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# Sample preparation prior to molecular amplification: Complexities and opportunities

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Molecular amplification using Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) is currently considered as the gold standard to detect enteric human pathogenic viruses such as norovirus and hepatitis A virus in food and water. However, the molecular-based detection requires an adequate sampling strategy and a sample preparation specific for viruses. Sampling for enteric human viruses in water and food should not necessarily follow bacterial sampling plans. The development of a reference detection method including sample preparation as proposed in ISO/TS 15216 represents a milestone to facilitate the evaluation of the performance and eventually validation of future virus detection methods. The potential viral infectivity linked to a positive PCR result is a remaining issue and pretreatments allowing the differentiation of infectious viruses would be useful for future risk assessments.

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## Introduction

Molecular amplification using Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) is currently considered as the gold standard to detect enteric human pathogenic viruses such as norovirus (NoV) and hepatitis A virus (HAV) in food and water. Viruses cannot grow outside their specific host cells and therefore replication does not occur in the environment nor in foods. For this reason, enrichment, which is typically used for bacterial propagation for analytical purposes, cannot be applied to increase virus concentration [1,2]. Therefore, an adequate sampling strategy including sample preparation, specifically for viruses, is required. The food or water sample needs to be subjected to virus extraction and concentration steps followed by nucleic acid purification prior to molecular amplification. The complexities and the opportunities associated with the sampling, the sample preparation consisting of virus extraction and concentration steps and the preparation of ready-to-use nucleic acids will be discussed.

## Sampling

As for any other microbial contaminant, detection of human enteric viruses in water and food should start with a rational sampling plan in which sampling points, number and volumes of samples should be determined based on the aim of the testing, the anticipated prevalence and the desired accuracy [3]. Unfortunately, this can be challenging. While there is information available on the prevalence of NoV in oysters where outbreak- and non-outbreak-related positive samples were compared, there is only limited information on the prevalence of viruses in other foods such as different fruits and vegetables [4,5,6••]. This general lack of data may explain why there is no specific mention of sampling for human enteric viruses in any of the available standards from international bodies [7•].

Currently, sampling for viruses is based on sampling strategies for bacteria such as published by the International Commission for the Microbiological Specifications of Foods (ICMSF) [8]. As an example, for the enhanced monitoring for NoV and HAV in frozen strawberries imported from China to the European Union, a sampling plan is proposed requiring 5 samples to be taken throughout a batch [9•,10]. This could prevent highly contaminated berries from reaching the consumer. In a similar way, monitoring of NoV in shellfish appears to be a relevant approach to determine whether contamination is present above a particular level, in order to prevent highly contaminated shellfish batches reaching the market [6••].

Sampling for virological analyses of food should not necessarily follow the bacterial approach since important differences are evident, such as the generally low level of viral contamination, the inability to enrich viruses and the complexity and high cost of assays [7•]. Relevant and economically viable new approaches are needed for the food industry to incorporate sampling for enteric viruses, and the future standards for microbial sampling should include recommendations for enteric viruses.

For instance, viral sampling of fresh produce presents many difficulties and limitations, including especially low numbers of heterogeneously distributed viruses in combination with the high cost of the analysis. In such circumstances, testing environmental samples, for example irrigation or washing water at a production or

a processing stage, may prove more relevant than testing the produce directly [11••]. In comparison to food samples, sampling volumes and procedures for enteric virus detection in water are more comprehensively documented and related to water quality [3]. Following the same logic, it could be more useful to undertake virus screening on swabs from the hands of harvesting personnel or other environmental surfaces than to sample the produce or food directly [11••]. Such indirect evidence of virus contamination could be added to certain raw material specifications to ensure greater traceability and enhance the confidence in critical raw materials such as berries.

Alternatively, rather than attempting to monitor the presence of viral pathogens, sampling for a more prevalent index virus could be used to indicate the potential presence of human pathogenic viruses in water coming into contact with food or even the food itself. Candidate viruses would be those which are largely carried by healthy people and eliminated via the fecal route. Attention has focused on human Adenovirus (hAdV) which are excreted in large quantities by the populations of widely divergent geographical areas and are more resistant to environmental degradation than many other enteric viruses [12,13]. Other virus types such as polyomavirus and more recently, pepper mild mottle virus, have also been proposed [14,15]. The use of such 'indicator' viruses might represent a more reliable approach in terms of risk management and could be used by the food industry to build trust in certain supply chains.

## Sample preparation

Sample preparation for the detection of viruses in food and water requires two steps: (i) virus extraction and concentration from the sample and (ii) nucleic acid extraction and purification. The latter step no longer represents a bottleneck as reliable and reproducible commercial kits are available [7•,16]. However, the former step can be restrictive due to the high variability of virus recoveries and the low extraction efficiency [17–19]. Furthermore, the large spectrum of matrices and the broad diversity of existing virus extraction and concentration approaches add complexity [17]. Indeed, a great number of protocols with the aim of detecting viruses in foods are published. In fact, these protocols represent numerous variants on each other and can be grouped. Two distinct approaches are applied: (i) elution–concentration of virus particles or (ii) the direct viral RNA extraction from food [17]. Similarly, for water, several technologies are described to concentrate viruses; the number of protocols can be categorized mainly as (i) various adsorption–elution methods using electronegative or electropositive filters and (ii) ultrafiltration-based methods [20•]. The comparison of method performance for those available methods is lacking and the limited number of viral extraction studies that are available for any given combination of virus, water or food type are limited.

To monitor the efficiency of a virus extraction method, a process virus control needs to be included at the beginning of the virus extraction step. This control should be a virus with similar morphological and physicochemical proprieties and environmental persistence to the target virus [21]. ISO/TS 15216 [22••,23••], a reference method for high risk food categories (bivalve molluscan shellfish, soft fruits and salad vegetables, food surfaces and bottled water) developed by the CEN/TC 275-Food analysis, Horizontal Methods; Working Group 6, Technical Advisory Group 4 (CEN TAG4), proposed the use of a genetically modified mengovirus. Other candidates are reported in the literature, such as feline calicivirus [24], MS2 bacteriophage [19], and murine norovirus 1 (MNV-1) [21]. It is important to point out that MNV-1 is not easily accessible to private companies.

A meta-analysis comparing the recovery of the process virus control can be used to evaluate the performance of methods. Cashdollar [20•] carried out this type of analysis for water, but the compilation of different process control viruses resulted in a wide range of recoveries. It was not evident if the divergence in recoveries was due to the process virus control or the method itself. It was suggested that the virus itself, rather than the matrix, filter type or sample volume, is more important in predicting the performance of a method for detection of viruses in water [20•]. Additionally, the mechanism of virus adsorption to food or virus behavior in water is poorly understood since no systematic investigations have been performed. The latter would help to clarify the difference in recoveries observed between methods and virus types.

The recovery can be also impacted by the presence of inhibitory substances, such as polysaccharides, proteins and fatty acids compounds [25–28]. To mitigate RT-qPCR inhibition a 10-fold dilution of ribonucleic acid (RNA) is commonly applied. For the detection of HAV and MNV-1 in lettuce, Coudray *et al.* [18] obtained higher recoveries using a 10-fold dilution compared to an undiluted RNA. However, viral RNA copy number is close to the assay detection limit, the diluted RNA will give a negative result, demonstrating the importance of analyzing diluted and undiluted RNA [18], as recommended in ISO/TS 15216 [22••,23••]. These inhibitors should be removed and controlled before molecular detection to avoid false negative results.

An alternative means of comparing methods might be to consider the detection limit achieved by each method. It is difficult to make robust comparisons based on detection limits as these are defined differently in different studies e.g. RT-qPCR units (RT-qPCRU), Plaque Forming

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