Comparison of methods for quantification of norovirus in dried and fresh seaweed products

Report of the National Reference Laboratory for Foodborne Viruses

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Contents

1. Introduction

In recent years a small number of norovirus illness incidents linked to consumption of seaweed products have been reported both in the UK (UK Health Security Agency, personal communication) and overseas (Park et al, 2015; Sakon et al, 2018; Whitworth, 2019). At the request of the Food Standards Agency (FSA), the National Reference Laboratory (NRL) for Foodborne Viruses was asked to carry out a practical investigation on methods for detection and quantification of norovirus in various seaweed products, with the aim of establishing a method that would be suitable for use in a future outbreak investigation or survey.

ISO 15216-1, the international standard method for quantification of viruses in foods (International Organization for Standardization, 2017) includes leaf, stem and bulb vegetables amongst its target matrices, however this matrix category does not include either fresh or dried seaweed. In the absence of an international standard method explicitly recommended for virus testing in seaweed products it is therefore necessary for testing laboratories to identify and implement suitable candidate methods themselves.

Depending on the country, outbreak investigations have used a very wide variety of methods, some of which are radically different from the methods for analysis of foodborne viruses currently used at the NRL. We therefore decided to use the ISO 15216-1 method for vegetables as the starting point for our investigations.

The FSA asked the NRL to focus on two different types of products, namely:-

- Dried seaweed (nori – red algae of genus *Pyropia* – dried, fried in oil and seasoned with salt and other flavourings)
- Fresh seaweed of a variety of different species including
    - Dulse (red algae of genus *Palmaria*)
    - Sea lettuce (green algae of genus *Ulva*)
    - Kombu kelp (brown algae of genus *Laminaria*)
    - Egg wrack (brown algae of genus *Ascophyllum*)

Because of the very different physical qualities of the two different types of products, method investigations developed differently. These are detailed in separate results sections below.

2. Methods and Approach

The starting point for methodological investigations was the **ISO 15216-1 method for vegetables** (25g samples). Depending on the sample type, modifications were introduced to this method in order to improve the performance including:-

- addition of an inhibitor removal step at the end of the RNA extraction procedure (this step is routinely carried out and recommended by the NRL for soft fruit samples)

- reduction of the sample weight from 25g to 5g

After initial rounds of experiments using the **ISO 15216-1 method for vegetables**, two further methods were trialled for fresh seaweed samples only:-

- the **direct lysis method** (Rajiuddin et al, 2020)
- a **swabbing method** based on the ISO 15216-1 method for food surfaces (International Organization for Standardization, 2017)

For investigations using fresh seaweed, different species were used in different phases of the investigation depending on availability; all four species listed were included in the final method comparison however.

In all experiments, three subsamples of seaweed per sample type/method combination were contaminated with norovirus genogroup II (GII; from faecal material) and mengo virus as a process control in accordance with the guidance in ISO 15216-1. After virus and RNA extraction, samples were analysed by the reverse transcription – quantitative polymerase chain reaction assay (RT-qPCR). In accordance with the requirements of ISO 15216-1 for vegetable samples, undiluted and 1/10 diluted sample RNA was tested simultaneously (the standard states that results with diluted RNA can be used if undiluted RNA is inhibitory to RT-qPCR). In addition to quantification of norovirus GII, sample RNA was assessed for RT-qPCR inhibition using the external control (EC) RNA method, and for exaction efficiency by measuring mengo virus recovery as described in ISO 15216-1.

Detailed method protocols are provided in the Appendix.

3. Results

3.1. Dried Seaweed

Initial experiments with dried seaweed used the method for vegetables as described in ISO 15216-1 and a sample weight of 25g as described in that method. In this initial phase the 40 ml Tris Glycine Beef Extract (TGBE) buffer added to the sample was entirely absorbed by the 25g dried seaweed, producing a sludge that yielded very little eluate after the initial incubation. These extractions were abandoned as a result.

In the second phase the sample weight was reduced to 5g to try to counteract this complete absorption. Performance of this modified method (which included inhibitor removal) in terms of practicability was much improved with reduced absorption and sufficient eluate produced for testing after the initial incubation. Quality control (QC) parameters (extraction efficiency and RT-qPCR inhibition) for all three subsamples tested were in the acceptable range according to ISO 15216-1 (>1% extraction efficiency and <75% RT-qPCR inhibition) and high levels of norovirus GII were detected (Table 1, Figure

1). Testing using 1/10 diluted RNA did not significantly improve QC parameters or increase calculated levels of norovirus GII. This pattern of results is normal where samples are non-inhibitory.

Table 1; results for testing dried seaweed samples using reduced sample weight of 5g

Subsample	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
a	50.8	0.0	5.6	47.0	0.0	8.0
b	34.9	0.0	1.5	26.4	0.0	2.8
c	35.1	0.0	2.8	32.2	0.0	4.2

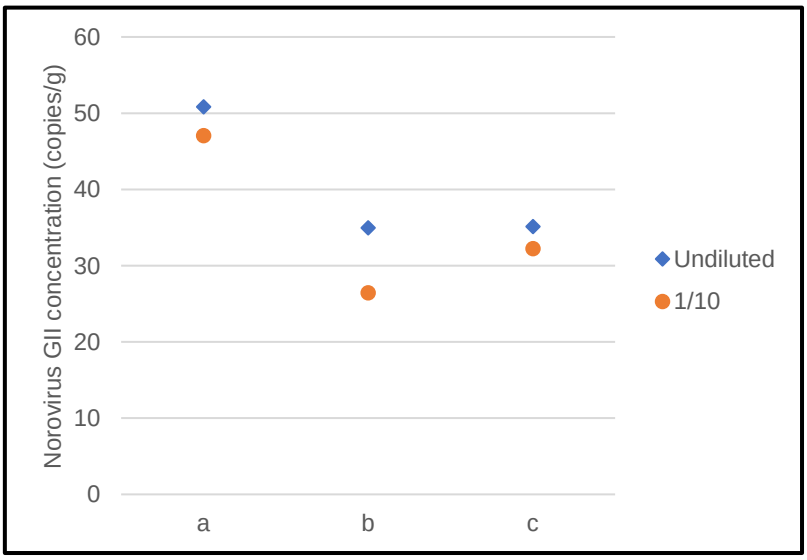

Figure 1; comparison of norovirus levels using undiluted and 1/10 diluted RNA for dried seaweed samples using reduced sample weight of 5g

3.2. Fresh Seaweed

The initial phase of testing of fresh seaweed samples used samples of two species, sea lettuce and kombu kelp, and the ISO 15216-1 method for vegetables. Initial experiments used a method without a specific step to remove PCR inhibitors. This step is included in UK NRL (UK NRL protocol for viruses in soft fruit) and European Union Reference Laboratory protocols for testing soft fruit for viruses (EURL protocol for viruses in soft fruit), but not generally recommended for testing vegetable samples. Results showed significant RT-qPCR inhibition for kombu kelp samples, and some problems with inhibition for sea

lettuce samples (Table 2). It was therefore decided to repeat RT-qPCR analysis after subjecting stored sample RNA to inhibitor removal. This step made some improvements to the QC parameters (samples were less likely to fail the inhibition parameter, and extraction efficiencies were increased in most cases), but for kombu kelp some samples still failed the inhibition parameter when undiluted RNA was tested. In all cases samples passed the inhibition QC parameter when diluted RNA was tested. Across both species, only two samples gave positive results for norovirus GII at very low levels. This was most likely due to the norovirus GII stock used to artificially contaminate the samples being too dilute.

Table 2; results for testing fresh seaweed samples using the ISO 15216-1 method with and without inhibitor removal. For norovirus GII concentration, a dash (-) indicates no detection. For QC parameters, values marked with an asterisk (*) are unacceptable according to ISO 15216-1.

Subsample	With inhibitor removal (y/n)	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
Sea lettuce a	n	-	0.0	59.7	-	1.4	43.6
Sea lettuce b	n	-	70.6	34.9	-	10.5	52.2
Sea lettuce c	n	-	81.9*	54.6	3.4	0.0	67.8
Sea lettuce a	y	-	0.0	54.2	-	0.0	84.0
Sea lettuce b	y	-	0.0	62.7	-	0.0	109.3
Sea lettuce c	y	-	0.0	78.7	-	0.0	118.6
Kombu kelp a	n	-	99.7*	1.7	-	82.7*	18.9
Kombu kelp b	n	-	97.5*	1.7	-	53.6	26.2
Kombu kelp c	n	-	98.4*	2.9	-	42.9	20.8
Kombu kelp a	y	0.4	98.8*	7.4	3.8	14.5	56.8
Kombu kelp b	y	-	95.3*	10.1	-	0.0	51.4
Kombu kelp c	y	-	0.0	13.0	-	2.7	42.2

It was therefore decided to repeat this analysis (including inhibitor removal) using additional sample types and a higher concentration of inoculum. This second phase of testing used samples of three species (egg wrack, dulse, and kombu kelp). The method worked best for kombu kelp samples with all three subsamples passing both QC parameters when tested with both undiluted and diluted RNA (Table 3). Egg wrack and dulse samples were both highly inhibitory when undiluted RNA was tested; all three dulse samples passed the inhibition QC parameter when 1/10 diluted RNA was tested, but only

one out of three egg wrack samples did. Recovery of norovirus GII varied between species and was largely consistent with the QC results. High levels were detected in both undiluted and diluted RNA from kombu kelp samples. For egg wrack and dulse, undiluted RNA provided a mixture of negative results and positive results at low levels, when diluted RNA was tested all samples gave positive results, albeit at levels lower than were detected in kombu kelp sample (Table 3, Figure 2).

Table 3; results for testing fresh seaweed samples using the ISO 15216-1 method with inhibitor removal, with increased levels of norovirus GII stock used for contamination. For norovirus GII concentration, a dash (-) indicates no detection. For QC parameters, values marked with an asterisk (*) are unacceptable according to ISO 15216-1.

Subsample	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
Egg wrack a	0.4	98.8*	0.1*	11.4	97.6*	2.2
Egg wrack b	0.2	99.8*	0.2*	3.5	95.0*	2.2
Egg wrack c	-	98.3*	0.1*	8.5	32.5	1.4
Dulse a	0.7	97.3*	8.4	3.3	11.1	41.6
Dulse b	-	99.8*	1.2	7.3	64.4	48.0
Dulse c	-	97.8*	3.2	6.9	53.0	33.1
Kombu kelp a	30.6	33.6	71.7	43.3	0.0	98.7
Kombu kelp b	17.9	0.0	78.3	45.8	0.0	71.2
Kombu kelp c	25.0	26.8	50.0	36.8	0.0	59.7

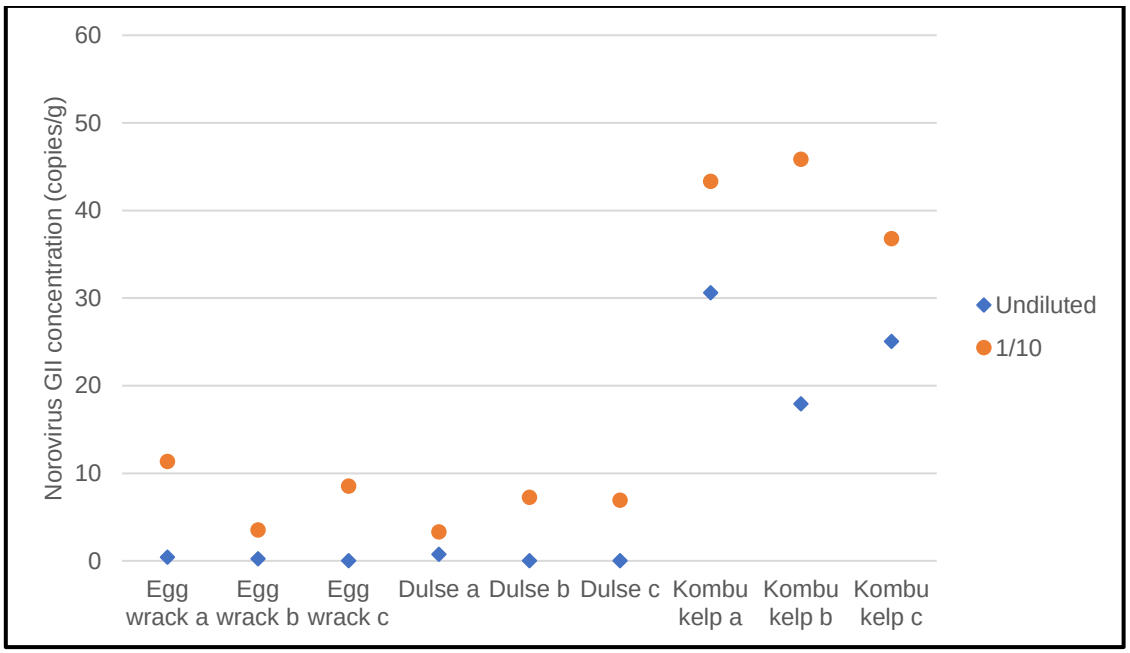

Figure 2; comparison of norovirus levels in undiluted and diluted RNA in samples of 3 species tested with the ISO 15216-1 method

At this stage results to date were reviewed in consultation with the FSA. As RT-qPCR inhibition was noted to some extent in all the fresh seaweed types tested it was decided to investigate additional alternative methods to determine if these might be used in preference.

After a review of the literature, it was decided to investigate two additional methods; a **direct lysis method**, used in a previous investigation of norovirus linked to seaweed consumption (Rajiuddin et al, 2020) and a simple **swabbing method**, based on the ISO 15216-1 method for hard surfaces (International Organization for Standardization, 2017).

Each of the three candidate methods were applied to parallel sets of three subsamples of four different fresh seaweed species (sea lettuce, egg wrack, dulse, kombu kelp). In each case an inhibitor removal step was included prior to analysis. Results divided by seaweed species are shown in Tables 4-7 and Figures 3-6.

Table 4; results for testing fresh sea lettuce samples using three different methods including inhibitor removal. For norovirus GII concentration, a dash (-) indicates no detection. For QC parameters, values marked with an asterisk (*) are unacceptable according to ISO 15216-1.

Sea lettuce; subsample and method	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
a (ISO 15216-1)	2.0	73.8	67.7	-	27.4	78.4
b (ISO 15216-1)	1.9	56.1	68.5	-	33.0	84.2
c (ISO 15216-1)	1.2	77.9*	65.9	-	0.0	62.4
d (direct lysis)	0.4	73.3	0.1*	-	10.1	0.1*
e (direct lysis)	1.3	72.4	2.4	-	7.1	5.8
f (direct lysis)	5.1	75.3*	1.9	4.5	34.7	8.9
g (swabbing)	13.7	3.3	223.7	4.0	0.0	198.6
h (swabbing)	9.5	23.3	252.1	11.6	21.7	226.8
j (swabbing)	15.6	22.3	247.1	15.4	0.0	215.1

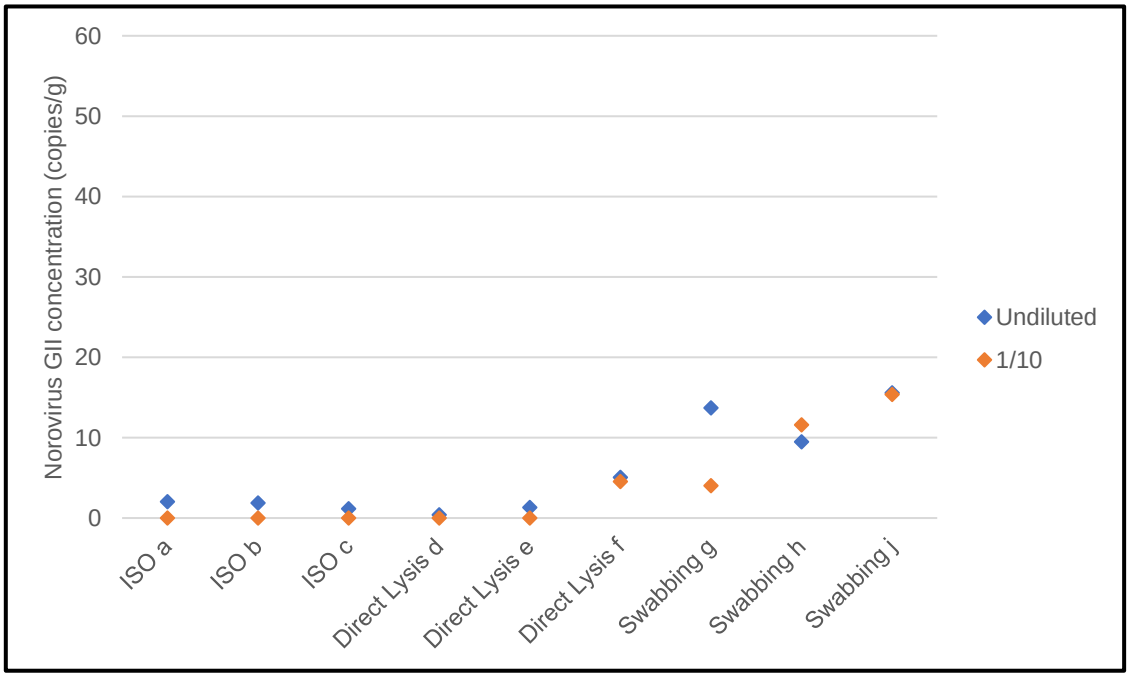

Figure 3; comparison of norovirus levels in undiluted and diluted RNA in sea lettuce samples tested with three methods

Table 5; results for testing fresh egg wrack samples using two different methods including inhibitor removal. For norovirus GII concentration, a dash (-) indicates no detection. For QC parameters, values marked with an asterisk (*) are unacceptable according to ISO 15216-1. Note: testing using a third method (direct lysis) was started but abandoned due to the high viscosity of virus extracts rendering RNA extraction impracticable.

Egg wrack; subsample and method	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
a (ISO 15216-1)	0.7	97.7*	2.1	19.2	62.1	12.6
b (ISO 15216-1)	2.3	98.2*	1.5	6.3	63.9	11.6
c (ISO 15216-1)	1.0	99.5*	0.5*	5.7	61.8	5.7
g (swabbing)	41.7	34.6	151.1	29.2	48.8	252.3
h (swabbing)	31.0	21.2	186.9	39.7	82.1*	193.3
j (swabbing)	19.3	45.3	160.4	25.7	63.0	206.6

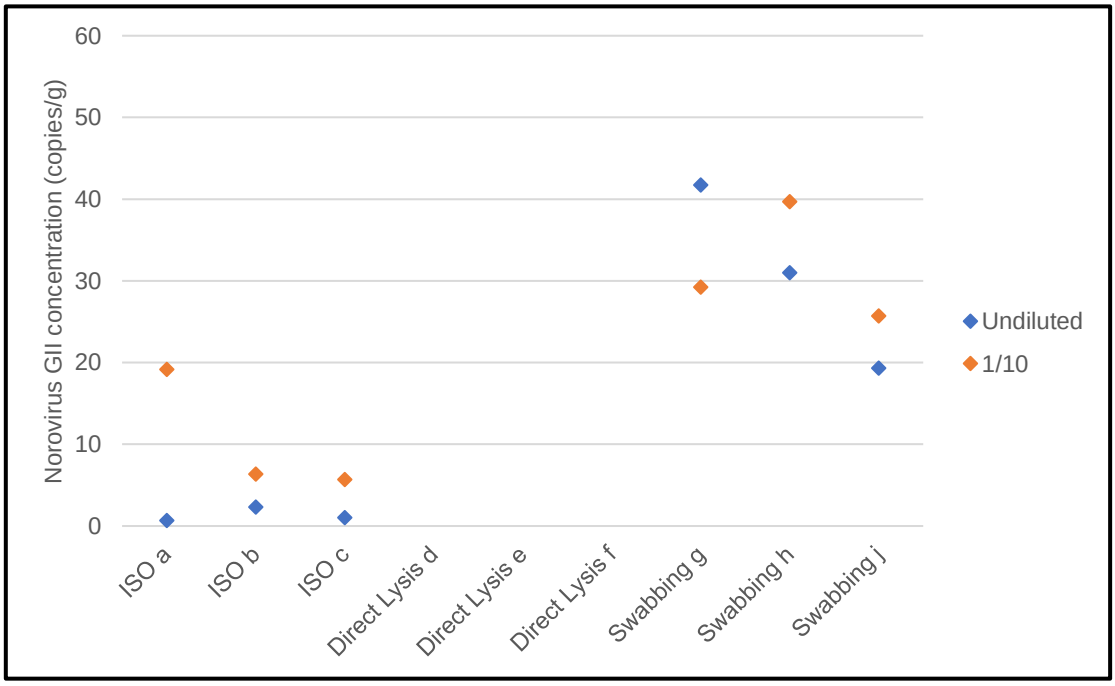

Figure 4; comparison of norovirus levels in undiluted and diluted RNA in egg wrack samples tested with three methods. Note: testing using the direct lysis method was started but abandoned due to the high viscosity of virus extracts rendering RNA extraction impracticable

Table 6; results for testing fresh dulse samples using three different methods including inhibitor removal. For norovirus GII concentration, a dash (-) indicates no detection. For QC parameters, values marked with an asterisk (*) are unacceptable according to ISO 15216-1.

Dulse; subsample and method	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
a (ISO 15216-1)	55.9	11.7	58.0	49.8	37.7	64.1
b (ISO 15216-1)	29.5	53.1	52.4	27.5	50.7	67.1
c (ISO 15216-1)	40.3	58.3	51.1	27.0	0.0	59.2
d (direct lysis)	8.8	24.4	5.0	17.3	0.0	5.7
e (direct lysis)	11.0	2.0	5.9	18.1	0.0	6.7
f (direct lysis)	10.1	11.3	4.5	23.0	3.3	3.6
g (swabbing)	11.6	37.9	72.2	-	31.0	80.3
h (swabbing)	14.5	19.1	69.4	17.3	34.6	75.2
j (swabbing)	5.3	30.1	72.2	17.6	24.8	78.2

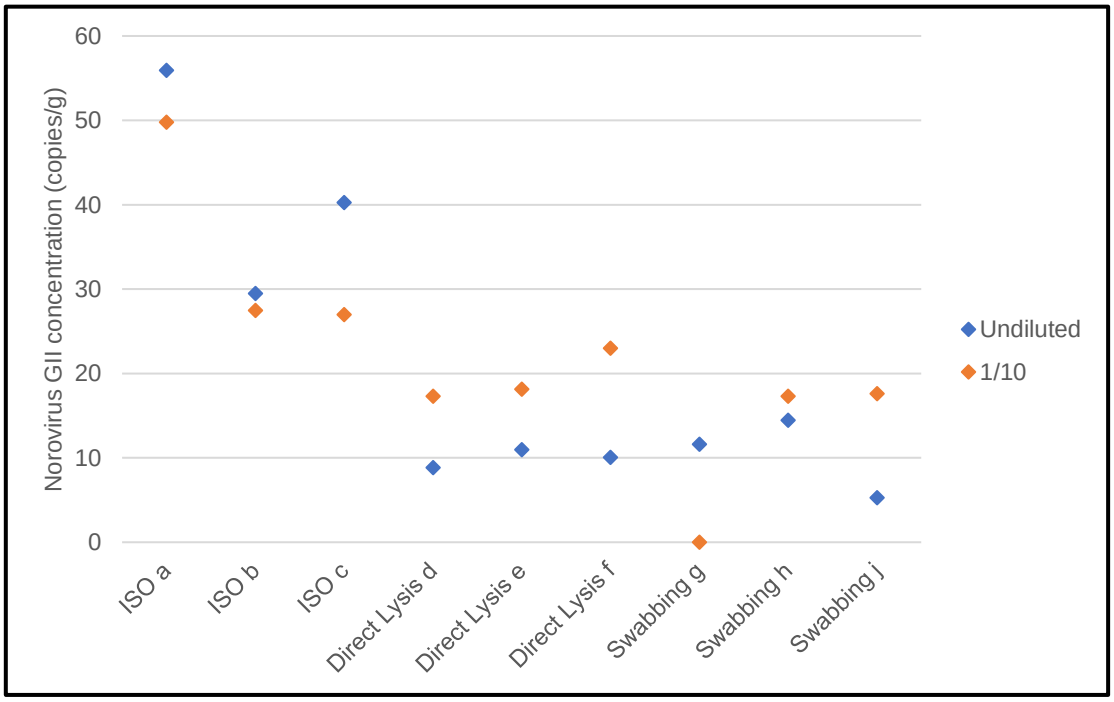

Figure 5; comparison of norovirus levels in undiluted and diluted RNA in dulse samples tested with three methods

Table 7; results for testing fresh kombu kelp samples using three different methods including inhibitor removal. For norovirus GII concentration, a dash (-) indicates no detection. For QC parameters, values marked with an asterisk (*) are unacceptable according to ISO 15216-1.

Kombu kelp; subsample and method	Norovirus GII concentration (copies/g) using undiluted RNA	Norovirus GII inhibition (%) using undiluted RNA	Extraction efficiency (%) using undiluted RNA	Norovirus GII concentration (copies/g) using 1/10 diluted RNA	Norovirus GII inhibition (%) using 1/10 diluted RNA	Extraction efficiency (%) using 1/10 diluted RNA
a (ISO 15216-1)	17.9	85.5*	22.5	54.5	52.1	22.5
b (ISO 15216-1)	12.9	91.4*	17.7	42.6	35.4	24.7
c (ISO 15216-1)	37.8	88.5*	15.3	52.4	64.7	16.2
d (direct lysis)	-	99.7*	0.1*	4.7	79.0*	0.9*
e (direct lysis)	-	>99.9*	<0.1*	-	94.0*	1.3
f (direct lysis)	-	>99.9*	<0.1*	-	93.6*	-
g (swabbing)	10.2	11.8	142.3	8.8	0.0	141.4
h (swabbing)	28.9	83.1*	201.1	53.2	0.0	193.3
j (swabbing)	25.2	68.8	139.5	22.2	47.1	173.8

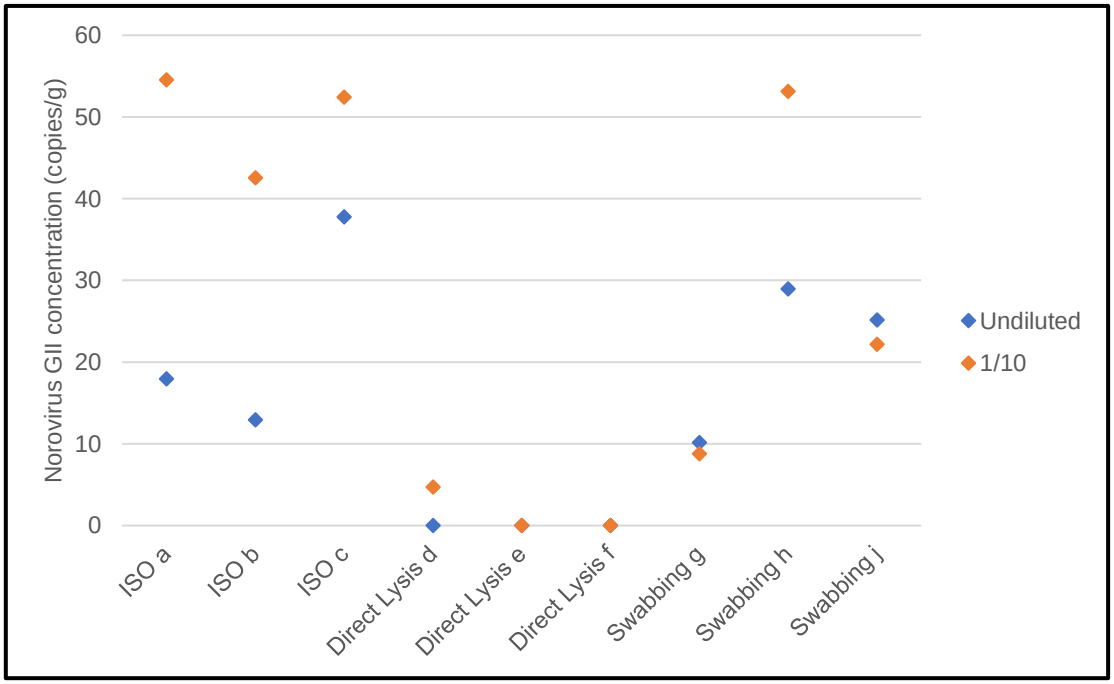

Figure 6; comparison of norovirus levels in undiluted and diluted RNA in kombu kelp samples tested with three methods

Results varied considerably depending on the method and seaweed species tested. The ISO 15216-1 method performed best with dulse samples, with recovery of high levels of norovirus GII, and acceptable QC parameters using both undiluted and diluted RNA. For the other species the method performed less well, with high to very high levels of inhibition noted using undiluted RNA. In all cases, samples gave acceptable QC results when 1/10 diluted RNA was tested, however.

Comparing with the ISO 15216-1 method as a baseline, the alternative direct lysis method gave somewhat similar results for sea lettuce (based on recovery of norovirus GII and the inhibition QC parameter; recovery of the mengo virus process control was markedly reduced), but for the other three seaweed species this method produced much worse results than the ISO 15216-1 method. This was particularly significant for egg wrack samples, where the direct lysis method was completely impracticable due to the high viscosity of the virus extract interfering with the RNA extraction, and for kombu kelp samples, where undiluted sample RNAs were extremely inhibitory, and high inhibition was noted even after RNA dilution.

Results using swabbing were better than with the ISO 15216-1 method for sea lettuce and egg wrack samples, both in terms of levels of norovirus recovered and QC parameters. For dulse samples, QC parameters were similar for the two methods, but recovery of the target GII norovirus was better using the ISO 15216-1 method. For kombu kelp QC parameters were better for samples extracted using the swabbing method, but recovery of norovirus GII was similar with the two methods. Taken together results for dulse and kombu kelp samples indicate that compared with the ISO 15216-1 method, the swabbing method is relatively more effective at recovering the process control virus, than the target norovirus GII (i.e. the ratio of recovery of mengo virus to recovery of norovirus GII is higher for swabbing than for the ISO 15216-1 method).

4. Discussion

The aim of this study was to establish a method for quantification of norovirus in different seaweed products, that could be used in a future outbreak investigation or survey. Due to their very different physical qualities, dried and fresh seaweed products were considered separately in the study. In both cases the starting point for investigations was the ISO 15216-1 method for quantification of norovirus in leaf, stem and bulb vegetables.

For dried seaweed, a problem arose due to absorption of virus elution buffer (40ml) by the sample (25g) when using the standard ISO 15216-1 method. This issue was effectively addressed by reducing the sample weight to 5g. Relative risks of dried and fresh seaweed products are unknown, however the equivalent wet weight of 5g dried nori is likely to be considerably greater than 25g; use of the smaller sample weight should not present a significant risk in terms of false negative results, therefore. Unlike fresh seaweed RT-qPCR inhibition did not appear to be a major issue with these types of samples; all results

were obtained using an inhibitor removal step however (to harmonise the method as much as possible with fresh seaweed).

With fresh seaweed, RT-qPCR inhibition was noted in the earliest experiments and continued as a problem even after the introduction of an inhibitor removal step (used routinely for soft fruit samples but not vegetables). For all four fresh seaweed species, RT-qPCR inhibition above the acceptable threshold (75%) was noted in one or more subsamples when undiluted RNA extracted using the ISO 15216-1 method (with inhibitor removal) was analysed. The extent of inhibition varied markedly between different species, for example all six tested subsamples of egg wrack exceeded the acceptable threshold, while only one out of six sea lettuce subsamples did. There also appeared to be considerable batch-to-batch variation in inhibition within a species. In one experiment, all three dulse subsamples exceeded the threshold while all three kombu kelp subsamples were acceptable; in the following experiment this pattern was reversed.

As recommended in ISO 15216-1, samples were tested in parallel using undiluted and 1/10 diluted RNA. According to the ISO, results obtained using diluted RNA are used where undiluted RNA fails the inhibition QC parameter. In our experiments RNA dilution was effective in reducing inhibition to acceptable levels in most cases; 25 out of 27 fresh seaweed samples provided acceptable inhibition when undiluted RNA extracted using the ISO 15216-1 method was analysed. However, by its nature, RNA dilution of this type reduces the sensitivity of the test as the effective quantity of sample analysed is reduced ten-fold. To reduce possible false negatives, it would be preferable if the need to use results from diluted RNA was minimised.

For this reason, two alternative extraction methods were identified and trialled. The direct lysis method which has previously been recommended for use with seaweed (Rajiuddin et al, 2020) produced results that were in all cases no better than the ISO 15216-1 method, and in most seaweed species were markedly worse. Based on these results this method cannot therefore be recommended by the UK NRL for routine seaweed analysis.

The other method chosen was a simple swabbing procedure based on the ISO 15216-1 method for food surfaces. This swabbing method produced better results than the ISO 15216-1 method for vegetables in terms of recovery of target norovirus GII for two seaweed species (sea lettuce and egg wrack), similar results for one species (kombu kelp), and somewhat worse results for one species (dulse). Quality control parameters (extraction efficiency and RT-qPCR inhibition) were similar or better for the swabbing method compared with the ISO 15216-1 method for vegetables. In addition, the swabbing method is quicker, more straightforward, and requires fewer reagents and consumables than the ISO 15216-1 method for vegetables.

Based solely on the results obtained the swabbing method therefore appeared to have advantages over the ISO 15216-1 method for vegetables, however some other considerations suggested caution. Firstly, by its nature the swabbing method targets only the surface of the sample, whereas the ISO 15216-1 method for vegetables, with its extended period of agitation of the sample in elution buffer, will in theory also allow for

elution of viruses from internal structures of the sample in at least some species. At present there is little evidence as to whether virus particles on environmentally contaminated seaweed samples (such as those implicated in outbreaks) are found only on the surfaces, or are predominantly internalised, however it has been shown that viral contamination of the interior of some types of vegetables can occur (Chancellor et al, 2006; Dicaprio et al, 2012). If this is also the case with seaweed, use of the swabbing method may increase the likelihood of false negative results.

Secondly, we noted that while effective swabbing of the entire sample surface was possible with kombu kelp, which is flat and ribbon-like, it was much more difficult to effectively swab all surfaces of the other seaweed samples which had a more complex structure. The apparent good results with the swabbing method may reflect the fact that it was only possible to effectively contaminate exposed surfaces of the sample, i.e. those surfaces that were also easy to swab. In environmentally contaminated samples, even if contamination occurs at the seaweed surface, it may be found on both accessible and inaccessible areas; in this case again, use of the swabbing method may increase the likelihood of false negative results.

5. Conclusion

For dried seaweed samples use of the ISO 15216-1 method for vegetables with a reduced sample weight of 5g, and an additional step for inhibitor removal appears to be adequate for testing and can be recommended by the UK NRL for routine analysis.

For fresh seaweed samples, results were more complex. The alternative swabbing method appeared to work best for most sample types, but there are significant doubts about its suitability for environmentally contaminated rather than artificially contaminated samples. For this reason, we would recommend use of the ISO 15216-1 method for vegetables with an additional step for inhibitor removal for routine seaweed analysis. This recommendation comes with the caveat that in a significant proportion of samples (depending on the seaweed species), dilution of the sample RNA (with concurrent reduction in test sensitivity) will be required to obtain acceptable QC results, and that in a smaller proportion of samples even this dilution may not produce acceptable QC results.

6. References

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Appendix – Method Protocols

For preparation of reagents, and methods for RNA extraction, removal of PCR inhibitors and RT-qPCR (TaqMan) analysis, refer to the UK NRL generic protocol for quantification of norovirus and hepatitis A virus in soft fruit NRL protocol for viruses in soft fruit (cefas.co.uk).

Protocol for artificial contamination of dried and fresh seaweed samples with norovirus GII

- Prepare sample(s) of 5 g of dried or 25g of fresh seaweed.
- For ISO 15216-1 and direct lysis methods, chop each sample into pieces of approximately 2.5 x 2.5 cm.
- Pipette 50 µl of diluted norovirus GII stock (prepared by dilution of faecal material in PBS) onto the surface of each sample in “spots” of 10 µl and leave to air dry/adsorb to the surface at room temperature for 20 min.

Protocol for dried or fresh seaweed analysis using the ISO 15216-1 method for vegetables, with inhibitor removal

- Pool together sufficient aliquots of mengo virus process control material for use with all samples under test (allow 10 µl per sample plus 25 µl excess).
- Retain a 20 µl subsample of pooled mengo virus process control material for RNA extraction and preparation of the standard curve. Store at −15 °C.
- Transfer each chopped dried or fresh seaweed sample (artificially contaminated with norovirus GII as described above) to a mesh filter bag inside a 400 ml beaker. Add 40 ml TGBE buffer and 10 µl of mengo virus process control virus material to each sample.
- Incubate at room temperature with constant rocking at approximately 60 oscillations min−1 for 20 min. Decant the eluate from the filtered compartment into a centrifuge tube.
- Clarify by centrifugation at 10,000 x g for 30 min at 5 ºC. Decant the supernatant into a clean tube and adjust the pH to 7.0 ± 0.5 with HCl (5 M).
- Measure the volume of the sample and add 0.25 volumes of 5 x PEG/NaCl solution (to produce a final concentration of 10 % PEG, 0.3 M NaCl), homogenise by shaking for 60 s then incubate with constant rocking at around 60 oscillations min−1 at 5 ºC for 60 min.
- Centrifuge at 10,000 x g for 30 min at 5 ºC (split volume across two centrifuge tubes if necessary).
- Decant and discard the supernatant, then centrifuge at 10,000 x g for 5 min at 5 ºC to compact pellet.

- Discard the supernatant and resuspend pellet in 500 µl PBS. If a single sample has been split across two tubes resuspend both pellets stepwise in the same aliquot of PBS.
- Process the suspension (virus extract) immediately, or store at 4°C for up to 48 h or at −15 °C or below for longer periods.
- Subject the entire virus extract to RNA extraction using the miniMAG equipment, including removal of PCR inhibitors, according to sections 7.2 and 7.3 of the UK NRL generic protocol. Separately carry out a process control reference RNA extraction (500 µl water and 10 µl retained mengo virus process control material) following the same method.
- Analyse sample RNA for norovirus GII (including assessment of inhibition using EC RNA) and mengo virus according to sections 7.4 to 7.8 of the UK NRL generic protocol.

Protocol for fresh seaweed analysis using the direct lysis method, with inhibitor removal

- Pool together sufficient aliquots of mengo virus process control material for use with all samples under test (allow 10 µl per sample plus 25 µl excess).
- Retain a 20 µl subsample of pooled mengo virus process control material for RNA extraction and preparation of the standard curve. Store at −15 °C.
- Transfer each chopped fresh seaweed sample (artificially contaminated with norovirus GII as described above) to a suitable tube or bottle and add 10 ml NucliSens lysis buffer, 10 µl mengo virus process control virus material, 1 ml pectinase from *Aspergillus aculeatus* (>3800 units) and 400 µl Plant RNA Isolation Aid (Invitrogen).
- Shake at 200 oscillations min−1 at room temperature for 10 min.
- Transfer liquid to a clean centrifuge tube by pipetting, then clarify by centrifugation at 10,000 x g for 10 min at room temperature.
- Transfer supernatant to a clean centrifuge tube and add 140 µl well-mixed NucliSens magnetic silica solution. Mix by vortexing briefly.
- Incubate for 10 min at room temperature, centrifuge for 2 min at 1,500 x g then carefully discard supernatant by e.g. aspiration.
- Add 400 µl NucliSens wash buffer 1 and resuspend the pellet by pipetting/vortexing.
- Subject the resuspended pellet to RNA extraction using the miniMAG equipment, including removal of PCR inhibitors, according to sections 7.2.1 and 7.3 of the UK NRL generic protocol.

  Note: with this method, initial RNA extraction steps as described in section 7.2 of the UK NRL generic protocol are modified as described above.

- Separately carry out a process control reference RNA extraction (500 µl water and 10 µl retained mengo virus process control material) according to sections 7.2 and 7.3 of the UK NRL generic protocol.

- Analyse sample RNA for norovirus GII (including assessment of inhibition using EC RNA) and mengo virus according to sections 7.4 to 7.8 of the UK NRL generic protocol.

Protocol for fresh seaweed analysis using the swabbing method, with inhibitor removal

- Pool together sufficient aliquots of mengo virus process control material for use with all samples under test (allow 10 µl per sample plus 25 µl excess).
- Retain a 20 µl subsample of pooled mengo virus process control material for RNA extraction and preparation of the standard curve. Store at −15 °C.
- Using a sterile cotton swab pre-moistened in PBS, swab the entire surface of each fresh seaweed sample (artificially contaminated with norovirus GII as described above), applying a little pressure to detach virus particles.
- Add 10 µl of mengo virus process control material to the swab. Immediately after the addition of process control virus material, immerse the swab in a tube containing 490 µl lysis buffer, then press against the side of the tube to release liquid. Repeat the immersion and pressing cycle three or four times to ensure maximum yield of virus.
- Incubate tube at room temperature for 10 min, then add 50 µl well-mixed NucliSens magnetic silica solution. Mix by vortexing briefly.
- Incubate for 10 min at room temperature, centrifuge for 2 min at 1,500 x g then carefully discard supernatant by e.g. aspiration.
- Add 400 µl NucliSens wash buffer 1 and resuspend the pellet by pipetting/vortexing.
- Subject the resuspended pellet to RNA extraction using the miniMAG equipment, including removal of PCR inhibitors, according to sections 7.2.1 and 7.3 of the UK NRL generic protocol.

  Note: with this method, initial RNA extraction steps as described in section 7.2 of the UK NRL generic protocol are modified as described above.

- Separately carry out a process control reference RNA extraction (500 µl water and 10 µl retained mengo virus process control material) according to sections 7.2 and 7.3 of the UK NRL generic protocol.
- Analyse sample RNA for norovirus GII (including assessment of inhibition using EC RNA) and mengo virus according to sections 7.4 to 7.8 of the UK NRL generic protocol.

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