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Analytical Methods for Virus Detection in Water and Food

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Abstract:

Potential ways to address the issues that relate to the techniques for analyzing food and environmental samples for the presence of enteric viruses are discussed. It is not the authors' remit to produce or recommend standard or reference methods but to address specific issues in the analytical procedures. Foods of primary importance are bivalve molluscs, particularly, oysters, clams, and mussels; salad crops such as lettuce, green onions and other greens; and soft fruits such as raspberries and strawberries. All types of water, not only drinking water but also recreational water (fresh, marine, and swimming pool), river water (irrigation water), raw and treated sewage are potential vehicles for virus transmission. Well over 100 different enteric viruses could be food or water contaminants; however, with few exceptions, most well-characterized foodborne or waterborne viral outbreaks are restricted to hepatitis A virus (HAV) and calicivirus, essentially norovirus (NoV). Target viruses for analytical methods include, in addition to NoV and HAV, hepatitis E virus (HEV), enteroviruses (e.g., poliovirus), adenovirus, rotavirus, astrovirus, and any other relevant virus likely to be transmitted by food or water. A survey of the currently available methods for detection of viruses in food and environmental matrices was conducted, gathering information on protocols for extraction of viruses from various matrices and on the various specific detection techniques for each virus type.

Keywords: Enteric viruses – Gastroenteritis – Hepatitis – Detection – Concentration

Introduction

The transmission of viruses through consumption of or contact with contaminated water and food is well recognised. Transmission of viruses associated with the consumption of contaminated bivalve shellfish, particularly oysters which are eaten uncooked is regularly reported[1-3]. Other foods including raspberries[4-12] and salads[13] have caused outbreaks after being contaminated by polluted water or virus infected food handlers. Polluted water, both drinking water and recreational water have been shown to have transmitted viruses[14-16]. Because many people may consume a batch of food or come into contact with the contaminated material outbreaks involving large numbers infected people are common. The outbreaks of viral gastro-enteritis are known to be mainly caused by norovirus (NoV) and outbreaks of viral hepatitis are caused by Hepatitis A virus (HAV)[17-21] and in the case of water, more rarely Hepatitis E virus (HEV)[22].

In all the above cases it is important to have effective tools with which to analyse the food or water matrix for its viral content. The following sections address the issues of what and how to take samples of food and water, how to release the virus from each matrix type, non-molecular virus detection, the methods for nucleic acid extraction and molecular detection techniques.

Sampling for viruses associated with food

When sampling procedures for food/fresh produce are considered the questions which arise are: i) is a specific weight of a particular fruit or vegetable representative, ii) is one item, e.g. tomato, per crate representative, iii) is one leaf of a lettuce or cabbage representative, and if so which leaf, iv) should the food item be analyzed whole or chopped, and v) will a single sample suffice or should the test be repeated in triplicate?

In order to assess the real role of food in virus transmission, cost-effective standardized or comparable methods need to be developed for application in reference laboratories. The infectious dose for viruses such as HAV and NoV is estimated to be about 10-100 infectious particles, therefore, although the viral load on fresh produce, minimally processed and ready-to eat foods may be low it may still be a source of infection and illness. Data on shellfish is still scarce but some publications reported NoV concentrations ranging from 10^2 to 10^4 copies per gram of digestive tissues[23-27]. HAV has recently been quantified in naturally contaminated shellfish samples showing titers ranging from 10^3 to 10^5 HAV genomes per gram of clam[28,29], and it has also been reported titers of 0.2-224 infectious particles per 100 g shellfish meat[30].

Therefore methods for the detection of viruses on food samples have to have a high level of analytical sensitivity and specificity. With regard to sample size, it should represent the whole batch or crop, and that USEPA, ISO and national QA regulations must be followed. There is however very little information in the literature, and in USEPA and CEN/ISO guidelines, regarding sampling procedures for the viral analysis of food. References to sampling for microbiology usually refer to the statistical representativeness of the samples, and economic and logistical considerations usually limit the number, type and location of samples to be taken[31]. Sampling for virological analyses of food will not necessarily follow the bacterial approach since the low level of contamination, and the complexity and cost of assays are greater. The USEPA Manual of Methods for Virology[32] only addresses the virological examination of water, with no mention of the virological examination of food. The Health Protection Agency (HPA) of the United Kingdom has a standard operating procedure (SOP) for the preparation of samples for the microbiological examination of food samples (BS 5763). In this procedure a 25-g sample of food, which is homogenized, is used but this SOP only

relates to subsequent bacteriological analyses where the results are reported as colony forming unit per gram or milliliter (CFU/g or mL). Many of the published or methods under development for the detection of viruses on fruit and vegetables use a 10 g – 100 g sample size (a detailed overview of these methods have been described by Croci and co-authors[33], however there is no mention of how many 10 g – 100 g samples need to be taken in from a crate, field or truckload of the particular food matrix to have a statistically representative sample. Obviously, sampling procedures vary according to food matrix type and must have to take the quantity of sample, seasonality, rainfall and probable amount of contamination or pollution into account.

From the literature it is also not clear whether the food sample should be analyzed as a whole or chopped. As most viral contamination would be from external sources during spraying or irrigation, a critical factor influencing the decision to analyze vegetables whole or chopped would be whether the claims that viruses can enter plants through root damage are substantiated or not. It appears that internal contamination of the leaves of tomato plants and green onions can occur[34,35], and that the internal contamination is of a much lower level than external contamination[36,37].

The detection of viruses inside a plant crop could potentially be an indicator of higher levels of external contamination and would be significant as these viruses would not be removed or inactivated by washing or UV irradiation[36]. Sampling of foods implicated in an outbreak of viral disease would be focused on the particular batches consumed.

Sampling for viruses in water

With regard to the virological analysis of water a similar question arises as to those encountered with the virological analysis of food, namely “What is a representative sample”? When sampling procedures for irrigation and washing water are considered

the questions which come up are: i) will a specific volume of water be representative, and ii) will the water quality influence the sampling procedure?

Microbes pose the most significant waterborne health-risk[36] with waterborne diseases being misdiagnosed or underdiagnosed[38]. Contamination of surface water with enteric viruses through disposal of human waste is a concern for public health, especially if these surface waters are used for recreational water, irrigation water and the production of drinking water[39]. The surveillance of irrigation water and water for washing the fresh produce is therefore essential to facilitate correct management procedures for the protection of fruit and vegetable growers and the health of farm workers and the consumers. In order to monitor the virological quality of water, an efficient combination of techniques has to be applied for the optimal recovery and detection of the low titres of viruses present in water[40,41].

Sampling and analytical procedures for the virological analysis of water are well documented[32,42-45]. A variety of techniques have been described for the recovery of viruses from water – each with their own advantages and disadvantages as the physico-chemical quality of the water, including but not limited to the pH, conductivity, turbidity, presence of particulate matter and organic acids can all affect the efficiency of recovery of viruses[46]. Viral recovery and concentration techniques include ultrafiltration[41,47-49], adsorption-elution using filters or membranes[40,50,51], glass wool[52,53] or glass powder[54,55], two-phase separation with polymers[56], flocculation[57,58] and the use of monolithic chromatographic columns[59-62]. The use of the glass wool adsorption-elution procedure for the recovery of enteric viruses from large volumes of water has proven to be a cost-effective method and has successfully been applied for the routine recovery of human enteric viruses from large volumes of water in the South African setting[63-71]. This method can be adapted for the in-line recovery of viruses from water[72]

which circumvents transporting of large volumes of potentially highly polluted water great distances to a central laboratory which would be expensive and a potential health hazard.

It is important to acknowledge that no single method may universally be recognized as superior: efficiency, constancy of performance, robustness, cost, and complexity are all factors to be considered for each method and performance characteristics must be continually monitored.

Sampling aerosols and surfaces

There is concern over the potential transmission of viruses into the food chain through aerosols and from surface contamination. The crucial issues are sample collection and preparation for different virus detection techniques: different methods have been developed based on the attachment properties to surfaces of airborne particles[73].

The most used air samplers are based on impact on solid surfaces, impingement and filtration: all of them have been successfully used for virus detection, but have advantages and disadvantages:

Impact samplers: an air flow with a fixed speed is directed to impact on a solid surface, generally an agar medium[74]. Some equipment (Andersen sampler) has multiple stages, and can sample separately particles with different size. After sampling virus can be eluted from the solid medium; purified and prepared for subsequent virus analysis. This sampling method is easy, but dehydratation or impact trauma can affect the virus survival; flow rate and sampling duration are critical.

Impinger samplers: the air is forced to flow through a narrow orifice to make bubbles in a liquid medium[75]. After sampling the medium can be concentrated or directly decontaminated, purified and analysed. The recovery efficiency of this method is high

because it avoids dehydratation but flow rate and the composition of the collection fluid are again critical for virus recovery[76,77].

Filter samplers: the air pass through a filter and airborne particles are retained as a function of their aerodynamic size and surface properties, such as electrostatic charge. For aerosol sampling, membranes with 1-3 μ pore size can retain droplets with an aerodynamic size <500nm more efficiently than other samplers[73]. Viruses in an aerosol are associated with particles and can be collected. The membrane material can be polytetrafluoroethylene (PTFE), cellulose, polycarbonate, or gelatine[78]. The last one is easier for viral sampling because it can be directly dissolved in an appropriate liquid medium. This sampling method is easy to use, but the flow rate, the sampling duration and the membrane composition have to be strictly controlled to avoid dehydratation.

Further methods for the analysis of viruses in aerosols include cyclone[79] or electrostatic precipitators[80], and, in the last years, the fear of bioterrorism stimulated the study of new methodologies (like mass spectrometry)[81] able to identify pathogens in air, but their application to the routine environmental analysis is still far in the future and will require very large result data bases from many environmental samples.

To better understand the fate of virus dispersed through air, surface monitoring should be also performed, because of the settling of droplets with greater size. Surface sampling has its major indication in health care settings[82] and in food production[83] to assess not only viral contamination, but also efficiency and correct application of disinfection procedures. To this aim, a definite surface area (i.e. 10 cm^2) is swabbed, then the swab is eluted and the eluate processed as a liquid sample. For biomolecular tests some swabs can be submitted directly for nucleic acid extraction. Alternative methods are contact plates that can be eluted.

Virus release from food matrices

The food matrix and the route of contamination involved, determines the way of virus release prior to nucleic acid extraction. Viral particles need to be extracted from homogenized tissues in case of intrinsic contamination (i.e. oysters tissues, pig liver), or eluted from the surface of the food item (i.e. contamination by irrigation water or food handling).

Shellfish are filter feeders and concentrate enteric viruses from their environment while feeding. The majority of accumulated virus is found in the pancreatic tissue, also called the digestive diverticula. Mechanical entrapment and ionic bonding are among the mechanisms that have been suggested to explain observed differences in accumulation of different viruses and among different oyster species[84,85]. Another potential mechanism for the uptake and concentration of viruses in shellfish has been proposed based upon the observation of specific binding of a NoV genogroup I to shellfish tissues[86].

Several efficient methods are now available for shellfish analysis, for example Atmar et al. (1995) proposed the dissection of digestive tissues for virus extraction[87]. Testing the stomach and digestive gland for virus presented several advantages in comparison with testing whole shellfish: less time-consuming procedure, increased test sensitivity, and decrease in the sample-associated interference with RT-PCR[87]. Since the initial description of analyzing only digestive tissues, a number of variations have been published, and most have analyzed the same weight (1.5 to 2 g) of digestive tissues. Viruses are eluted using various buffers (e.g. chloroform-butanol or glycine) before concentration by polyethylene glycol or ultracentrifugation[23,88-90]. Direct lysis of virus particles is used more and more frequently. For example, proteinase K, or Trizol and

lysis of shellfish tissues using Zirconia beads and a denaturing buffer have all been used for virus elution[91,92].

Methods that have currently been developed and optimized for virus detection from fruit and salad vegetables focus on elution of the virus from the surface[33]. A number of washing procedures and buffer systems have been described for the recovery of viruses from fruits and vegetables. The average recovery rates vary depending on the food matrix and virus[33]. Dubois et al. (2007)[93] described a protocol that included the rinsing of fruit and vegetable surfaces with a buffer of pH 9.5, supplemented with 100 mM Tris, 50 mM glycine and 1% beef extract, a protein and nucleic acid rich substance. This buffer ruptures the electrostatic and hydrophobic interactions between fruit or vegetable surfaces and virus. In the case of soft fruits, pectinase has to be added to prevent formation of a gelatinous substance[94,95].

Some authors have reported the presence of viral particles trapped inside vegetables taken up intracellularly through the roots[34-37]. This mechanism warrants further examination, and if confirmed it will change future approaches for the detection of viruses from vegetables.

Virus concentrations on food are likely to be low, indicating that the virus which is present in the relatively large volumes of elution buffer, needs to be concentrated prior to detection. The choice of virus concentration method is dependent on the food matrix and eluant. Frequently used concentration methods include precipitation by polyethylene glycol (PEG), ultrafiltration and ultracentrifugation.

Immunological methods have also been applied to concentrate virus in food[96-99]. However, NoV immunoconcentration is unlikely to be adapted widely for norovirus detection due to the difficulties in obtaining antibodies and its variability at the capsid level. Immunological methods have also been applied to concentrate virus in food.

Nucleic acid extraction and purification

Following virus elution or concentration a variety of subsequent nucleic acid extraction and purification protocols may be employed. Recently a number of methods using kits have been published. A wide variety of commercial kits has been applied for nucleic acid purification, offering reliability, reproducibility and they are quite easy to use. Most of these kits are based on guanidinium lysis, and then capture of nucleic acids on a column or bead of silica (commonly called Boom's method[100]). Although most perform well, differences can be found depending on the virus and/or matrix analyzed[39,101-105]. In the last years automated nucleic acid extraction platforms have been developed by commercial companies, which have been shown to be suitable for the analysis of virus in water samples[39,106].

Virus detection

Virus detection is mainly based on two principles: the detection of infectious viruses by propagation in cell culture or the detection of the viral genomes by molecular amplification techniques such as PCR or RT-PCR. Detection by cell culture is mainly based on the formation of cytopathic effects; followed by quantification of the viruses by plaque assay, most probable number or tissue culture infectious dose 50 ($TCID_{50}$). Virus typing may be done by immunofluorescence or neutralization assays. However, efficient cell culture systems are not available for all viruses, and others grow slowly or do not produce a cytopathic effect.

In the last decade, real-time PCR assays have revolutionized nucleic acid detection by the high speed, sensitivity, reproducibility and minimization of contamination. These methods are widely used in the field of food virology and are continuously evolving. For instance, Sanchez et al. (2007) summarized published real-time RT-PCR methods for HAV detection in food[107] and since then several new methods have become

available[108,109]. It is essential that the specificity, the range of viruses detectable, and sensitivity of real-time RT-PCR assays are demonstrated. All these points are interconnected and depend mostly on the target sequences for primers and probe. The selected targets must guarantee an absolute specificity and must reach equilibrium between high sensitivity, broad reactivity, and reliability of quantification.

Real-time RT-PCR procedures enable not only the qualitative but also the quantitative detection, which opens the possibility of quantitative hazard risk assessment analysis critical for several public health actions or food ban regulations. Quantification can also be performed as most probable number by conventional PCR[39,110]. Qualitative real-time PCR producing a ‘positive or negative‘ result is most appropriate when testing matrices that are unlikely to be contaminated with virus as it is least expensive and straightforward. Quantitative real-time PCR is required when a sample such as shellfish is likely to contain viruses and the degree of contamination needs to be ascertained.

Molecular assays by conventional PCR i.e. gel-based remain useful as larger volumes of sample can be tested, larger PCR-products can be obtained and it is less expensive. The alternative molecular technique nucleic acid sequence based amplification (NASBA) was shown to be less prone to environmental PCR inhibitors present in large volumes of surface water samples[111].

Although the detection of enteric viruses in food is mainly done by molecular techniques, there are several limitations. The method is prone to inhibition, favoring false negative results, and demonstrating the need for proper quality controls. Several ways have been described to overcome this inhibition, such as the analysis of samples dilutions, smaller sample sizes, adaptation of the PCR by e.g. the addition of Tween, BSA or commercial reagents[39,95,112].

One of the major limitations of PCR is its inability to differentiate between infectious and noninfectious viruses. Various approaches to overcome this limitation have been evaluated. Of them, integrated systems based on the molecular detection of viruses after cell culture infection are the most promising techniques[113-115], a detailed overview of these approaches can be found elsewhere[116]. The ICC-PCR assay is based on a selective enumeration of infectious viruses in combination with a rapid molecular detection, circumventing long incubation periods for cytopathic effect formation. Such ICC-PCR assays have been successfully utilized for the detection of several enteric viruses in environmental samples[115]. Other alternatives, such as a protease and RNase pretreatment have successfully been used to differentiate between infectious and non-infectious virus[117-120] although Baert and collaborators did not find correlation for murine norovirus[121].

Quality controls

One of the most critical challenges is the implementation of novel molecular-based methods for the detection of enteric viruses in the routine food analytical laboratories. However, obstacles that influence routine virus detection in foods include the low efficiency of concentration and nucleic acid extraction procedures and, the presence of inhibitors to the molecular reactions. It seems obvious that harmonization of the molecular techniques, as well as addressing quality control and quality assurance (QA/QC) issues is required before adoption of the procedures by routine monitoring laboratories. QA/QC measures include the use of positive and negative controls thus tracing any false negative or positive result, respectively. Most false negatives are consequence of inefficient virus and/or nucleic-acid extraction, and of inhibition of the RT reaction. Most false positives result from cross-contamination.

The first dilemma is to choose between an actual internal control and an added external control for the extraction procedure. For the diagnosis of an RNA virus, the use of an internal control based on the detection of the expression of a housekeeping gene, ideally containing introns, through the amplification of its mRNA in the target tissues is a clear first choice. However this is an unrealistic approach for its application in food virology, which involves an increasingly heterogeneous selection of food matrices. For instance only in shellfish, a pair of primers to amplify an mRNA for a specific hepatopancreas transcribed gene would be required for each species. It is impossible to apply this for the range of foodstuffs susceptible to be assayed for viruses, which leads to compromise in the use of an external control, applicable to all matrices under assay. Table 1 depicts the complete list of terms and definitions in standardized molecular detection assays for virus detection in food[122-129].

One of the most important issues is the the control of nucleic acid extraction efficiency. Recently, the use of a nonpathogenic virus, mengovirus MC0[130] and feline calicivirus[131-134] have been proposed as process control. Although the latter one has been reported to be an inappropriate surrogate for norovirus in acid conditions[135]. Quantitative standardized procedures presently enable to perform QMRA in food samples[136,137].

Several authors have reported that the number of infectious viruses did not correlate with the number of genomes detected by real-time RT-PCR in water samples[121,131,133,138,139]. This is more evident when water or food undergoes a removal/inactivation process.

It is a matter of debate whether the detection of enteric viruses in food or water by PCR or real-time PCR should be considered a safety issue and confirmation of a public health risk. However, one can argue that if viruses were found, even if not infectious, it

would be an indication that the food or water is contaminated and that viruses were present in the food or water at some point.

Table 1: Terms and definitions in standardized molecular detection assays for virus detection in food. .

Process control virus	A virus added to the sample portion at the earliest opportunity prior to virus extraction to control for extraction efficiency.
Process control virus RNA	RNA released from the process control virus in order to produce standard curve data for the estimation of extraction efficiency.
Negative RNA extraction control	Control free of target RNA carried through all steps of the RNA extraction and detection procedure to monitor any cross-contamination events.
Negative process control	Control free of target RNA carried through all steps of the virus extraction, RNA extraction and detection procedure to monitor any cross-contamination events.
Hydrolysis probe	An oligonucleotide probe labelled with a fluoresecent reporter and quencher at the 5’ and 3’ ends respectively. Hydrolysis of the probe during real-time PCR due to the 5'-3' exonuclease activity of Taq DNA polymerase results in an increase in measurable fluorescence from the reporter.
Negative RT-PCR control	An aliquot of highly pure water used as template in a real-time RT-PCR reaction to control for contamination in the real-time RT-PCR reagents.
External control RNA	Reference RNA that can serve as target for the real-time PCR assay of relevance, e.g. run-off transcripts from a plasmid carrying a copy of the target gene, which is added to an aliquot of sample RNA in a defined amount to serve as a control for amplification in a separate reaction.
Cq value	Quantification cycle; the PCR cycle at which the target is quantified in a given real-time PCR reaction. This corresponds to the point at which reaction fluorescence rises above a threshold level.
Theoretical limit of detection (tLOD)	A level that constitutes the smallest quantity of target that can in theory be detected. This corresponds to one genome copy per volume of RNA tested in the target assay but will vary according to the test matrix and the quantity of starting material.
Practical limit of detection (pLOD)	The lowest concentration of target in a test sample that can be reproducibly detected (95% confidence interval), as demonstrated by a collaborative trial or other validation (Annex L).
Limit of quantification (LOQ)	

The lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy, as demonstrated by a collaborative trial or other

Summary

The analysis of food and water matrices for the detection of viruses is now well established to the extent that European Standards are in draft:

Microbiology of food and animal feeding stuffs- Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR- Part 1: Method for quantitative determination

Microbiology of food and animal feeding stuffs- Horizontal method for detection of hepatitis A virus and norovirus in food using real-time RT-PCR- Part 2: Method for qualitative detection

Validation studies are expected to be undertaken for each of the process stages before the Standard is confirmed. This will ensure that the highest level of quality assurance is achieved. Developmental studies on matrices not covered by the Standard will continue to be required to reach consensus on the optimum techniques necessary to ensure effective systems.

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References

Reference List

1. M. Koopmans and E. Duizer, Int. J. Food Microbiol. 90**,** 23 (2004).
2. M. A. Widdowson, A. Sulka, S. N. Bulens et al., Emerg. Infect. Dis. 11**,** 95 (2005).
3. Anonymous, The EFSA Journal 1496**,** (2010).
4. B. Cotterelle, C. Drougard, J. Rolland et al., Euro Surveill 10**,** (2005).
5. F. S. Le Guyader, C. Mittelholzer, L. Haugarreau et al., Int. J. Food Microbiol. 97**,** 179 (2004).

6. C. D. Gaulin, D. Ramsay, P. Cardinal, and M. A. D'Halevyn, Can. J. Public Health 90**,** 37 (1999).

7. B. Korsager, S. Hede, H. Boggild, B. Bottiger, and K. Molbak, Euro Surveill 10**,** (2005).

8. K. O. Hedlund, E. Rubilar-Abreu, and L. Svensson, J. Infect. Dis. 181**,** S275-S280 (2000).

9. G. Falkenhorst, L. Krusell, M. Lisby, S. B. Madsen, B. Bottiger, and K. Molbak, Euro Surveill 10**,** (2005).

10. M. Hjertqvist, A. Johansson, N. Svensson et al., Euro Surveill. 11**,** (2006).

11. G. Fell, M. Boyens, and S. Baumgarte, Bundesgesundheitsblatt. Gesundheitsforschung. Gesundheitsschutz. 50**,** 230 (2007).

12. A. Ponka, L. Maunula, C.-H. von Bonsdorff, and O. Lyytikainen, Euro Surveill 4**,** 66 (1999).

13. R. Vivancos, A. Shroufi, M. Sillis et al., Int. J. Infect. Dis. 13**,** 629 (2009).

14. R. O. Cannon, J. R. Poliner, R. B. Hirschhorn et al., J. Infect. Dis. 164**,** 860 (1991).

15. H. W. Lawson, M. M. Braun, R. I. Glass et al., Lancet 337**,** 1200 (1991).

16. L. Maunula, I. T. Miettinen, and C. H. von Bonsdorff, Emerg. Infect. Dis. 11**,** 1716 (2005).

17. C. N. Ramsay and P. A. Upton, Lancet 1**,** 43 (1989).

18. T. M. Reid and H. G. Robinson, Epidemiol. Infect. 98**,** 109 (1987).

19. L. Calder, G. Simmons, C. Thornley et al., Epidemiol. Infect. 131**,** 745 (2003).

20. M. T. Niu, L. B. Polish, B. H. Robertson et al., J. Infect. Dis. 166**,** 518 (1992).

21. Y. J. Hutin, V. Pool, E. H. Cramer et al., N. Engl. J. Med. 340**,** 595 (1999).

22. N. Jothikumar, K. Aparna, S. Kamatchiammal, R. Paulmurugan, S. Saravanadevi, and P. Khanna, Appl. Environ. Microbiol. 59**,** 2558 (1993).

23. T. Nishida, O. Nishio, M. Kato et al., Microbiol. Immunol. 51**,** 177 (2007).

24. F. S. Le Guyader, F. H. Neill, E. Dubois et al., Int J Food Microbiol 87**,** 107 (2003).

25. F. S. Le Guyader, F. Bon, D. DeMedici et al., J CLIN MICROBIOL 44**,** 3878 (2006).

26. F. S. Le Guyader, S. Parnaudeau, J. Schaeffer et al., Appl. Environ. Microbiol 75, 618 (2009).

27. F. S. Le Guyader, J. C. Le Saux, K. Ambert-Balay et al., J. Clin. Microbiol. 46, 4011 (2008).

28. M. I. Costafreda, A. Bosch, and R. M. Pinto, Appl. Environ. Microbiol. 72, 3846 (2006).

29. R. M. Pinto, M. I. Costafreda, and A. Bosch, Appl. Environ. Microbiol. 75, 7350 (2009).

30. F. P. Williams and G. S. Fout, Environ. Sci. Technol. 26, 689 (1992).

31. C. J. Murray, in *Manual of Environmental Microbiology.*, 2nd ed., Edited by C. J. Hurst *et al.* (ASM Press, Washington D.C., 2002), p. 166.

32. EPA, (U.S. Environmental Protection Agency, Cincinnati, 1984), Chap. Publication no. EPA/600/4-84-013.

33. L. Croci, E. Dubois, N. Cook et al., Food Anal. Methods 1, 73 (2008).

34. G. Oron, M. Goemanns, Y. Manor, and J. Feyen, Water Research 29, 1069 g(1995).

35. D. D. Chancellor, S. Tyagi, M. C. Bazaco et al., J. Food Prot. 69, 1468 (2006).

36. M. J. Carter, J. Appl. Microbiol. 98, 1354 (2005).

37. A. Urbanucci, M. Myrmel, I. Berg, C. H. von Bonsdorff, and L. Maunula, Int. J Food Microbiol. 135, 175 (2009).

38. P. L. Meinhardt, Journal of Water and Health 4, 27 (2006).

39. S. A. Rutjes, R. Italiaander, H. H. van den Berg, W. J. Lodder, and A. M. Roda Husman, Appl. Environ. Microbiol. 71, 3734 (2005).

40. M. Gilgen, D. Germann, J. Lüthy, and P. Hübner, Int. J. Food Microbiol. 37, 189 (1997).

41. H. Soule, O. Genoulaz, B. Gratacap-Cavallier, P. Chevallier, J. X. Liu, and J. M. Seigneurin, Water Res. 34, 1063 (2000).

42. C. P. Gerba, in *Methods for recovering viruses from the environment*, Edited by G. Berg (CRC Press, Inc., Boca Raton, Florida, 1987), p. 1.

43. C. J. Hurst and K. A. Reynolds, in *Manual of Environmental Microbiology*, 2nd ed., Edited by C. J. Hurst *et al.* (ASM Press, Washington D.C., 2002), p. 244.

44. American Public Health Association, American Water Works Association, and Water Environment Federation, in *Standard Methods for the*

Examination of Water and Wastewater, 21st ed., Edited by A. D. Eaton *et al.* (United Book Press Inc., Baltimore MD, 2005), p. 9.

45. P. Wyn-Jones, in *Human Viruses in Water*, Edited by A. Bosch (Elsevier, Amsterdam, 2007), p. 177.

46. G. P. Richards, M. A. Watson, and D. H. Kingsley, J. Virol. Methods 116**,** 63 (2004).

47. M. Divizia, A. L. Santi, and A. Pana, J. Virol. Methods 23**,** 55 (1989).

48. M. Divizia, P. De Filippis, A. Di Napoli, A. venuti, R. Perez-Bercof, and A. Pana, Water Res 23**,** 1155 (1989).

49. D. Garin, F. Fuchs, J. M. Crance et al., Epidemiol. Infect. 113**,** 541 (1994).

50. J. Passagot, J. M. Crance, R. Deloince, H. Laveran, D. Beytout, and R. Fontanges, Water Res. 19**,** 1167 (1985).

51. S. Senouci, A. Maul, and L. Schwartzbrod, Zentralblatt fur Hygiene und Umweltmedizin 198**,** 307 (1996).

52. P. Vilaginès, B. Sarrette, G. Husson, and R. Vilaginès, Water science and technology 27**,** 306 (1993).

53. P. Vilaginès, B. Sarrette, H. Champsaur et al., Water science and technology 35**,** 445 (1997).

54. R. Gajardo, J. M. Diez, J. Jofre, and A. Bosch, J. Virol. Methods 31**,** 345 (1991).

55. C. Menut, C. Beril, and L. Schwartzbrod, Water science and technology 27**,** 294 (1993).

56. K. J. Schwab, R. De Leon, and M. D. Sobsey, Water Sci. Technol. 27**,** 211 (1993).

57. A. Nasser, D. Weinberg, N. Dinoor, B. Fattal, and A. Adin, Water Sci. Technol. 31**,** 63 (1995).

58. H. Backer, Clin. Infect. Dis. 34**,** 355 (2002).

59. K. Branovic, D. Forcic, J. Ivancic et al., J. Virol. Methods 110**,** 163 (2003).

60. P. Kramberger, N. Petrovic, A. Strancar, and M. Ravnikar, J. Virol. Methods 120**,** 51 (2004).

61. K. Kovac, I. Gutierrez-Aguirre, M. Banjac et al., J. Virol. Methods 162**,** 272 (2009).

62. I. Gutierrez-Aguirre, M. Banjac, A. Steyer et al., J. Chromatogr. A 1216**,** 2700 (2009).

63. M. B. Taylor, N. Cox, M. A. Vrey, and W. O. Grabow, Water Res 35**,** 2653 (2001).

64. J. Van Heerden, M. M. Ehlers, A. Heim, and W. O. K. Grabow, J APPL MICROBIOL 99**,** 234 (2005).

65. J. Van Heerden, M. M. Ehlers, W. B. Van Zyl, and W. O. K. Grabow, Water Sci. Technol. 50**,** 39 (2004).

66. J. C. Vivier, M. M. Ehlers, and W. O. K. Grabow, Water Res. 38**,** 2699 (2001).

67. J. C. Vivier, M. M. Ehlers, W. O. K. Grabow, and A. H. Havelaar, Water Supply 2**,** 1 (2002).

68. J. C. Vivier, M. M. Ehlers, and W.-O. K. Grabow, Water Research 2004; 38**,** 11 (2004).

69. W. B. Van Zyl, P. J. Williams, W.-O. K. Grabow, and M. B. Taylor, Water Sci. Technol. 2004**,** 1 (2004).

70. W. B. Van Zyl, N. A. Page, W. O. Grabow, A. D. Steele, and M. B. Taylor, Appl. Environ. Microbiol. 72**,** 4554 (2006).

71. J. M. E. Venter, University of Pretoria, 2004.

72. W. O. K. Grabow, M. B. Taylor, and M. Wolfaardt, WRC Report Report No. 496/1/96, 1996.

73. D. Verreault, S. Moineau, and C. Duchaine, Microbiol Mol. Biol. Rev. 72**,** 413 (2008).

74. T. F. Booth, B. Kournikakis, N. Bastien et al., J Infect. Dis. 191**,** 1472 (2005).

75. O. V. Pyankov, I. E. Agranovski, O. Pyankova et al., Environ. Microbiol. 9**,** 992 (2007).

76. C. J. Hogan, Jr., E. M. Kettleson, M. H. Lee, B. Ramaswami, L. T. Angenent, and P. Biswas, J Appl. Microbiol. 99**,** 1422 (2005).

77. J. R. Hermann, S. J. Hoff, K. J. Yoon, A. C. Burkhardt, R. B. Evans, and J. J. Zimmerman, Appl. Environ. Microbiol. 72**,** 4811 (2006).

78. N. C. Burton, S. A. Grinshpun, and T. Reponen, Ann. Occup. Hyg. 51**,** 143 (2007).

79. S. Alexandersen, Z. Zhang, S. M. Reid, G. H. Hutchings, and A. I. Donaldson, J Gen. Virol. 83**,** 1915 (2002).

80. B. E. Moore, B. P. Sagik, and C. A. Sorber, Appl. Environ. Microbiol. 38**,** 688 (1979).

81. M. V. Johnston, J Mass Spectrom. 35**,** 585 (2000).

82. A. Carducci, M. Verani, B. Casini et al., Lett. Appl. Microbiol. 34**,** 189 (2002).

83. K. Scherer, D. M+ñde, L. Ellerbroek, J. Schulenburg, R. Johne, and G. Klein, Food and Environmental Virology 1**,** 42 (2009).

84. W. Burkhardt, III and K. R. Calci, Appl. Environ. Microbiol 66**,** 1375 (2000).

85. G. R. Di, J. Liston, and J. Matches, Appl. Environ. Microbiol. 33**,** 19 (1977).

86. F. Le Guyader, F. Loisy, R. L. Atmar et al., Emerg. Infect. Dis. 12**,** 931 (2006).

87. R. L. Atmar, F. H. Neill, J. L. Romalde et al., Appl. Environ. Microbiol. 61**,** 3014 (1995).

88. K. J. Schwab, F. H. Neill, F. Le Guyader, M. K. Estes, and R. L. Atmar, Appl. Environ. Microbiol. 67**,** 742 (2001).

89. S. Fukuda, Y. Sasaki, and M. Seno, Appl. Environ. Microbiol 74**,** 3912 (2008).

90. S. A. Milne, S. Gallacher, P. Cash, D. N. Lees, K. Henshilwood, and A. J. Porter, J Food Prot. 70**,** 1475 (2007).

91. N. Jothikumar, J. A. Lowther, K. Henshilwood, D. N. Lees, V. R. Hill, and J. Vinje, Appl. Environ. Microbiol. 71**,** 1870 (2005).

92. F. Lodder-Verschoor, A. M. de Roda Husman, H. H. van den Berg, A. Stein, H. M. van Pelt-Heerschap, and W. H. van der Poel, J Food Prot. 68**,** 1853 (2005).

93. E. Dubois, C. Hennechart, G. Merle et al., Int. J. Food Microbiol. 117**,** 141 (2007).

94. A. Rzezutka, M. Alotaibi, M. D'Agostino, and N. Cook, J. Food Prot. 68**,** 1923 (2005).

95. S. Butot, T. Putallaz, and G. Sanchez, Appl. Environ. Microbiol. 73**,** 186 (2007).

96. S. Bidawid, J. M. Farber, and S. A. Sattar, J. Virol. Methods 88**,** 175 (2000).

97. S. Kobayashi, K. Natori, N. Takeda, and K. Sakae, Microbiol. Immunol. 48**,** 201 (2004).

98. X. C. Shan, P. Wolffs, and M. W. Griffiths, Appl. Environ. Microbiol. 71**,** 5624 (2005).

99. P. Tian and R. Mandrell, J. Appl. Microbiol. 100**,** 564 (2006).

100. R. Boom, C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and N. J. van der, J. Clin. Microbiol. 28**,** 495 (1990).

101. M. K. Hourfar, M. Schmidt, E. Seifried, and W. K. Roth, Vox Sang. 89**,** 71 (2005).

102. J. H. Knepp, M. A. Geahr, M. S. Forman, and A. Valsamakis, J. Clin. Microbiol. 41, 3532 (2003).

103. T. Kok, S. Wati, B. Bayly, D. Devonshire-Gill, and G. Higgins, J. Clin. Virol. 16, 59 (2000).

104. F. S. Le Guyader, S. Parnaudeau, J. Schaeffer et al., Appl. Environ. Microbiol. 75, 618 (2009).

105. M. Burgener, U. Candrian, and M. Gilgen, J. Virol. Methods 108, 165 (2003).

106. S. Perelle, L. Cavellini, C. Burger et al., Journal of Virological Methods 157, 80 (2009).

107. G. Sanchez, A. Bosch, and R. M. Pinto, Lett. Appl. Microbiol. 45, 1 (2007).

108. A. Houde, E. Guevremont, E. Poitras et al., J Virol. Methods 140, 80 (2007).

109. N. Casas, F. Amarita, and I. Martínez de Marañon, Int. J Food Microbiol 120, 179 (2007).

110. S. A. Rutjes, F. Lodder-Verschoor, W. H. van der Poel, Y. T. van Duijnhoven, and A. M. de Roda Husman, J Food Prot. 69, 1949 (2006).

111. S. A. Rutjes, H. H. van den Berg, W. J. Lodder, and A. M. Roda Husman, Appl. Environ. Microbiol. 72, 5349 (2006).

112. W. A. Al-Soud and P. Radstrom, J. Clin. Microbiol. 39, 485 (2001).

113. R. M. Pinto, J. M. Diez, and A. Bosch, J. Med. Virol. 44, 310 (1994).

114. R. M. Pinto, R. Gajardo, F. X. Abad, and A. Bosch, Environmental Science and Technology 29, 2636 (1995).

115. K. A. Reynolds, C. P. Gerba, M. Abbaszadegan, and L. L. Pepper, Can. J. Microbiol. 47, 153 (2001).

116. R. A. Rodriguez, I. L. Pepper, and C. P. Gerba, Appl. Environ. Microbiol. 75, 297 (2009).

117. S. Nuanualsuwan and D. O. Cliver, Appl. Environ. Microbiol. 69, 350 (2003).

118. S. Lamhoujeb, I. Fliss, S. E. Ngazoa, and J. Jean, Appl. Environ. Microbiol. 74, 3349 (2008).

119. S. Nuanualsuwan and D. O. Cliver, J. Virol. Methods 104, 217 (2002).

120. J. R. Topping, H. Schnerr, J. Haines et al., J. Virol. Methods 156, 89 (2009).

121. L. Baert, C. E. Wobus, C. E. Van, L. B. Thackray, J. Debevere, and M. Uyttendaele, Appl. Environ. Microbiol. 74, 543 (2008).

122. J. Hoorfar, N. Cook, B. Malorny et al., J. Clin. Microbiol. 41, 5835 (2003).

123. D. Rodríguez-Lázaro, B. Lombard, H. Smith et al., Trends Food Sci. Technol. 18, 306 (2007).

124. M. I. Costafreda, A. Bosch, and R. M. Pinto, Appl. Environ. Microbiol. 72, 3846 (2006).

125. Anonymous, International Organization for Standardization, Geneve Switzerland (2005).

126. J. Hoorfar and N. Cook, Methods Mol. Biol. 216, 51 (2003).

127. J. Hoorfar, B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach, J. Clin. Microbiol. 42, 1863 (2004).

128. D. Rodriguez-Lazaro, M. D'Agostino, M. Pla, and N. Cook, J. Clin. Microbiol. 42, 5832 (2004).

129. D. Rodriguez-Lazaro, M. Pla, M. Scortti, H. J. Monzo, and J. A. Vazquez-Boland, Appl. Environ. Microbiol. 71, 9008 (2005).

130. K. Mattison, J. Brassard, M. J. Gagne et al., Int. J Food Microbiol 132, 73 (2009).

131. S. Butot, T. Putallaz, and G. Sanchez, Int. J. Food Microbiol. 126, 30 (2008).

132. J. L. Cannon, E. Papafragkou, G. W. Park, J. Osborne, L. A. Jaykus, and J. Vinje, J. Food Prot. 69, 2761 (2006).

133. J. Hewitt and G. E. Greening, J. Food Prot. 67, 1743 (2004).

134. R. M. Pinto, M. I. Costafreda, and A. Bosch, Appl. Environ. Microbiol. 75, 7350 (2009).

135. R. M. Pinto, M. I. Costafreda, and A. Bosch, Appl. Environ. Microbiol. 75, 7350 (2009).

136. B. Gassilloud, L. Schwartzbrod, and C. Gantzer, Appl. Environ. Microbiol. 69, 3965 (2003).

137. C. Arnal, J. M. Crance, C. Gantzer, L. Schwartzbrod, R. Deloince, and S. Billaudel, Zentralblatt fur Hygiene und Umweltmedizin 201, 279 (1998).

138. S. Butot, T. Putallaz, R. Amoroso, and G. Sanchez, Appl. Environ. Microbiol. 75, 4155 (2009).

139. L. Baert, M. Uyttendaele, M. Vermeersch, C. E. Van, and J. Debevere, J. Food Prot. 71, 1590 (2008).