cq cut-off value, they did agree that laboratories should have clear guidelines for interpretation of high Cq values.***

Test accuracy is influenced by analytical sensitivity (limit of detection) and specificity, with small changes in either influencing the likelihood of false positive and false negative results. Positive predictive value (PPV), a measure of the value of the test in predicting contamination in screening assays, is influenced by these parameters but also by the prevalence of contamination. ***Most studies suggest low virus contamination frequency (prevalence) and Cq values close to assay limit of detection for berry products subjected to routine testing, thus compounding the chance/probability of false positive results in routine berry testing. In this regard, further confirmation steps (e.g., sequencing, replicate testing, multi-target RT-qPCR, etc.) might be called for, particularly for samples that present with high Cq values that fail to be replicated.*** These steps will require increased laboratory capacity, expense, and lengthen the time-to-result.

Mathematical modeling demonstrated that, even with a highly sensitive and specific test, the combination of small numbers of samples, low prevalence of contamination, low virus concentration, and in many instances, heterogeneous virus distribution in a contaminated lot or batch of berries, gives way to an elevated likelihood of obtaining false negative results. This differs from the situation observed in surveillance and monitoring studies with bivalve molluscan shellfish, for which contamination is internally localized, contamination prevalence is higher, and samples with lower Cq values are more common. It also differs from samples testing positive in outbreak investigation, for which great efforts are usually taken to identify viral contamination. ***Product characteristics and test performance differences complicate direct comparison of berry test results to those obtained for other products or in other circumstances.***

Assuming that the Cq signal is a true positive, ***the Expert Panel believed that foodborne viral RNA should not be detected in a berry sample and may constitute some significance, although the public health magnitude of that is unknown. Although not unanimous, a few members were also of the opinion that the presence of viral RNA in a berry sample suggests that human fecal contamination has occurred somewhere along the production-processing chain. This stance implies that a valid RT-qPCR signal can be treated/considered as a microbiological indicator of product safety. At the time of this writing, there is no international consensus on this position which merits further scientific deliberation.***

The Expert Panel members agreed that detection of viral RNA in food and environmental samples does not inform on their infectivity. This presents complications when extrapolating lot or batch disposition from sample positivity or negativity. The Expert Panel concurred that the lower the Cq value, and/or the more subsamples testing positive, the more likely it is that the sample contains some infectious virus. However, as stated above, low Cq values and/or replicate positives are rare. While some QMRAs have been undertaken to evaluate risk of contamination or illness under different contamination scenarios, none have addressed the role for, or interpretation of, testing. ***Without further knowledge on the relationship between a positive test result and virus infectivity, it is not possible to accurately determine the significance of a positive test result with respect to human health risk, although it should merit scrutiny within the supply chain.***

Assuming the quality and accuracy of the testing is high, there are situations in which testing provides useful results to industry. However, over the last few years, there has been an increasing push toward end-product testing, largely driven by private-label and brand owners purchasing product from upstream frozen processers in the supply chain. ***In general, end-product testing is one means by which to manage potential contamination risk, which could influence the likelihood of exposure. The Expert Panel concluded, however, that it should not be used in isolation, but rather as a holistic approach that includes attention and adherence to prerequisite programs and best practices in production and processing. Risk management can include testing, but not rely solely on it.***

So, what might a holistic approach look like? The published QMRAs specific to the berry supply chain confirm that contaminated waters and infected food handlers (at production, harvest, or primary processing) remain the most important sources of virus contamination, highlighting the need for mitigation strategies from the very beginning of the food supply chain at primary production and processing.

Because elimination of viral contamination is difficult if not impossible for frozen berries, prevention of contamination is crucial. A first step in this process consists of developing awareness of all players on virological risks associated with berries in this complex supply chain. Details of such are outlined in the FAO/WHO CODEX Guidelines on the Application of General Principles of Food Hygiene to the Control of Viruses in Foods (Annex 2) (CAC/GL 79–2012; available at: https://www.fao.org/fao-who-codexalimentarius/codex-texts/guidelines/en/). The following were identified as overarching critical prevention strategies:

- High quality agricultural water must be used for irrigation, dilution of pesticides, and handwashing in berry fruit production. Untreated human fecal waste, individual or sewage (collective), must be avoided in production and processing. Water that has been impacted by human feces, including that pumped out of nearby rivers, must not

be used for these purposes. Unfortunately, at the current time, there are no standard or affordable methods to screen agricultural waters for foodborne virus contamination.

- Contamination risks vary with different irrigation methods, especially for strawberries and similar fruit growing near the ground level. In these cases, drip irrigation should be favored.
- Pickers (hand harvesters) must understand the viral risks associated with poor hand hygiene and should undergo a training program on hygiene practices at the orchard. Training typically should include procedures on how to properly wash hands, how to maintain good personal hygiene, and how to react in cases of vomiting and/or diarrhea in the primary production area. Workers must be encouraged to voluntarily exclude themselves from work if ill with vomiting and/or diarrhea. Certain management practices (e.g., paid sick leave, incentives for truthfulness) help facilitate worker compliance. As is the case for agricultural waters, there are no standard methods to screen workers for infection, or to test hands for foodborne viral contamination. Such testing might also pose an ethical dilemma.
- An adequate number of toilet facilities should be located close to the fields/orchards, accompanied by adjacent hand-washing facilities. In remote areas where mobile toilets are used, particular attention should be paid to preventing any sewage leaks containing human feces into the growing areas, or around sources of municipal and agricultural water, and/or irrigation lines. Workers should not be subjected to direct or indirect financial penalties for restroom breaks.
- Similarly, food workers at primary processing who handle berries during sorting, washing, cooling and and/or freezing must be trained on the importance of hand hygiene and gloving. Appropriate clean, and well-stocked restroom facilities must be available and readily accessible.
- Implementing the necessary awareness requires easily understandable and practical guidance documents that can be used for training and assessment, with the goal of continuous improvement. Training materials and programs should be simple, informative and meaningful for farmers, including for the smaller entities and those located in low to middle income countries. Training material should include graphical representations and be translated into local languages. Food trade associations can play an influential role in building knowledge across the supply chain.
- Supply chain-level risk management tools (developed by industry and academia) addressing risks presented specifically by foodborne viruses, and broader food safety certification programs (e.g. GlobalGAP), may also be useful in helping suppliers to progress towards more credible and relevant certifications.

Analytical testing of finished products has a role as part of a broader program to assure the safety of the berry supply chain. Although not yet investigated, testing could theoretically have value in helping to validate the efficacy of targeted interventions, such as water disinfection or semi-automated handwashing, in reducing the likelihood of foodborne virus contamination. However, routine end-product virus testing is not likely to do much in the way of preventing potentially contaminated product from reaching the market. In short, it is a "blunt" tool: it is difficult to pinpoint a defined food lot that is contaminated, and testing cannot be relied upon to definitively rule out virus contamination in berries destined for the market. It must also be noted that the requirement of end-product testing has its own detractions, including high cost and lengthy time-to-result that may necessitate prolonged product retention times in frozen storage or result in higher priced products. Instead, end-product testing should be viewed as a means by which to corroborate a breakdown in food safety management, and as such, to build an effective preventive approach based on a "assessment-education-continuous improvement" process. This will allow for assurance of the safety of berries across the supply chain and include all actors that can facilitate a virus-free product.

The Expert Panel identified the following research needs specific to detection technologies:

- Pursue further development of infectivity assays, and/or appropriate proxies, for potential incorporation into testing and/or test confirmation protocols.
- Identify the nature (i.e., enclosed within a capsid or free, genome complete or partial) of viral RNA in berries and associated environmental samples (e.g., waters) testing positive by RT-qPCR.
- Related to the above, conduct root cause analysis in instances of outbreaks and when berry samples test positive for foodborne viral RNA using RT-qPCR. This could lead to targeted interventions that can be applied during production and processing.
- Conduct quantitative risk assessments that consider the value of testing, taking into account sampling plan(s), frequency/prevalence of contamination, features of the test method, and interpretation of results, with an eye toward the relationship between positive test results and virus infectivity. Such risk assessments can be used to inform targeted monitoring approaches, as well as to aid in decision-making for lots/batches testing positive for foodborne viral RNA.
- Develop and adopt standardized methods that can be used to detect foodborne viral contamination in relevant environmental samples, particularly waters and hands of operators/harvesters, that might be used for testing in support of future GAPs and/or HACCP-like preventive control programs.
- Continue the search for effective microbiological indicators that can be used in place of human enteric virus testing to identify products, environments, and/or circumstances which might elevate the risk of viral contamination of berries.

CRediT authorship contribution statement

Lee-Ann Jaykus: Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization. **Sabah Bidawid:** Writing – review & editing. **Albert Bosch:** Writing – review & editing. **Sophie Butot:** Writing – review & editing, Conceptualization. **Nigel Cook:** Writing – review & editing, Writing – original draft. **James Lowther:** Writing – review & editing, Writing – original draft, Data curation. **Neda Nasheri:** Writing – review & editing. **Rosa M. Pintó:** Writing – review & editing, Conceptualization. **Donald W. Schaffner:** Writing – review & editing, Writing – original draft, Formal analysis. **Magnus Simonsson:** Writing – review & editing. **Branko Velebit:** Writing – review & editing, Writing – original draft, Data curation. **Jan Vinjé:** Writing – review & editing, Writing – original draft. **Sophie Zuber:** Writing – review & editing, Conceptualization. **Sanjay Gummalla:** Writing – review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Sabah Bidawid, Sophie Butot, James Lowther, and Sophie Zuber declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work

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Neda Nasheri reports financial support was provided by Health Canada. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111436.

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Data availability

Data will be made available on request.

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