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RESEARCH

# Short Communication — Stability of "Free" Norovirus RNA on Fresh Produce

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## Abstract

The accuracy and dependability of results generated through molecular detection of specific target sequences of RNA, commonly used for detecting viruses in food, have been extensively debated within the scientific community. Such concerns have been raised by researchers, clients, and regulators alike, highlighting the need for further investigation and clarification. In particular, there has been debate about the possibility of molecular methods to detect enteric viruses, such as norovirus, in foods, producing false positive RT-PCR results due to the presence of "free" target RNA sequences in the food supply chain environment. This study aimed to investigate this issue by evaluating the recovery of "free" norovirus RNA from lettuce leaves. The "free" RNA was produced using heat treatment and chemical extraction methods. The study findings indicate that recovery of heat-extracted RNA from the lettuce leaves decreased markedly within 24 h, while recovery of chemically extracted RNA remained stable and even increased over time. The results of this study suggest that positive molecular detection of viruses in food is more likely to be associated with intact and potentially infectious virus particles, rather than "free" unprotected RNA. These findings have significant implications for the food industry and regulatory bodies in terms of the interpretation, accuracy and reliability of molecular detection methods for virus detection in food.

**Keywords** Norovirus · RNA stability · Infectious virus particle · RT-PCR · Food safety · Molecular detection

## Introduction

Detection of norovirus and hepatitis A virus on fresh (and frozen) produce such as leafy greens and soft berry fruits has become a more common procedure in many countries worldwide where there is the capability and expertise necessary to carry out such analysis. As a result of local food safety authorities carrying out routine monitoring of at risk foods, useful alerts and warnings have been issued to the global food industry by means of the Rapid Alert System for Food and Feed (RASFF). The consequences of such alerts may include the withdrawal of the product from market or potentially a recall. The availability of a full ISO standard for the detection of Norovirus and Hepatitis A virus in food (Anonymous. 2019) has increased the capability of the food industry to expand its testing regimes to include viruses. There are still relatively few food testing laboratories which have the expertise to carry out the method and interpret the results correctly, compared with those that carry out traditional bacteriological testing. However, concerns have been raised within the scientific community regarding the interpretation of results produced by this method.

When testing foods for norovirus using this molecular based method, the inability to unambiguously determine whether a positive result is derived from an infectious or non-infectious virus particle is a cause for concern for many in the food industry when it comes to being able to apply risk-based intervention strategies for enteric virus transmission through food supply chains (Anonymous. 2014a, b). Baert et al. (Baert et al. 2011) concluded that detection of viruses may indicate contact with NoV in the fresh produce chain and that potential risk for infection cannot be excluded, but the exact risk remains unknown due to the use of RT-PCR (Reverse Transcription Polymerase Chain Reaction). They recommended that studies should be designed which can determine the probability of infection related to the presence or levels of NoV genomic copies. Adding to the issue are reports that RT-PCR results from the amplification

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of small viral genomic sequences such as those associated with the detection of norovirus and hepatitis A virus may not represent the presence of actual infectious virus particles on foods (Knight et al. 2012). The theory is that false positive signals could be produced by the detection of target sequences prevailing in the food supply chain environment (the "free" RNA) which have made their way onto the surface of the food. This raises significant challenges for both analysts and businesses in interpreting the data and making informed decisions on control measures in response to positive results. Prior investigations into detecting naked viral RNA on food surfaces (Escudero et al. 2012) have used RNA extracted from norovirus obtained from faecal suspensions as well as a surrogate virus, murine norovirus. Viral RNA extraction kits were used to produce free purified RNA. After being diluted in nuclease free water, these viral eluates were placed on lettuce leaves, and the RNA was eluted with PBS until it was no longer detectable by RT-PCR. These studies found that murine norovirus RNA remained stable for up to 14 days, while human norovirus RNA remained stable for less than 4 days. However, it should be noted that the RNA used in these experiments would have had some degree of protection from the elution buffer, and the lettuce leaves were not subjected to a full detection method. Thus, these results may not be representative of routine analysis of food types as is currently carried out, and the claim that "free" RNA can be detected for extended periods may not be reliable. The aim of this study was to challenge the theory that "free" RNA in the fresh produce supply chain could be responsible for positive results in virus detection assays, and instead investigate the likelihood that positive results are much more likely due to the presence of intact and potentially infectious viruses. This paper presents the findings of this experiment.

## Experimental Design

The hypothesis is that truly "free" RNA obtained through heat treatment of a virus suspension would rapidly deteriorate upon dissociation from the viral capsid, while protected RNA extracted chemically (using an RNA protective eluate) would exhibit little deterioration. The heat-treated sample, which produces free RNA by breaking apart the virus capsid at a high temperature and exposing the RNA remains in an unprotected state and more closely resembles RNA found in nature or on a food surface, whereas RNA which has been extracted chemically prevents rapid deterioration and therefore produces an unnaturally stable version of "free" RNA on a food surface. Two different methods were therefore used to release the RNA: (1) heating the virus suspension at 95 °C for 5 min to inactivate virus particles and release nucleic acids, resulting in a suspension of "free" RNA, and (2) chemical lysis using a nucleic acid extraction kit, resulting in a clean suspension of "free" RNA suspended in an elution buffer designed to stabilize the RNA for storage and limit degradation before RT-PCR.

After treatment, the suspensions were inoculated onto 25 g lettuce leaves and allowed to air dry for 2 h in a safety cabinet. Three samples were immediately tested (Time 0), while the remaining 3 were tested after being stored at 20 °C for 24 h (Time 24) and 48 h (Time 48 h) to mimic typical shelf conditions within a retail environment (in UK retail stores, unless leafy greens/salad leaves are packaged in sealed bags, they are not usually refrigerated). Our hypothesis was that at time 0, similar levels of RNA recovery would be observed from both heat-treated and chemically treated suspensions due to the RNA having little time to deteriorate. However, after 24 h on lettuce leaves, we expected a significant reduction in detectable "free" RNA from heat-treated samples compared to the chemically treated samples, since these RNA extraction eluates are designed to protect RNA. To replicate a standard food sample analysis, the full ISO-based method involving virus removal and concentration, based on ISO15216:1 was performed according to a UKAS accredited method. Nucleic acid extraction was conducted using the NucliSens® Magnetic Extraction Kit (bioMerieux Ltd) rather than simply resuspending RNA from the lettuce leaf surface as has been done in other studies (Knight et al. 2012).

## Materials

Norovirus GII LENTICULE® Discs (Public Health England), NucliSens Lysis Buffer (bioMerieux Ltd) Ceeram Tools Norovirus GII Detection Kit (bioMerieux Ltd) Ceeram Tools Norovirus GII quantification Kit (bioMerieux Ltd), NucliSens® Magnetic Extraction Kit (bioMerieux Ltd), Thermomixer set to 95 °C (Eppendorf), miniMAG® semi-automated nucleic-acid extraction system (bioMerieux Ltd), 96 well PCR plates (Applied Biosystems), 7500 FAST PCR instrument (Applied Biosystems), general reagents and consumables required for the detection method, open-headed lettuce purchased from supermarket.

### Preparation of Virus Suspension and Extract

Norovirus GII LENTICULE® Discs (Microbiological Reference Material for Internal Quality Control) were obtained from Public Health England. The discs contained genome copies ranging from $6.2 \times 10^2$ to $2.3 \times 10^3$ per Lenticule and were stored at − 20 °C. To prepare for use, six discs were defrosted for 10 min, added to 1 mL Phosphate Buffered Saline, and left for at least 10 min until fully dissolved. As the suspensions were considered an infectious biological agent, all rehydration steps were performed within a Biosafety Level 2 safety cabinet.

BioMerieux Ltd. supplied the commercially available virus nucleic acid extraction and detection/quantification kits. The detection kits included a Mastermix solution containing a non-target Internal Amplification Control (IAC) to confirm the correct amplification process, a tube of Enzyme mix, positive PCR RNA control material, and negative PCR water control. A standard curve was produced using the protocol from Ceeram Tools Norovirus GII Quantification Kit. The RT-PCR conditions used were according to the kit manufacturer's instructions: 45°C/10min; 95°C/10min; and 45 cycles of 95°C/15sec to 60°C /45 sec. The limit of detection is between 1 and 10 genome copies per reaction.

## Results

The recovery of norovirus from lettuce leaves was evaluated using two different methods: heat treatment and chemical extraction. The mean recovery of norovirus genome copies per ml for heat-treated samples was 305 gc at time 0 h, which decreased to 21 gc at 24 h and 35 gc at 48 h (Fig. 1).

For chemically treated samples, the mean recovery was 336 gc per ml at time 0 h, which increased to 477 gc at 24 h and 1514 gc at 48 h (Fig. 2).

Figure 3 shows the comparison between the recoveries of both treatments after 0 and 24 h. The paired *t*-test was used to analyze the heat-treated data and chemical-treated data separately.

The results showed that the reduction in norovirus RNA in the heat-treated samples after 24 h was statistically significant ($P < 0.05$), while the increase in RNA in the chemically treated samples was not statistically significant ($P > 0.05$). The paired *t*-test was also used to compare the heat-treated and chemical-treated data after 24 h, and the results were statistically significant ($P < 0.05$). These findings suggest that the heat-extracted free RNA degraded significantly in just 24 h, while the chemical extraction yielded protected RNA that was stable over the same period. The

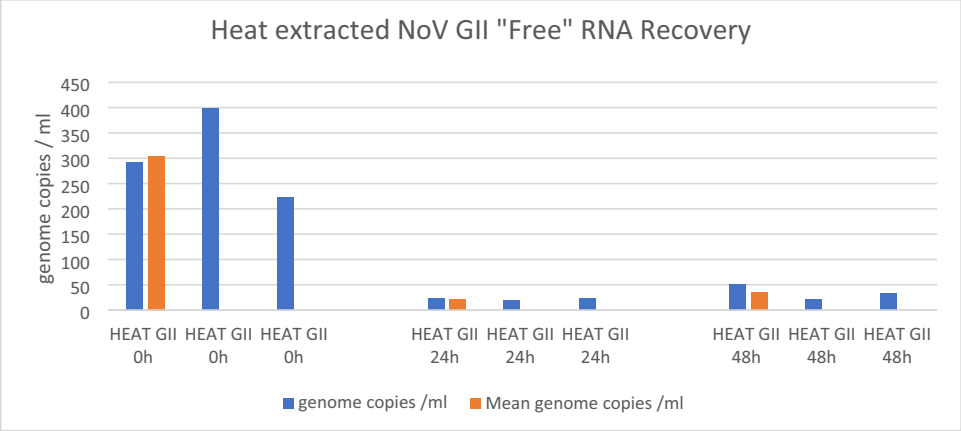


**Fig. 1** Recovery of 3 NoV GII heat extracted RNA suspensions from lettuce leaves tested at time 0 h, 24 h and 48 h

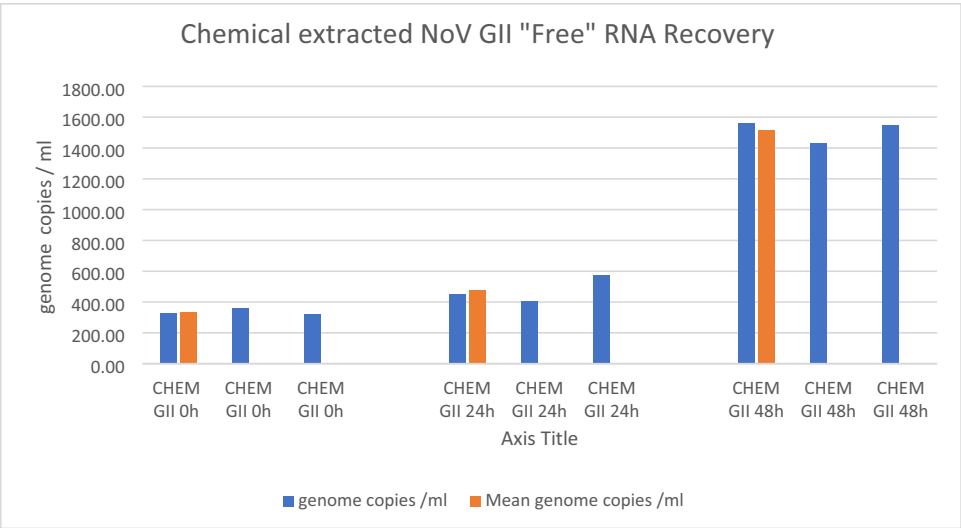


**Fig. 2** Recovery of 3 NoV GII chemical RNA extracted RNA suspensions from lettuce leaves tested at time 0 h, 24 h and 48 h

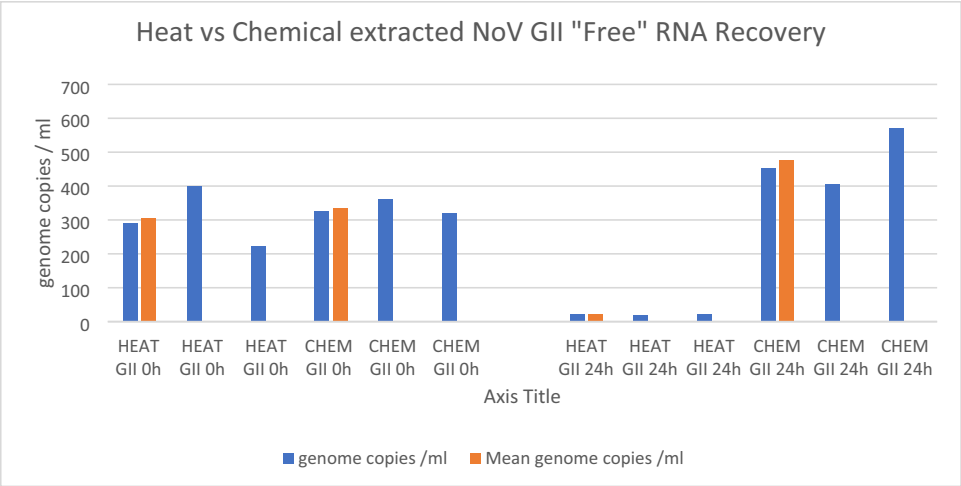


**Fig. 3** Recovery comparison of 3 NoV GII heat and chemical extracted RNA from lettuce leaves tested at time 0 h and 24 h

heat extracted-RNA demonstrated a 1.16 log reduction after 24 h, while the chemically extracted RNA demonstrated a − 0.15 log reduction. Finally, the recovery of the chemical extraction increased over time (24–48 h), likely due to the protected RNA being given increased time to bind to the lettuce leaf surfaces. These results show that the heat and chemical methods to produce free RNA have different effects on the stability of norovirus RNA on the surface of lettuce leaves, suggesting that similar experiments using chemically recovered RNA do not truly represent unprotected "free" RNA and thus may produce overestimated persistence characteristics.

## Discussion

The absence of routine monitoring of fresh or frozen produce for the presence of enteric viruses is a topic that requires discussion. Despite the potential risk posed by these viruses, no regulations or microbiological criteria have been established for their presence in food. This is likely due to the lack of suitable infectivity assays and a molecular detection method being the only available option. Additionally, the routine use of this method is constrained by a limited number of laboratories with the necessary technical capabilities, leading to challenges in interpretation of results by the food industry. Food safety authorities have yet to take a stance on the routine testing of foods for enteric viruses, and positive results may require action or control measures that the industry is often reluctant to take.

The question of whether a positive norovirus RT-PCR signal in fresh or frozen produce samples indicates the presence of an infection-causing virus is a common inquiry in the food industry. However, it is essential to recognize the broader implications of a positive molecular result. It is unacceptable to have human-derived pathogenic material in food, so any positive result should not be taken lightly. Often, the complexity of interpreting RT-PCR signals leads the food industry to consider results as uninterpretable, resulting in no action being taken. However, it is crucial to recognize that a positive RT-PCR result may derive from infectious viruses. Evidence is lacking to prove otherwise. Therefore, when something unexpected is found in food, the focus of investigations should be on determining the cause. The potential to cause illness is of secondary importance when there are no control measures in place to inactivate viruses. It is therefore important to assume the worst-case scenario to prevent any harm to the public.

While it is currently unknown whether a positive result necessarily indicates the presence of an infectious virus, this is not dissimilar to the lack of certainty in determining whether other pathogens such as *Salmonella* or *E. coli* will cause illness if the food is consumed. Therefore, the key question should not be solely focused on the infectivity of a positive result, but rather on identifying the origin and path of norovirus or hepatitis A virus in fresh or frozen produce and addressing any flaws in the food supply chain that permitted contamination to occur. By shifting the focus to these areas, it will be possible to develop effective control strategies for preventing contamination in the first place, and to move away from the focus on infectivity.

**Author Contribution** Martin D'Agostino performed the experimental work and authored the paper in its entirety.

**Data Availability** Raw RT-PCR data is available upon request.

## Declarations

**Conflict of Interest** Martin D'Agostino declares that he has no conflict of interest.



## References

Anonymous (2014a) Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (Salmonella and Norovirus in berries). EFSA J, 12(6). https://doi.org/10.2903/j.efsa.2014.3706

Anonymous (2014b) Scientific Opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (Salmonella and Norovirus in leafy greens eaten raw as salads). EFSA J, 12(3). https://doi.org/10.2903/j.efsa.2014.3600

Anonymous (2019) Microbiology of the food chain - horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR. (BS EN ISO 15216–2:2019). https://doi.org/10.3403/30364243

Baert L, Mattison K, Loisy-Hamon F, Harlow J, Martyres A, Lebeau B, Stals A, Van Coillie E, Herman L, Uyttendaele M (2011) Review: norovirus prevalence in Belgian, Canadian and French fresh produce: a threat to human health? Int J Food Microbiol 151(3):261–269. https://doi.org/10.1016/j.ijfoodmicro.2011.09.013

Escudero BI, Rawsthorne H, Gensel C, Jaykus LA (2012) Persistence and transferability of noroviruses on and between common surfaces and foods. J Food Prot 75(5):927–935. https://doi.org/10.4315/0362-028x.jfp-11-460

Knight A, Li D, Uyttendaele M, Jaykus L-A (2012) A critical review of methods for detecting human noroviruses and predicting their infectivity. Crit Rev Microbiol 39(3):295–309. https://doi.org/10.3109/1040841x.2012.709820