THE DEVELOPMENT OF QUANTITATIVE MOLECULAR ASSAYS FOR DETERMINATION OF ENTERIC VIRAL LOAD WITHIN ENVIRONMENTAL SAMPLES

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Introduction

Human enteric viruses are frequently present in sewage effluents. Following marine discharge these viruses may be concentrated and retained by filter-feeding bivalve shellfish. Outbreaks of gastroenteritis following the consumption of shellfish contaminated with the enteric Norwalk-like viruses (NLV's) are well documented. Standards for the receiving waters, which control discharge of sewage into the marine environment, are based on bacterial faecal indicator levels. However, previous studies have shown that inactivation rates of viruses may not necessarily comply with those of faecal indicator bacteria. Limited data is available on the survival characteristics of the cultivable enteric viruses such as hepatitis A virus and enterovirus in the marine environment. However, it cannot be assumed that behaviour amongst other human enteric viruses, such as the NLV's will be similar. Currently no information is available regarding the survival characteristics of NLV's within sewage treatment processes or after discharge into the marine environment. Unlike other enteric viruses, such as the enteroviruses it is not possible to quantitate NLV's by traditional tissue culture methods. Current detection methods for these viruses therefore rely on non-quantitative electron microscopy or RT-PCR. The absence of quantitative methods severely hampers studies on NLV survival characteristics. This poster describes the development of real-time fluorogenic 5' nuclease assays for the quantitative detection of enteric viruses in sewage effluent and other environmental samples as a first stage towards carrying out such viral survival studies.

Materials and Methods

Virus stocks: Enteric adenovirus, enterovirus (poliovirus) and NLV were used in initial work to develop molecular quantitation. Adenovirus type 41 was kindly supplied by A. Allard, Sweden. Poliovirus type I obtained from the American Type Culture Collection was grown and titrated in BGM cells as previously described (Lees *et al.*, 1994). NLV, Grimsby strain was prepared from clinical faecal material as a 10% extract in phosphate buffered saline (PBSa)

Purification and extraction of viral RNA: NLV, adenovirus and poliovirus RNA was extracted by a modification of the Boom method as previously described (Lees *et al.*, 1994).

RT-PCR: A nested RT-PCR was used for adenovirus and NLV as previously described for NLV (Green *et al.*, 1998). Primer sequences for adenovirus were as described by Allard *et al.*, 1992. cDNA for the fluorogenic 5' nuclease assay was prepared by reverse transcription of extracted RNA using random primers as previously described (Green *et al.*, 1998).

Fluorogenic 5' nuclease assay: Primer-probe sets for adenovirus, poliovirus and NLV were designed using Primer Express™ software (PE Biosystems). Inhibition levels were analysed through the utilisation of an 18S Ribosomal RNA kit (PE Biosystems).

PCR reaction mix contained 12.5µl TaqMan Universal PCR master mix (PE Biosystems) primer and probe concentrations optimised according to the manufacture's instructions (PE Biosystems), and nuclease free water to a final volume of 23µl. The reaction mix was added to 2µl of target cDNA/DNA and amplified at 50°C for 2 seconds, 95°C for 10 seconds, and then 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Quantitative results were derived from a threshold cycle value (CT value). This value is the cycle number at which exponential amplification commences within a given PCR reaction.

Sewage processing, purification and extraction of viral RNA: Samples of crude sewage effluent were received from Dorchester sewage treatment works and frozen at -20°C until analysis. Methods were evaluated for optimum virus recovery and RT-PCR inhibitor removal from sewage effluents. There are a number of published methods for extraction of sewage and other environmental samples prior to PCR. These include methods by Manuala *et al.*, 1999 utilising phenol/chloroform extraction, Tsai *et al.*, 1993 utilising membrane filtration followed by chloroform -isoamyl alcohol purification, Girones *et al.*, 1993 utilising ultracentrifugation followed by elution in glycine at high pH and extraction using a silica capture matrix. A modification of the Girones *et al.*, 1993 method was used in initial studies evaluating methods for use with the fluorogenic 5' nuclease assay. Method B was a minor modification to the published method, Method A omitted the glycine extraction stage with the ultracentrafugation pellet resuspended in PBSa and directly extracted using silica capture matrix. Further methods will be evaluated in future work.

Amplification inhibition: PCR amplification inhibition by sample matrix components was measured using depression of 18S ribosomal RNA amplification as a marker of PCR reaction efficiency. 18S ribosomal RNA was assayed and quantified by the fluorogenic 5' nuclease assay using a kit according to manufacturer's guidelines (PE Biosystems).

Results

Fluorogenic 5' Nuclease Assay:
The Fluorogenic 5' nuclease assay exploits the 5'-3' exonuclease activity of *Taq* polymerase, which during PCR primer extension results in cleavage of a fluorescently labelled probe, incorporated into the PCR reaction mix. Fluorescence emission by the cleaved probe is directly proportional to the amount of target template in the sample, and is measured in real-time by the 5700 Sequence Detection System (PE Biosystems). The Threshold Cycle (CT) value at which exponential amplification commences is proportional to the amount of initial template target, with higher initial template amounts giving a lower CT value. Each viral primer and probe set was assessed for specificity and sensitivity by extracting and amplifying a titration series in PBSa. Figure 1 shows the fluorogenic 5' nuclease assay results for each virus dilution with a fitted linear regression line for each virus. Neat poliovirus 1 and adenovirus 41 samples contained 9.3 x 10^4 pfu and 3.67 x 10^4 pfu virus respectively. Culture quantitation data is not available for the non-cultivable NLV Grimsby strain.

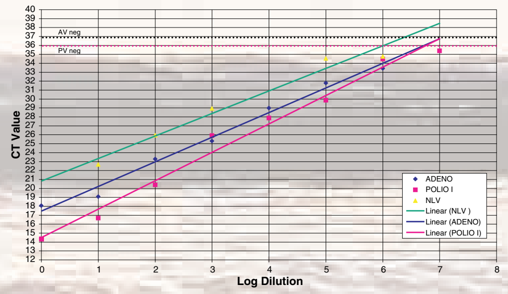

Figure 1 fluorogenic 5' nuclease assay curves for log dilution series of NLV Grimsby strain, adenovirus 41 and poliovirus type 1. Linear regression R^2 values were 0.98 for poliovirus, 0.99 for adenovirus and 0.97 for NLV Grimsby strain.

As expected CT values for each varied in accordance to the amount of viral initial template present with poliovirus commencing amplification approximately 4 cycles earlier than adenovirus in line with initial concentrations. NLV Grimsby strain was detected substantially later than the other viruses indicating lower titres of virus in the clinical material. Linear regression analysis demonstrated high correlation coefficient values (>0.97) for all virus titration series indicating a good fit with consistent assay performance. In comparison with culture assays, the fluorogenic 5' nuclease assay detected poliovirus to an approximate titre of 0.1 pfu and adenovirus to an approximate titre of 0.01 pfu. These results indicate high assay sensitivity compared to conventional tissue culture techniques possibly reflecting detection of non-infectious viral template.

Sensitivity of fluorogenic 5' nuclease assay:
The sensitivity of the fluorogenic 5' nuclease assay was compared against conventional nested RT-PCR for adenovirus and NLV Grimsby strain. Figure 2 shows fluorogenic 5' nuclease assay DNA amplification plots of serial dilutions of adenovirus in PBSa compared with conventional nested PCR against the same extracts. The results show that the fluorogenic 5' nuclease assay was up to an order of magnitude more sensitive than conventional nested PCR as judged by visualisation of amplification products in ethidium bromide stained gels. A similar sensitivity comparison was performed for the NLV Grimsby strain. The results shown in figure 3 suggest that the fluorogenic 5' nuclease assay was similar to or more sensitive than, conventional nested RT-PCR for detection of NLV in a 10% faecal extract sample.

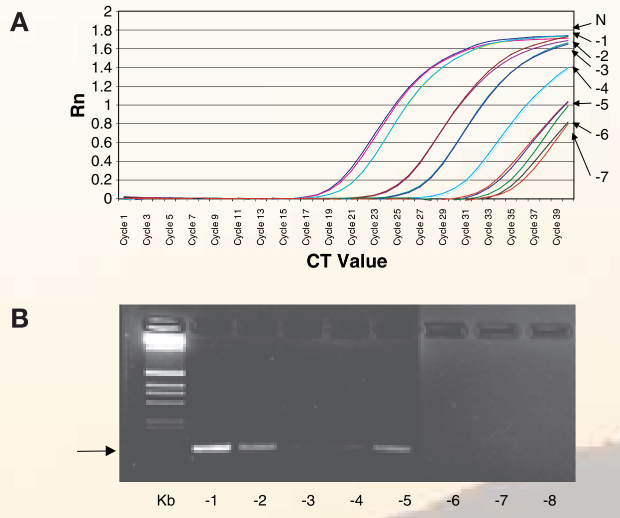

Figure 2 Comparison of fluorogenic 5' nuclease assay sensitivity compared with nested PCR for the detection of adenovirus. Kb denotes 1 kb molecular weight ladder. -1 to -7 indicate serial dilutions of adenovirus assayed by A- the fluorogenic 5' nuclease assay and B- conventional nested PCR. Arrow denotes correct size amplicon (166bp)

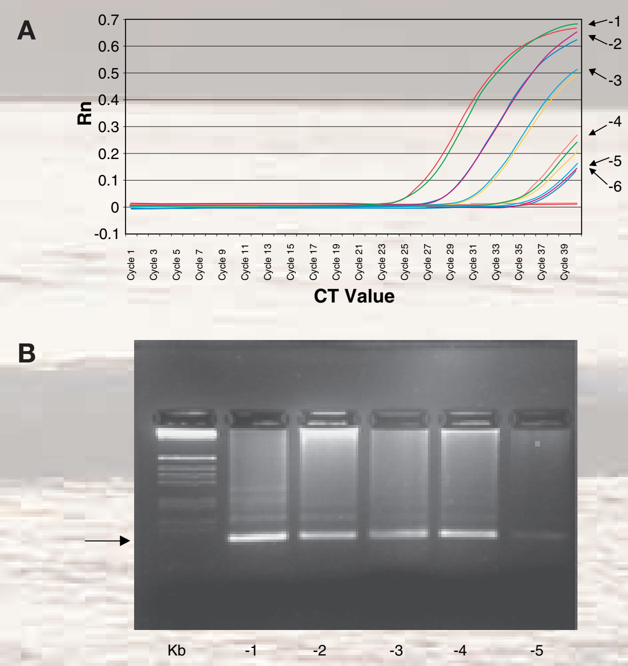

Figure 3 Comparison of fluorogenic 5' nuclease assay sensitivity compared with nested RT-PCR for the detection of NLV's. Kb denotes 1 kb molecular weight ladder. -1 to -5 indicate serial dilutions of NLV Grimsby strain assayed by A- the fluorogenic 5' nuclease assay and B- conventional nested RT-PCR. Arrow denotes correct size amplicon (114bp)

Application of fluorogenic 5' nuclease assay to sewage samples:
Environmental samples such as sewage are known to contain potent PCR amplification inhibitors. Prior to application of the fluorogenic 5' nuclease assay to quantitation of virus in such samples, it is necessary to establish suitable processing methods for removal of amplification inhibitors. Residual amplification inhibitors would depress amplification efficiency and thus invalidate quantitative PCR results. To facilitate evaluation of extraction methods a procedure was established for measurement of PCR amplification inhibitors in a sample matrix. The assay relied on quantitative measurement of the efficiency of amplification of a common 18S ribosomal RNA target using the fluorogenic 5' nuclease assay. The total RNA from a 40ml crude sewage effluent sample was extracted and purified by the extraction methods under evaluation and cDNA produced by reverse transcription using random primers as described. The cDNA samples were then serially diluted 1:5 with PBSa to a dilution of approximately 1:16000 and 18S ribosomal RNA measured by the fluorogenic 5' nuclease assay according to manufactures instructions. CT values for each dilution were plotted and evaluated for fit against an ideal straight line (no amplification inhibitors present).

Figure 4 shows a plot for a sample extracted according to method A and a plot for the same sample extracted according to method B. It can be clearly seen that method A failed to completely remove amplification inhibitors whereas method B produced a sample with no obvious inhibitors present. The depression of amplification from the ideal straight line can be measured and used as a quantitative index of assay performance for removal of inhibitors. Furthermore, the total amount of 18S ribosomal RNA recovered is a measure of assay recovery efficiency, which should reflect assay sensitivity for viruses. It can be seen that method A recovered approximately 2 orders of magnitude more total RNA than method B which may explain the higher levels of inhibition observed. These results suggest that neither method is yet fully optimised for both maximum RNA recovery and amplification inhibitor removal. Further work will apply the 18S ribosomal RNA assay to evaluation and optimisation of sample processing methods prior to initiation of work on detection of enteric viruses within effluent samples.

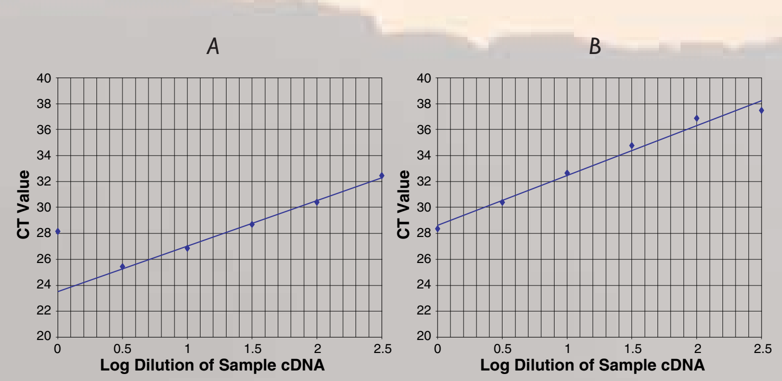

Figure 4 Measurement of PCR amplification inhibition by sample matrix components using the 18S ribosomal RNA assay. Results are shown for a sewage sample processed by method A (inhibitory) and by method B (non-inhibitory)

Discussion

Initial experiments utilising cultured stocks of adenovirus 41 and poliovirus 1 and NLV Grimsby strain faecal material indicated that the fluorogenic 5' nuclease assay produced specific, reproducible and quantifiable results. It should however be noted that the high sensitivity and specificity required of the fluorogenic 5' nuclease assay, coupled with the high sequence diversity amongst the NLV group, makes synthesising a primer and probe set to detect more than one NLV strain problematical. Grimsby NLV strain was chosen for primer and probe design as a common strain in circulation within the community. The fluorogenic 5' nuclease assay was shown to be as, or more sensitive than conventional nested PCR for detection of both adenovirus and NLV Grimsby strain.

This study also reports for the first time the development of a method to quantify levels of PCR inhibitors and its application to effluent samples. This approach will be utilised for further work on the optimisation of sample extraction procedure for both maximal amplification inhibitor removal and efficiency of RNA recovery.

Further work will focus on the application of the fluorogenic 5' nuclease assay to the quantitation of enteric viral load within untreated and treated sewage effluents, and the study of the efficiency of viral removal/inactivation within sewage treatment processes.

Acknowledgements

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