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Norovirus and Hepatitis A virus in shellfish, soft fruits and water

Albert Bosch*
Virus Entèrics, Department of Microbiology
University of Barcelona
Avda. Diagonal 645
08028 Barcelona, Spain
Phone: (+34) 934034620
Fax: (+34) 934034629
E-mail: abosch@ub.edu

Sabah Bidawid
, Microbiology Research Division, Health Canada
Food Directorate, Bureau of Microbial Hazards Sir F.G. Banting Research Centre, RmE405, PL # 2204E
251 Promenade Sir Frederick Banting Driveway Tunney's Pasture Ottawa, Ont., Canada K1A 0K9
Phone: + (613) 957-0908
Fax: + (613) 941-0280
sabah.bidawid@hc-sc.gc.ca

Françoise S. Le Guyader
Laboratoire de Microbiologie-LNR, Departement EMP,IFREMER
1 rue de l'Ile d'Yeu
BP 21105
44311 Nantes cedex 03, France
Phone : +33 (0) 240374052
Fax : +33 (0) 40374073
E-mail : Soizick.Le.Guyader@ifremer.fr

David Lees,
European Community Reference Laboratory,
CEFAS Laboratory,
Barrack Road, The Nothe,
Weymouth, Dorset, DT4 8UB,UK
Phone : +44 (0) 1305 206600
Fax : +44 (0) 1305 206601
E-mail : david.n.lees@cefas.co.uk

Lee-Ann Jaykus,
Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University Raleigh, NC 27695-7624,USA
Phone: (919) 513-2074
Fax: (919) 513-0014
E-mail: leeann_jaykus@ncsu.edu

*Corresponding autor. Send proofs to :

Albert Bosch
Department of Microbiology
University of Barcelona
Avda. Diagonal 645
08028 Barcelona
Spain

1. Introduction

Viruses such as hepatitis A virus (HAV), noroviruses (NoV), sapoviruses, enteroviruses, astroviruses, adenoviruses, rotaviruses and hepatitis E virus have all been implicated in food- and/or water-borne outbreaks of illness. As a matter of fact, there is a potential for any virus which causes illness after ingestion, to be transmitted by food. However, in practice most reported incidents of viral foodborne illness are due to the human NoV, and HAV.

In the European Union, viral agents were reported to be responsible for approx. 12% of the foodborne outbreaks reported to the European Food Safety Authority (EFSA, http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902515341.htm) during 2007 and were identified as the second most common causative agent group, after *Salmonella*. In the United States, the importance of viral agents (i.e., NoV) to the overall burden of foodborne disease is increasingly being recognized based on results from outbreak investigations. In a report on outbreak-associated illnesses compiled by the Centers for Disease Control and Prevention for the period 1998-2002 (Lynch et al., 2006), viral pathogens, predominantly norovirus, caused 33% of outbreaks and 41% of cases; the proportion of outbreaks attributed to viral agents increased from 16% in 1998 to 42% in 2002. Viral pathogens accounted for an increased proportion of outbreaks each year during this reporting period and a higher proportion of outbreaks of known etiology during this reporting period than preceding reporting periods. This is probably reflecting the increased availability of improved viral diagnostic tests. In most countries, however, the extent of the problem is not known.

Human viruses can contaminate food either through contamination at source, principally through sewage pollution of the environment, or in association with food processing through inadequate hygiene practices of operatives or systems. Consequently many different food items such as vegetables, shellfish and a great variety of ready-to-eat foods like sandwiches, cold meat, pastries etc. have been implicated in foodborne viral infections. Bivalve shellfish are commonly involved in outbreaks of foodborne viral diseases. Shellfish are filter feeders and if shellfish-growing waters are polluted with human sewage, the shellfish accumulate viruses infectious for humans. The difficulties in detecting virus in shellfish pose further problems, as well as the fact that correlation between levels of bacterial indicator organisms and the extent of viral contamination is poor.

Contamination of the food with fecal material can occur at any step during its production. For example, contamination of shellfish usually occurs prior to harvesting. Contamination of berries (e.g., raspberries) may occur prior to harvesting (e.g., due to irrigation with fecally-contaminated water), during harvesting (contamination by infected field workers) or during processing prior to distribution (contamination in the factory by infected food handlers or by spraying with contaminated water). Other foods implicated in outbreaks, including salads, sandwiches, and deli meats, have been contaminated at the site of preparation by infected food handlers.

Secondary transmission of NoV infection is common (often >30%), allowing amplification of an outbreak, particularly in closed settings. Such outbreaks are commonly recognized in healthcare institutions (e.g., hospital or nursing home) and on cruise ships (Lopman et al., 2004; Verhoef et al., 2010). Because NoV are relatively resistant to inactivation by many common disinfectants, outbreaks in these settings may require closure of the unit or ship for more extensive cleaning and disinfection.

Water is another frequent vehicle for enteric virus infections. Since current water treatments do not ensure their complete removal, viruses become contaminants of the water environment in numbers high enough to represent a public health threat, although low enough to pose serious difficulties for their detection. Poor water quality continues to pose a major threat to human health. Billions of cases of gastrointestinal

illness occur annually worldwide. The World Health Organisation (WHO) declared that diarrhoeal disease alone contributes to an estimated 4.1 % of the total DALY (disability adjusted life years) of global burden of disease and is responsible for the deaths of 1.8 million people every year (World Health Organization, 2004). It was figured that 88% of that burden is attributable to unsafe water supply, sanitation and hygiene, and it is mostly in children from developing countries. A significant amount of disease could be prevented, especially in developing countries, through better access to safe water supply, adequate sanitation facilities and better hygiene practices.

2. Viruses in food and water

Viruses are present in food and water samples at low to very low levels compared with human clinical samples. However, unlike many enteric bacteria causing foodborne disease, enteric viruses can initiate an infection in very low numbers; the NoV and HAV infectious doses are generally accepted to be around 10-100 virus particles (Teunis et al., 2008; http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm071294.htm). Thus sufficient sensitivity is a significant issue in the development of virus detection methods for food and water samples.

An expert meeting convened under the auspices of WHO/FAO and the World Organization for Animal Health (OIE) reviewed available evidence and grouped food and waterborne viruses according to their ability to cause high morbidity, severe disease, or a significant threat thereof.

Subsequently, available evidence for a specific hazard / food-commodity combination was reviewed, by considering available information on estimates of the incidence of food-borne disease linked to a specific commodity, and the level of evidence for the importance of that commodity in causing viral food-borne illness. This resulted in several virus-commodity combinations for which prevention and control measures should be considered:

- Noroviruses and hepatitis A in bivalve molluscan shellfish
- Noroviruses and hepatitis A in fresh produce
- Noroviruses and hepatitis A in prepared foods
- Rotaviruses in water for food preparation
- Emerging viruses in selected commodities

The present chapter will only deal with NoV a and HAV detection in bivalve mollusks, soft fruits and water.

3. Fecal pollution indicators

Fecal pollution indicators have a long history, dating back to the early twentieth century, of use for monitoring and managing the pathogen risk associated with fecal pollution. World-wide, legislative standards controlling the sanitary production of bivalve mollusks and drinking water utilize fecal indicators. For bivalves mollusks, fecal indicators are measured either in the shellfish themselves (EU system) or in their growing waters (USA system).

Both systems require the grading of areas according to the degree of fecal pollution which determines their sanitary quality and hence determines the approval to harvest, the marketability and the extent of food processing required for the product (Lees, 2000). The acceptability of bivalve mollusks in international trade, as set out by Codex Alimentarius, is also judged by their compliance with fecal indicator end-product

standards. *E. coli* is now generally targeted for shellfish flesh monitoring (e.g. EU system), whereas coliforms or fecal coliforms are analyzed for water monitoring (e.g. USA system). Standardized methods are stipulated in both the USA and EU for *E. coli* analysis (Anonymous, 2005).

For bivalve mollusks, these extensive controls do appear to adequately manage the risk from bacterial pathogens associated with fecal pollution (Lees, 2000). However, the historical approach of monitoring for fecal indicators, and setting legislative standards, does not appear to adequately control the risk from human enteric viruses, since outbreaks associated with both norovirus and hepatitis A virus continue to occur on a regular basis (Pintó et al., 2009; Shieh et al., 2007; Westrell et al., 2010).

A number of studies have evaluated alternative indicators more physically and behaviorally similar to enteric viruses, such as various bacteriophage species (Dore et al., 2000). However, the main thrust internationally is now towards detection of the virus pathogens directly in the food commodity of concern.

Unfortunately, in the USA, there are no equivalent guidelines for the acceptability of waters used for the production and processing of soft fruit. Some producers will evaluate waters periodically for total aerobic bacteria, coliforms, and/or *E. coli* but there is no legal mandate to do so.

As is the case for shellfish and their harvesting waters, the relationship between the traditional fecal indicators and the presence of human enteric viruses in production and processing waters is tenuous at best. Alternative indicators like the male specific coliphages may not work, although large scale studies of their efficacy have not yet been undertaken (Endley et al., 2003). As is the case for shellfish, most international efforts are focusing on direct detection of virus in contaminated products or waters as an alternative to the standard fecal indicators.

4. Virus detection in bivalve mollusks

The low virus concentrations present in shellfish require the use of laboratory techniques that efficiently recover most of viruses present from shellfish tissues. The greatest concentration of human enteric viruses being concentrated in the stomach and digestive diverticula, almost all sensitive methods are based on dissected digestive tissues. Two main options are available: i) methods based on an elution-concentration step of viral particles, ii) direct elution of virions.

In the elution-concentration methods, the initial steps consist of virus elution from shellfish tissues, recovery of viral particles, and then virus concentration. Some methods use acidic adsorption prior to virus elution but for most methods viruses are eluted using various buffers (e.g., chloroform-butanol or glycine) before concentration by polyethylene glycol precipitation or ultracentrifugation (Costafreda et al., 2006; DePaola et al., 2010; Le Guyader et al., 2009; Terio et al., 2010). In the direct elution of virus, particles using Proteinase K (Lowther et al., 2010) or even direct lysis of nucleic acid using Trizol or other lysis buffer are used (Nenonen et al., 2009).

Since viruses detected in shellfish are non culturable, the current detection system is the reverse-transcription polymerase-chain-reaction (RT-PCR). Thus, the second important parameter is nucleic acid extraction and purification. In-house methods such as Proteinase K capsid lysis, and then purification of nucleic acid using phenol-chloroform and cetyltrimethylammonium bromide (CTAB) precipitation, trizol treatment, Boom-based methods or cesium chloride cushion, have been proven efficient to detect viruses in shellfish in various studies including shellfish samples implicated in outbreaks (Atmar et al., 1995; DePaola et al., 2010; Le Guyader et al., 2008; Lowther et al., 2010). Currently, several methods have been adapted to use commercial kit based on guanidium thiocyanate lysis and then nucleic acid purification using either a

column or paramagnetic silica (Costafreda et al., 2006; Lees, 2000; Terio et al., 2010). The adaptation of these methods to utilize readily available reagents, but with minimum loss of assay sensitivity, has become a priority. The use of a kit, sometimes used with automated apparatus and real-time RT-PCR, or quantitative RT-PCR (qRT-PCR), constitute a major step for standardization. This approach is fundamentally important for shellfish safety and is under evaluation for future European regulation.

5. Vitus detection in soft fruits

There has been several high profile foodborne disease outbreaks linked, directly or indirectly, to fresh produce, including soft fruits (strawberries, raspberries, tomatoes) over the last decade (Centers for Disease Control and Prevention, 1997; Gaulin et al., 1999; Ponka et al., 1999). There is a need for reliable methods to detect viruses in foods remains, since current techniques are limited and none are yet commercialized.

Most of the developmental work on the detection of viral contamination of foods has been done using the molluskan shellfish matrix. While these reports have provided insights, soft fruits are different than oysters or clams. In particular, viral contamination in soft fruits is distributed on the surface of the product rather than localized within the fruit. Also, soft fruits lack fat, are high in both simple and complex carbohydrates, and have rough or irregular surfaces. Early studies targeting virus detection in soft fruits (Bidawid et al., 2000), focused on the detection of HAV in strawberries.

Croci and coworkers (2008) authored a comprehensive review of the methods used to detect enteric viral contamination of fresh produce. These authors described the sequential steps of such detection as (i) virus elution/clarification; (ii) virus concentration; (iii) nucleic acid extraction; and (iv) detection. Each of these are discussed below and summarized in Table 1.

The purpose of the elution/clarification step is to facilitate the removal of virus from the surface of the product (elution). This is usually achieved using eluant solutions of elevated pH and/or solutions which are supplemented with amino acids and/or organic matter, such as those present in beef extract or fetal bovine serum. The principle behind elution is that pH increase reduces that ability of viruses to adhere to matrix components, resulting in release of the virus into a suspension that is relatively free of organic matter. Clarification, or the process of removing the residual matrix components, can be accomplished by physical separation methods such as filtration or centrifugation. In this case, the virus-containing filtrate or supernatant, respectively, is recovered for further processing. The decision to use filtration or centrifugation for the clarification process depends on a number of factors, including the specific food matrix, the availability of relevant equipment (for centrifugation), and the experience of the testing facility.

The clarified eluent is usually of relatively high volume (>25 ml, frequently much more),while in naturally contaminated products, the level of virus contamination is anticipated to be quite low. Therefore, one or more additional concentration steps are needed. Both chemical and physical concentration approaches have been used. Chemically based methods rely on the capacity of viruses to behave as proteins in solution. For example, reducing the pH of the solution to below the isoelectric point of the virus favors precipitation (so-called acid precipitation), as does the addition of the protein precipitant polyethylene glycol (PEG).

These are relatively non-specific methods, which also favor the precipitation of residual matrix components not removed by clarification. The resulting precipitant is usually collected by relatively low speed centrifugation. Ultracentrifugation has been used for virus concentration, but due to the high cost of investment this technique is not a practical option for most food microbiology testing laboratories. Ultrafiltration has also been used but filters have a tendency to clog quickly unless ultrafiltration is

preceded by enzyme treatments intended to degrade large matrix-associated molecules. Nevertheless, investigators have had success pre-treating clarified eluants from soft fruits with pectinase prior to the application of ultrafiltration (Butot et al., 2007).

Sample volume reductions after a concentration step usually range from 10 to 1000-fold, resulting in concentrates of 100 µl-10 ml Overall, the smaller the volume the better, as most commercial RNA extraction methods are intended for samples <1 ml in volume. Although heat release of viral RNA (99ºC/5 min) was used in the early days of methodological development, this has largely been replaced with nucleic acid extraction, as extraction provides additional sample concentration and also removes residual matrix-associated inhibitors. Increasingly, RNA extraction methods are being supplemented with secondary purification steps, such as silica binding followed by nucleic acid elution. Many of the extraction methods have been commercialized over the last decade. A summary of current methods and their detection limits are provided in Table 2. This is by no means complete, but it does provide an indication of the wide variety of methods being used in the research setting. The method of Butot et al. (2007) as applied to soft fruits has received attention in Europe, while there has been interest in the re-circulating ligand capture methods in the US (Papafragkou et al., 2008). Few have done comprehensive comparisons of these methods. All methods have their own advantages and disadvantages. For example, immunocapture methods tend to be simple, requiring fewer sample manipulations with lower risk of virus loss, aæthough they may be hindered by high specificity since broadly reactive antisera are not available for the human NoV. Presently, there is no universal extraction method that can be applied to all foods, particularly when contamination levels are low.

6. Virus detection in water

The basic steps in virological analysis of water are sampling, concentration, decontamination/removal of inhibitors, and specific virus detection. Sample concentration is a particularly critical step since the viruses may be present in such low numbers that concentration of the water samples is indispensable to reduce the volume to be assayed to a few milliliters or even microliters.

In drinking water, the virus levels are likely to be so low that optimally hundreds, or even thousands, of liters should be sampled to increase the probability of virus detection. Methods for virus concentration from water samples are depicted in Table 3 and reviewed elsewhere (Wyn-Jones, 2007). A good concentration method should fulfill several requirements: it should be technically simple, fast, provide high virus recoveries, be adequate for a wide range of enteric viruses, provide a small volume of concentrate, and be inexpensive. No single method meets all these requirements.

Basically, all available procedures have been evaluated using samples spiked with known viruses. It is known that the recovery efficiency recorded with experimentally contaminated water dramatically decrease when the method is applied in actual life situations. Additionally, none of the existing concentration procedures has been tested with all of the medically important virus groups; normally, a few specific enteric viruses have been employed to conduct the evaluation trials.

Most of the procedures for concentrating and extracting viruses make use of the properties of the viral proteinaceous macromolecules. Certain protein structures confer on viruses in an aquatic environment the properties of a hydrophilic colloid of an amphoteric nature whose electric charge varies according to the pH and the ionic force of the environment. Viruses can therefore be adsorbed onto and then detach themselves from different substrates which are positively or negatively charged depending on their pH. Methods based on the adsorption of viruses from the sampled

water onto a suitable solid surface, from which they may subsequently be eluted into a much smaller volume, are preferred for use with large-volume samples.

Criteria based on the experience and expertise of the user on a given method should be employed to select the most appropriate system. Positively-charged filters (Sobsey and Glass, 1980) and glass wool (Kiulia et al., 2010; Vilagines et al., 1993) based methods are still among the best possibilities. Sampling large volumes requires a two-step concentration procedure, with polyethylene glycon (PEG) (Lewis and Metcalf, 1988) and ultrafiltration (Rutjes et al., 2006) as preferred procedures for re-concentration of the primary eluates. Additionally, PEG (Lewis and Metcalf, 1988) as well as lyophilization (Villena et al., 2003) may be used for direct virus concentration in heavily polluted medium size samples, e.g., sewage, having this latter method the added advantage of removing substances inhibitory to RT-PCR (Gajardo et al., 1995).

7. Rapid enumeration and characterization of viruses

When virus detection procedures are mentioned, the recurrent issue of detecting infectious or physical particles comes into discussion. Whenever possible, infectious assays coupled with identification methods are preferred for direct assessment of human health risk. The detection of infectious enteroviruses and even astroviruses or rotaviruses may be achieved by cell culture techniques with the appropriate cell lines. However, despite recent reports of cell lines permissive to the propagation of wild-type HAV (Konduru and Kaplan, 2006) and human NoV (Straub et al., 2007), issues related to assay complexity, cost-effectiveness and even feasibility make their routine use difficult.

Nucleic acid amplification techniques are currently the most widely used methods for detection of viruses in food and water, which also enable to gather information of the virus genotypes occurring in the environment and in food products, thus providing most relevant epidemiological information, particularly important for the implementation and follow up of vaccination programs in the human population.

Although nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) techniques have been reported as highly sensitive and specific, respectively (Fukuda et al., 2006; Jean et al., 2004), PCR and RT-PCR remain as the current gold standard for virus detection. A further improvement comes from qRT-PCR, which enables not only qualitative determination but also, and particularly, quantitative diagnostic assays (Costafreda et al., 2006; Kageyama et al., 2003). Furthermore, recent evidence indicates that these are more sensitive than the conventional gel-based methods which were developed in the early 1990's. The detection sensitivity and the diagnostic specificity of molecular amplification are highly dependent on the efficiency of the upstream processing methods, and the purity and yield of the RNA.

An additional factor impacting the performance of qRT-PCR is the choice of primers which is, in fact, very dependent upon virus. A general rule of thumb is to use broadly reactive primers with high annealing temperature and relatively low degeneracy (to prevent non-specific amplification). This is easier for some viruses and less so for others. This task, while attainable for HAV, is easier said than done for NoV. Certainly RNA regions containing complex multi-domain structures, mostly involved in both translation and/or replication functions, must be highly conserved. For instance, HAV translation is cap-independent and the recruitment of the translation machinery takes place at the internal ribosome entry site (IRES) contained in the 5'non-coding region (NCR) and hence this latter region is particularly suitable to be employed as target for amplification in diagnostic assays (Costa-Mattioli et al., 2002a; Costafreda et al., 2006; Jothikumar et al., 2005a).

However, the genetic diversity of the human NoV makes universal primer design more challenging and the absence of a 5'NCR, and in particular, of IRES in the calicivirus genomic and sub-genomic RNAs makes the approach described for HAV unfeasible. Four "regions" of the NoV genome have been used for primer design (designated regions A, B, C, and D) but the ORF1/ORF2 junction (just downstream of region B) seems to be the most conserved and is frequently used for genogroup-specific detection (da Silva et al., 2007; Jothikumar et al., 2005b; Kageyama et al., 2003; Loisy et al., 2005; Svraka et al., 2007).

Yet, the significance of a genome copy still remains controversial, since the virus specific infectivity or infectious/physical particle ratio is highly variable in food and water samples. Several studies show lack of correlation between the number of genome copies and infectivity in food and water safety studies (Baert et al., 2008b; El Senousy et al., 2007). On the other hand, a study on seafood reported a correlation between self-reported gastroenteric illness in restaurant consumers and the presence of norovirus in the batch of oysters consumed (Lowther et al., 2010). As stated above, no alternative to molecular detection analysis exists for highly health significant waterborne viruses such as human NoV and HAV.

The aforementioned approaches for the development of broadly reactive detection methods do not provide the ability to fulfill another ultimate goal in diagnostics, which is the possibility to type the isolates, as might be appropriate in outbreak investigations. For HAV, the most widely used region is the amino terminal 2A region, which is included in a larger fragment corresponding to theVP1/2A junction region (Robertson et al., 1992). However, recent data point to the analysis of the entire VP1 region for improved type discrimination (Costa-Mattioli et al., 2002b; Tallo et al., 2003). For NoV strain comparison, primers corresponding to the capsid region can be used, although these sometimes have a higher degree of degeneracy. It is recommended that multiple primer pairs be used when screening food samples for NoV contamination, as one pair may perform better than another, depending on virus load and the matrix from which the sample was derived (Boxman et al., 2007; Vinje et al., 2003).

Although RT-qPCR provides both amplification and confirmation in a single test, definitive epidemiological association in outbreak investigations relies on direct sequencing of amplicons obtained from clinical and environmental/food samples. Microarray technologies may eventually speed this process. For example, Ayodeji et al (2009) recently reported a microarray based approach for the identification of common foodborne viruses, finding that the hybridization profiles generated for HAV are conducive to the identification of closely related strains. Similar studied on the use of microarrays for the simultaneous detection and genotyping of NoV were described by Pagotto et al. (2008).

Multiplexing of amplifications has been applied on occasions (Morales-Rayas et al., 2010) but may reduce detection sensitivity. Semi-nested amplifications are also commonly applied (Baert et al., 2008a) and these can improve detection limits but with an increased risk for cross-contamination leading to false positive results. In general, nested amplification is not well suited to real-time amplification approaches. Interestingly, Hu and Arsov (2009) recently developed a method which combines conventional PCR, nested PCR and real-time PCR in a multistep detection procedure. Specifically, the method involves two consecutive amplifications: the first is a conventional RT-PCR targeting the 5' NCR of HAV, the second being a real-time PCR using a nested primer pair specific for the first PCR product and a TaqMan probe. This method facilitated the detection of as little as 0.2 plaque-forming units (PFU) of HAV, which can be more sensitive than other RT-PCR techniques. An approach such as this can provide a useful method for detecting HAV at low levels, as long the risk of cross contamination is adequately controlled.

8. Standardization of virus detection methods

A major factor limiting the uptake of virus testing into regulatory food controls worldwide is the current absence of any standardized and validated methods. In 2004, the European Committee for Standardization (CEN) initiated the development of a standard method for detection of NoV and HAV in foodstuffs based on PCR (Lees, 2020). The draft standard developed by a working group of expert European laboratories is now well advanced and due for publication in 2012.

As mentioned above, food samples present a challenging matrix and the standard method needed to be capable of extracting low levels of contaminating virus and presenting them in a non-inhibitory extract to a sensitive PCR assay. Key aspects of the method were tested by inter-laboratory evaluations to ensure robust performance. The method targets the at-risk food matrices of molluskan shellfish, soft fruits, salad vegetables, bottled water and food surfaces (both the surfaces of foods and food preparation surfaces).

For bivalve mollusks dissected digestive diverticulum (digestive gland) is used as the starting material with further enzymatic digestion using Proteinase K (Jothikumar et al., 2005b). For food surfaces swabbing is employed followed by elution into sample buffer (Scherer et al., 2009). For both soft fruit and salad vegetables viruses are eluted with agitation followed by recovery using PEG/NaCl precipitation (Dubois et al., 2007). For bottled water viruses are adsorbed to a positively charged membrane, eluted and then concentration by ultrafiltration (Butot et al., 2007).

Following initial sample treatment, all food matrices are then further processed by a common nucleic acid purification and PCR platform. Nucleic acid purification utilizes guanidine thiocyanate to denature viral coat proteins in combination with magnetic silica particles to bind released nucleic acid, which is then purified through successive washing stages before final elution in a small volume.

RT- PCR utilizes a one-step approach using specific primers in order to simplify the procedure as much as possible. However, commercial one-step kits must utilize enzymes specifically engineered for use with low abundance targets. TaqMan PCR real-time chemistries are stipulated for the amplification since: (i) the closed tube format is less susceptible to contamination; (ii) is logistically efficient; incorporates a probe based confirmation step; (iv) is quantitative; and (iv) is more amenable to standardization than conventional PCR.

To maximize the detection limit, real-time PCR assays are done separately for NoV genogroup I, NoV genogroup II, and HAV. Cross-reactive real-time PCR primers and probes are directed in the ORF1-ORF2 junction region for NoV (da Silva et al., 2007; Jothikumar et al., 2005b; Loisy et al., 2005) and in the highly conserved 5' non-coding region for HAV (Costafreda et al., 2006). The method is highly sensitive in order to detect the low levels of virus found in environmentally contaminated samples and hence also vulnerable to both cross-contamination (false positives) and potential matrix interferences (false negatives).

Thus a comprehensive suite of controls was also developed to cover: positive and negative process controls; negative RNA extraction control; positive RT-PCR and RT-PCR inhibition controls; negative and positive PCR controls. The positive process control measures the recovery of virus during the whole extraction and test procedure using a heterologous non-enveloped positive-sense ssRNA virus spiked into the test sample and assayed in parallel with the target viruses. During the development of the method inter-laboratory studies by the working group successfully utilized the MC0 strain of Mengo virus (Costafreda et al., 2006; Pintó et al., 2009) as a process control.

The negative process control is a known negative sample that is taken through the entire extraction procedure and analyzed. The RT-PCR inhibition control checks for potential matrix suppression by comparison of amplification of an external RNA template added to test material and a control well. Taken together the controls

generate data on all aspects of the assay and are utilized to determine the acceptability of test performance against established quality control criteria.

The draft standard incorporates two parts covering both quantitative and qualitative detection. The differences principally relate to the necessary suite of controls and the calibration curves required for determining virus template concentrations. Quantification is based on a plasmid DNA calibration curve for each assay (NoV GI, NoV GII, HAV) with plasmid DNA concentration measured using spectrometry at 260 nm. Results are reported in the standardized form of detectable virus genome copies per gram of material tested. Qualitative assays will report presence or absence with reference to their limit of detection. Formal validation studies are planned to characterize the method according to the international requirements.

9. Conclusions and future trends

Overall, detection of viruses in foods is done infrequently and usually only in response to known or suspected foodborne disease outbreaks in which there is a strong epidemiological association with one or two candidate foods.

Despite the progress in the adoption of procedures for the detection of viruses in food and water, a decision to test a food product for virus contamination must be approached cautiously due to the following reasons. Although validation work is currently being pursued in Europe, including those focused on bivalve mollusks, soft fruits and water, at the time of this writing, there are no internationally agreed standardized and validated methods available for the detection of viruses in foods. Other limitations to the routine application of virus detection protocols are problems with demonstrating the association (or lack thereof) between a positive detection signal and virus infectivity; less than optimal recoveries during virus extraction that may raise detection limits to unacceptably high levels; and finally the expense and the need for highly trained personnel for implementing virus detection protocols.

Even when foods are tested for potential viral contamination, results should be interpreted with caution, i.e., a negative test result does not rule out product contamination, while a positive test result only suggests that contamination occurred at some point in the farm-to-fork chain but does not confirm the presence of infectious virus in the sample that was screened (D'Souza et al., 2010).

The risk analysis framework, laid down by the Codex Alimentarius during the past ten years, has made it increasingly possible to link food safety to public health via risk assessment. However, it is still unclear how these new concepts will be used in the future risk analysis. Quantitative risk assessments require quantitative data on viruses in food and validated standardized procedures should be employed in selected commodities or in outbreak situations, which is particularly relevant in the present situation of global food trade.

However, some issues deserve attention: (i) the representativeness of the analyzed sample, considering that only a tiny amount of material, usually from a very large batch sample, is analyzed; (ii) the type of sample to analyze (e.g. outbreak related, imports, etc.), since it is unrealistic to routinely test all types of food for viruses; and (iii) the need to address a key question: who will cover the costs of the virological assays (e.g. producers, importers, distributors, consumers,)? Despite these pending questions, standardized virological assays will enable the formulation of guidelines to ensure the virological safety of selected commodities in specific scenarios thus contributing to reduce the incidence of foodborne infections.

TABLE 1. Summary of the Principles of Virus Concentration , Purification, and RNA Extraction from Soft Fruits.

Sample Size	Candidate Elution Buffers	Candidate Clarification Methods	Candidate Virus Concentration Approaches	RNA Extraction Approaches
--Varies from a low of 2-3 cm2 slices or pieces to a high of 100 g --25-50 g is a reasonable sample size	--Phosphate buffered saline --1 M sodium bicarbonate --0.05 M Glycine—0.14 N saline, pH range 7.5-9.5 --Beef Extract (1-3%) --Elution agents frequently combined, such as supplementing glycine-saline with beef extract, or fetal bovine	--Filtration: Remove residual food matrix, recover filtrate. --Cheesecloth, guaze, plastic net --Filter stomacher bags Centrifugation: Sediment (with or without co-precipitant) food matrix; recover	--Ultracentrifugation (100,000-300,000 x g for 0-5 to 2 h) --Ultrafiltration --Polyethylene glycol precipitation (6-12%) followed by centrifugation Filtration with electronegative membrane filters followed by elution	--Most commonly used approaches are based on guanidinium isothiocyanate with/without secondary silica purification. Commercial kits available Proteinase K followed by phenol-chloroform-isoamylalcohol and ethanol precipitation. Commercial

	serum --Additional pectinase treatment	supernatant No co-precipitate: 2500-30,000 x g; 5000-10,000 x g benchmark With co-precipitate; Usually CatFlot T or TL, can reduce centrifugation speed to 2500-3000 x g		kits available

TABLE 2. Summary of Candidate Methodological Approaches to the Detection of Virus Contamination in Soft Fruits.

Commodity and Virus(s)	Sample Size	General Pre-Analytical Processing Approach*	Detection Limits	Reference
Fresh and frozen strawberries; HAV	25 g	Elution (with pectinase) Filtration/centrifugation PEG precipitation (with subsequent chloroform-butanol extraction)	2.0 log PFU/25 g	(Dubois et al., 2007)
Berries; Human NoV, HAV, rotavirus	15 g	Elution/Filtration with pectinase Centrifugation Ultrafiltration	1 $TCID_{50}$ (HAV), 54 RT-qPCR units (HuNoV), 0.02 $TCID_{50}$ (rotavirus) per 15 g	(Butot et al., 2007)
Strawberries and raspberries; HAV and Human NoV	50 g	Elution Filtration using cationically charged filters (nanoalumina) Elution	2-20 viral particles/g	(Morales-Rayas et al., 2010)

Fruit salad and raspberries; HuNoV	50 g	Multiple methods, but most effective consisted of: Elution (with pectinase) Centrifugation Chloroform-butanol ext. PEG precipitation	10^2-10^3 RT-PCRU per 50 g).	(Baert et al., 2008a)
Blended Strawberries and Tomato Sauce; HAV	30 g	Adsorption (acid ppt.) Elution and Vertrel ext. PEG Precipitation with Vertrel ext.	14 PFU/g (tomato sauce) 33 PFU/g (blended strawberries)	(Love et al., 2008)
Strawberries; HAV	25 g	Elution/filtration Recirculating magnetic capture with cationic beads	10^{-1} -10^2 PFU/25 g	(Papafragkou et al, 2008)

TABLE 3. Procedures for the concentration of viruses from water samples.

Method	Principle	Pros	Cons
Adsorption - elution methods	Ionic charge		
Negatively-charged filters		Good recoveries	Requires sample preconditioning
Positively-charged filters		Good recoveries	Costly
Glass powder		Cheap. Good recoveries	Fragile apparatus
Glass wool		Good recoveries	Differences depending on manufacturers
Precipitation methods	Chemical precipitation		
Organic flocculation		Efficient for dirty samples or as secondary concentration	Beef extract is inhibitory to RT-PCR enzymes
Ammonium sulphate		Efficient for dirty samples or as	High citotoxicity Inhibitory to

		secondary concentration	RT-PCR enzymes
Polyethylene glycol		Efficient for dirty samples or as secondary concentration	Intra-assay variability
Ultracentrifugation	Physical sedimentation	Efficient as secondary concentration	Costly
Lyophilisation	Freeze-drying	Efficient for dirty samples or as secondary concentration May remove RT-PCR enzymes inhibitors	Costly. Time-consuming
Ultrafiltration	Particle size separation	Good recoveries for clean samples	Costly. Time-consuming
Magnetic beads	Immunoaffinity	Good recoveries from small	Requires specific assay for each virus.

	volumes	Costly. Few data available

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