PROGRESS TOWARDS A SIMPLIFIED METHOD FOR DETECTION OF ENTERIC VIRAL PATHOGENS IN SHELLFISH BY RT-PCR

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Introduction:

Contamination of bivalve molluscan shellfish with human enteric viruses, such as Norwalk-like virus (NLV), is well documented as a major cause of food borne gastroenteritis. The detection of such viral pathogens in shellfish by molecular techniques is hindered by the presence of potent RT-PCR inhibitors. Most published methods employ complex sample processing procedures to remove such amplification inhibitors. Such procedures are generally expensive, time consuming and may lack robustness. This study describes progress towards the development of a simplified method for shellfish processing prior to RT-PCR. Several previous studies have demonstrated high levels of virus bioaccumulation in the digestive gland tissues of shellfish. Our approach was based on the utilisation of this tissue to maximise viral recovery and minimise input loads of shellfish tissue. However unlike previous studies, we targeted extraction of digestive gland contents, rather than utilising a whole gland homogenate. Digestive glands were dissected, coarsely chopped and the contents eluted in buffered peptone. This extract proved suitable for application of standard nucleic acid extraction protocols without further complex purification procedures. The cut digestive gland extraction procedure was evaluated by comparison with a previously published whole animal method. Results are discussed in the context of the development of a robust, simple method for detection of enteric viruses in shellfish using RT-PCR.

Method:

Sample selection: Shellfish were obtained from harvesting areas subject to varying degrees of pollution and virus extraction was performed using both the whole animal and digestive gland methods.

Virus extraction/purification and viral nucleic acid extraction: Processing of shellfish samples and virus extraction using the whole animal method was performed as previously described (Lees *et al*., 1994). Virus extraction using the "cut digestive gland" method was performed as follows: Shellfish were carefully opened ensuring flesh was kept intact. The peripheral flesh was cut away to reveal the digestive gland. The digestive gland was excised onto a petri dish and finely chopped with a razor blade, releasing contents. An equivalent volume of peptone pH 6.3 was added to the dissected material, vigorously mixed and agitated for 1 h. The homogenate was centrifuged at 1000*xg*/10 min, and the supernatant was removed and stored at -20°C until required for extraction of viral RNA. Viral nucleic acid was extracted from the products of both extraction methods as previously described (Lees *et al*., 1994).

Nested Reverse Transcription-PCR: Amplification of NLV target nucleic acid was performed using a nested RT-PCR procedure as previously described (Green *et al*., 1998).

Assessment of sample inhibition: Evaluation of RT-PCR inhibitors present within shellfish samples was achieved by the quantitative measurement of amplification depression using real time PCR against a common 18S ribosomal RNA control. Real time PCR was performed using the TaqMan GeneAmp 5700 sequence detection system (PE Biosystems) in combination with the TaqMan Ribosomal RNA control reagent kit (PE Biosystems). All procedures were performed in accordance with manufacturers guidelines.

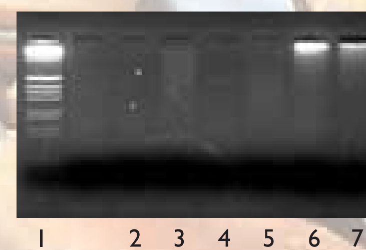
Results:

For comparison of the whole animal vs. cut digestive gland methods shellfish were obtained from sites displaying a range of faecal pollution, as judged by the *E. coli* content in shellfish and analysed for NLVs by both methods. The results (Table 1) suggest that virus recovery was more effective using the digestive gland method, compared with the whole animal method. Gel results are illustrated for two samples in Figure 1.

Table 1. Detection of NLV in shellfish using RT-PCR. Comparison of whole animal vs. cut digestive gland virus extraction.

Sample	Microbiological Result *E. coli*	PCR Result Whole animal	PCR Result Digestive gland
96/126	<20	-	-
96/246	>20	-	-
96/26	20	+	+
97/455	430	-	+
96/74	750	+	+
96/104	1300	+	+
98/4	1400	-	+
96/228	2400	-	+
96/264	2400	-	+
96/212	3500	-	-
96/247	5000	-	+
96/11	9100	+	+
96/211	18000	-	-
97/435	>18000	+	-
Percentage positive		35.7	64.3

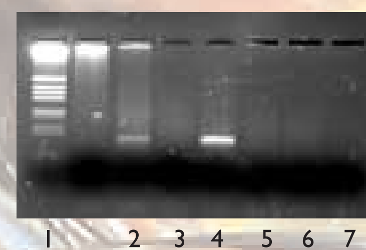
Whole Animal procedure



1 2 3 4 5 6 7

1. 1 Kb molecular marker
2. Neat 96/247 + 10μl glassmilk
3. 1:3 96/247 + 10μl glassmilk
4. Neat 96/264 + 10μl glassmilk
5. 1:3 96/264 + 10μl glassmilk
6. Neat PBSa + 10μl glassmilk (-ve)
7. Neat PBSa + 10μl glassmilk (-ve)

Cut digestive gland procedure



1 2 3 4 5 6 7

1. 1 Kb molecular marker
2. Neat 96/247 + 10μl glassmilk
3. 1:3 96/247 + 10μl glassmilk
4. Neat 96/264 + 10μl glassmilk
5. 1:3 96/264 + 10μl glassmilk
6. Neat PBSa + 10μl glassmilk (-ve)
7. Neat PBSa + 10μl glassmilk (-ve)

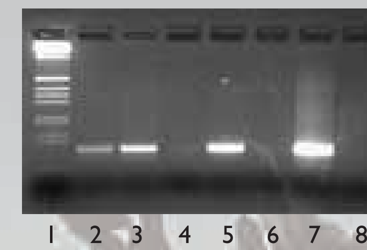
Figure 1 Gel electrophoresis of NLV RT-PCR amplicons from whole animal and cut digestive gland extraction methods.

Table 1 shows that this enhanced sensitivity was not effected by the level of contamination of the sample. The enhanced sensitivity of the digestive gland method suggested either more effective removal of amplification inhibitors or a greater efficiency of target recovery from the shellfish material. However, despite improved sensitivity compared with the whole animal method, problems were experienced in attempting to optimise nucleic acid recovery protocols, suggesting the possible presence of residual amplification inhibitors. This data is shown in Table 2 which shows dilutions of shellfish material (neat and 1:3 dilution in PBS) used in combination with a range of volumes of silica capture matrix.

Table 2 Detection of NLV by RT-PCR using various concentrations of silica capture matrix.

Sample	Harvesting Area	Volume of silica matrix 20μl Neat[1]	20μl 1:3[2]	10μl Neat	10μl 1:3	5μl Neat	5μl 1:3
99/174	Cat A	+	-	-	-	-	+
99/62	Cat A	-	+	+	-	-	-
99/622	Cat A/B	-	+	+	-	-	+
97/435	Cat B	+	+	-	-	-	-
97/455	Cat B	-	+	+	+	+	-
98/4	Cat B	-	+	-	+	+	+
99/632	Cat B	-	-	-	+	+	-
98/48	Prohibited	-	+	-	-	-	-
98/67	Prohibited	+	+	+	+	+	+
99/309	Prohibited	-	+	-	+	-	+
Percentage Positive		30	80	40	50	40	50

[1] Neat sample; [2] 1:3 dilution in PBS

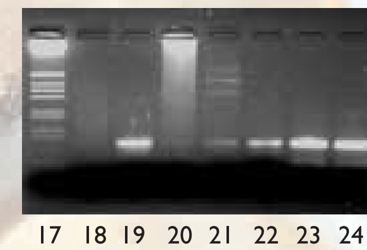


1 2 3 4 5 6 7 8

1. 1Kb molecular marker
2. Neat 97/435 + 20μl glassmilk
3. 1:3 97/435 + 20μl glassmilk
4. Neat 97/455 + 20μl glassmilk
5. 1:3 97/455 + 20μl glassmilk
6. Neat 97/4 + 20μl glassmilk
7. 1:3 97/4 + 20μl glassmilk
8. Negative control

9 10 11 12 13 14 15 16

9. 1Kb molecular marker
10. Neat 97/435 + 10μl glassmilk
11. 1:3 97/435 + 10μl glassmilk
12. Neat 97/455 + 10μl glassmilk
13. 1:3 97/455 + 10μl glassmilk
14. Neat 97/4 + 10μl glassmilk
15. 1:3 97/4 + 10μl glassmilk
16. Neat 97/435 + 5μl glassmilk



17 18 19 20 21 22 23 24

17. 1Kb molecular marker
18. 1:3 97/435 + 5μl glassmilk
19. Neat 97/455 + 5μl glassmilk
20. 1:3 97/455 + 5μl glassmilk
21. Neat 97/4 + 5μl glassmilk
22. 1:3 97/4 + 5μl glassmilk
23. Positive control
24. Positive control

Figure 2 Gel electrophoresis of NLV RT-PCR amplicons using various concentrations of silica capture matrix.

The Gel electrophoresis results for three samples are illustrated in Figure 2. The results show that samples gave various patterns with no obvious optimum concentration of capture matrix for maximum viral recovery. Furthermore, the degree of pollution of the sample appeared to play no obvious role in this variability.

To investigate this variability further and to facilitate final optimisation, an assay was developed for the quantitation of RT-PCR amplification inhibition. This relied on quantitative measurement of amplification depression using real time PCR (TaqMan®). The TaqMan assay relies upon the 5'→3' exonuclease cleavage by *Taq* polymerase of a fluroscently labelled probe incorporated into the PCR reaction mix. Probe cleavage is directly proportional to template concentration and is recorded in real time by the GeneAmp 5700 Sequence detection system (PE Biosystems). Threshold levels for exponential amplification of the PCR target (CT values) are recorded for quantitation purposes. Lower CT values denote higher initial template concentrations.

The level of amplification depression displayed by the shellfish samples was assessed against a common 18S ribosomal RNA control using the quantitative TaqMan assay. Serial dilutions of post-reverse transcription shellfish cDNA were prepared and amplified using a primer and probe set designed against the eukaryotic 18S rRNA gene. By comparing a plot of cDNA dilution against CT value for each combination of sample dilution/silica concentration, it was possible to assess 18S rRNA amplification depression in environmental shellfish samples. The results obtained are shown in figures 3 (a-d), with the CT values of the neat samples summarised in Table 3.

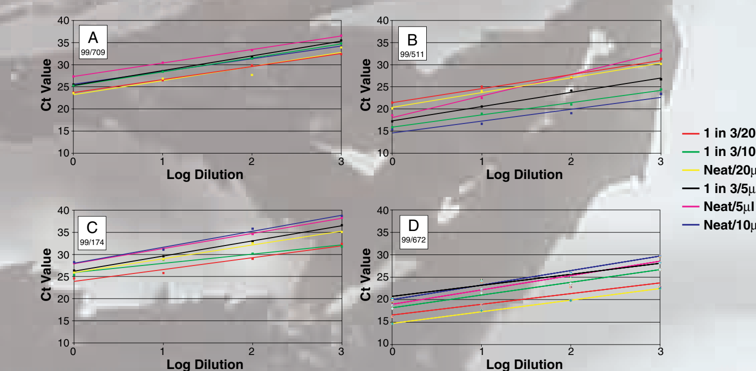


Figure 3 Assessment of 18S rRNA amplification depression in environmental shellfish samples using various concentrations of silica matrix/sample dilution: a. sample 99/709; b. sample 99/511; c. sample 99/174; d. sample 99/672.

Table 3 Detection of 18S rRNA using various concentrations of silica capture matrix quantified using real time PCR (TaqMan).

Sample	Volume of silica matrix 20μl Neat[1]	20μl 1:3[2]	10μl Neat	10μl 1:3	5μl Neat	5μl 1:3
99/174	14.76*	16.61	20.06	18.22	19.00	20.76
99/511	20.38	21.45	14.61	15.89	18.08	17.32
99/672	25.92	23.94	28.05	25.92	27.91	26.25
99/709	23.22	23.70	25.61	25.22	27.33	25.75

*CT Value: The Threshold cycle (CT) value is recorded as the cycle at which the exponential amplification of the PCR target is detected with the use of the GeneAmp 5700 Sequence detection system; [1] Neat sample; [2] 1:3 dilution in PBS.

The results suggest that the virus and nucleic acid extraction procedures employed were successful in removing PCR inhibitors present in the shellfish matrix, as no evidence was seen of PCR amplification depression in any of the samples. These results suggest that the inconsistency of the extraction method is not due to the presence of PCR inhibitors, but to variation in the recovery of viral nucleic acid. The 18S rRNA TaqMan assay will be further utilised to investigate approaches for further assay standardisation and optimisation.

Discussion:

This study describes the development of a simplified method for the detection of Norwalk-like virus in shellfish by RT-PCR. This simplified method using cut shellfish digestive gland was compared with a previously published whole animal assay. The results showed that the cut digestive gland method was more effective for the extraction and detection of NLV nucleic acids in shellfish. The increased level of sensitivity observed with this method suggested either a greater efficiency of virus recovery or better removal of the potent PCR inhibitors present in shellfish matrix. However, attempts to optimise the assay demonstrated inconsistencies in the optimal concentration of silica capture matrix for virus recovery.

An 18S rRNA assay was developed to investigate sample recovery and RT-PCR inhibition. This showed that assay inconsistency was not a result of the presence of residual amplification inhibitors. The 18S rRNA assay will be further utilised to optimise assay design for maximum nucleic acid template recovery.

In conclusion, the developed cut digestive gland method was successful in removal of sample-related inhibition and more sensitive for NLV detection than a previously published whole animal assay. However, further work is required for the optimsation of the procedure, particularly with regard to standardisation of the silica capture matrix protocol.

Acknowledgements

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References

Green, J., Henshilwood, K., Gallimore, C.I., Brown, D.W.G. and Lees, D.N. (1998) Application of a nested Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) assay for the detection of Small Round Structured Viruses (SRSVs) in environmentally contaminated molluscan shellfish. Appl.Environ.Microbiol. **64**, 858-867.

Lees, D.N., Henshilwood, K. and Dore, W.J. 1994. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. Appl.Environ.Microbiol. **60**, 2999-3005.

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