Improving Methodologies Used for the Enumeration of Viable F+ RNA Coliphage in Bivalve Shellfish

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Introduction

- Human norovirus (HuNoV) is a significant cause of bivalve shellfish related illness (epidemic and sporadic acute gastroenteritis).
- HuNoV is unculturable, therefore difficult to assess the infectivity risk.
- F+ RNA Coliphage (FRNAP) have been proposed as indicators of enteric viral contamination (1).
- Genogroup II FRNAP (GA) linked to human faecal sources, and so proposed as an indicator of HuNoV risk in environmental samples (2).
- The current membrane hybridisation method used to detect viable GA is labour intensive and slow (72 hour from sample to result).

Aim: Develop a cost effective most probable number (MPN) method with reduced hands-on and turnaround time to quantify viable GA by polymerase chain reaction (PCR) in bivalve shellfish.

Significance of Study

Globally HuNoV is responsible for an estimated 219,000 deaths and losses of $60.3 billion annually (3).

Routine testing for common enteric viral pathogens is not conducted in the UK.

Sewage discharges are prevalent estuaries where most bivalve shellfish are grown.

Bivalve shellfish are filter feeders which bioaccumulate micro-organisms and are therefore a reservoir of these enteric viruses and bacteria (Fig. 1).

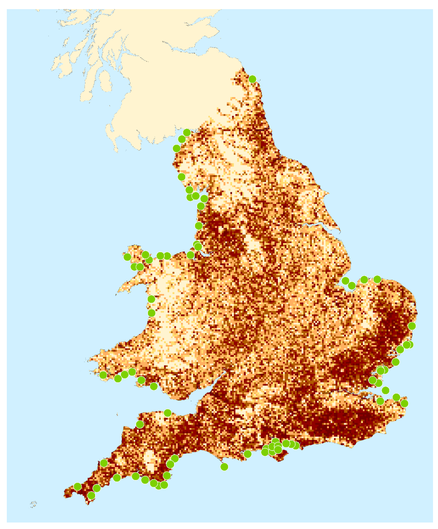


Figure 1. Map of sewage discharge density in England and Wales. Bivalve shellfish production areas are represented by green dots.

Methods

An 96-well format MPN-PCR method was developed for FRNAP. The 96-well format facilitates rapid sample handling compared to a 12 sample extraction method in test tubes.

A mixture of TYGB medium, calcium-glucose and antibiotics (ampicillin and streptomycin) was inoculated with pFamp host. This was incubated for 16 – 18 hours to grow the host to stationary phase.

Growth plate media consisted of a mixture of sterile water, CaGlu, antibiotics, modified TYGB and TYGB-Famp host. 200µl of homogenised digestive tissue (DT) dilutions was added to 800µl of growth media in a 4x3 MPN format (Fig 2.).

After an overnight (18-24 hour) incubation, RNA was extracted from 20µl well samples via a modified 96 well Nuclisens (Bio Merieux) magnetic bead extraction method (Fig 2.).

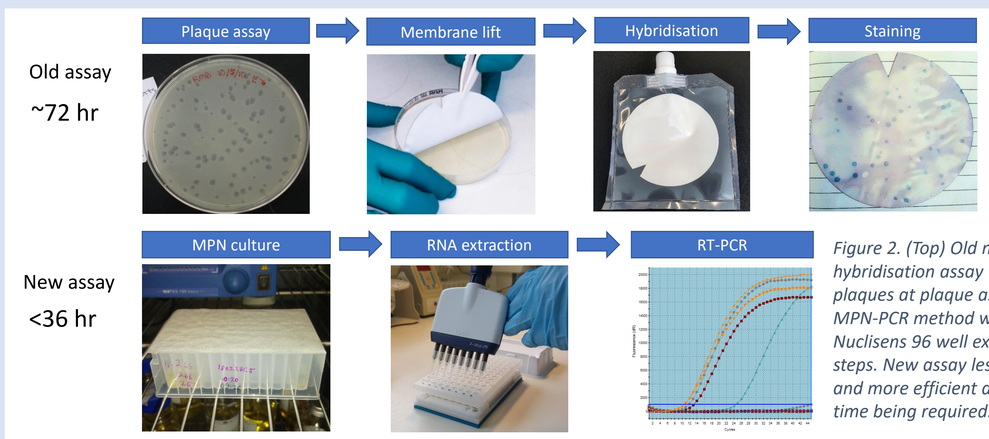


Figure 2. (Top) Old membrane hybridisation assay with observable plaques at plaque assay step (Bottom) MPN-PCR method with growth plate, Nuclisens 96 well extraction and RT-PCR steps. New assay less labour intensive and more efficient due to less hands on time being required.

One-step real time RT-PCR was used to distinguish between positive and negative GA growth in RNA extract.

Plaque assays (the first step in the old method) were used to enumerate the plaque forming units present within samples (Fig. 2). The assay was conducted with Tryptone Yeast Glucose 1% agar, pFamp host and Tryptone Yeast Glucose 2% agar plates.

Results and Discussion

MPN-PCR was found to be comparative to plaque assays. This evaluation was conducted using norovirus outbreak samples, artificially bioaccumulated samples and pure GA dilutions.

GA positive and GA negative samples were identified by one step real time RT-PCR testing of MPN growth plate RNA extracts.

Presence/absence data were compared to most probable number table to determine the level of viable GA FRNAP in each sample.

Plaque assay counts were recorded to allow a comparison of the initial stage of membrane hybridisation (Log PFU/g) with the MPN-PCR method (Log MPN/g).

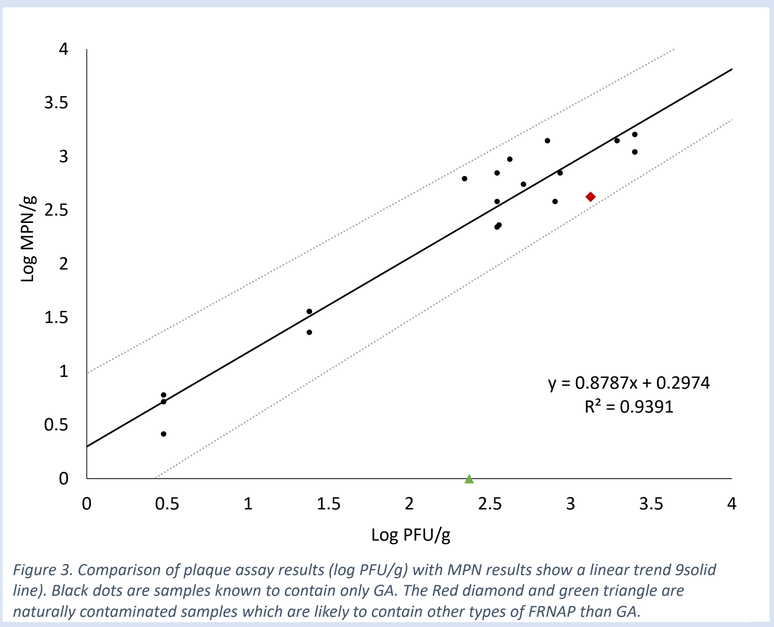


Figure 3. Comparison of plaque assay results (log PFU/g) with MPN results show a linear trend 9solid line). Black dots are samples known to contain only GA. The Red diamond and green triangle are naturally contaminated samples which are likely to contain other types of FRNAP than GA.

Linear regression (Fig. 3) indicates that the MPN-PCR compares well to plaque assays.

This evaluation is further supported by plaque results for all samples which were known to only contain GA, falling within the 95% confidence intervals for MPN tests.

Two samples were observed to not fall within the 95% confidence intervals for the MPN method. These were naturally contaminated samples which are likely to contain other types of FRNAP than GA.

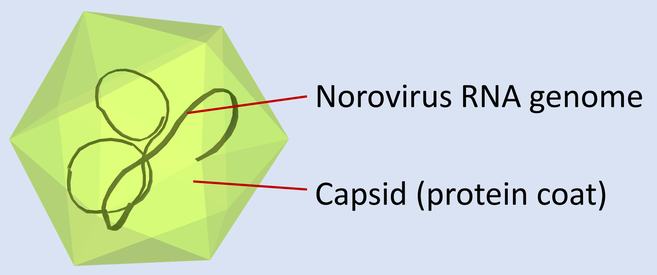
One of these samples (Fig. 3 green triangle) was taken from an area dominated by non-human faecal sources. The other (Fig. 3 red diamond) was taken from an area dominated by human faecal sources. This may support the use of FRNAP types as source tracking tools.

Further testing is required to fill data gaps

Further development of the method to include other FRNAP types and built in quality controls is ongoing

Summary

- MPN-PCR enumerates viable GA within shellfish samples as effectively as the membrane hybridisation method but does so with greater efficiency of resources.
- Real time RT-qPCR facilitates a definite distinction between positive and negative GA samples.
- Therefore, this method will be a useful tool for determining levels of risk associated with the consumption of shellfish.
- Further evaluation of the method will be conducted with bioaccumulation experiments and ongoing testing of HuNoV outbreak samples.



References: **(1)** Jofre, J. (2009) 'Is the replication of somatic coliphages in water environments significant?', *Journal of Applied Microbiology* 106(4), pp. 1059–1069. doi: 10.1111/j.1365-2672.2008.03957.x. **(2)** Hartard, C, Leclerc, M, Rivet, R, Maul, A, Loutreul, J, Banas, S, Boudaud, N and Gantzer C (2018). F-Specific RNA Bacteriophages, Especially Members of Subgroup II, Should Be Reconsidered as Good Indicators of Viral Pollution of Oysters. *Applied Environmental Microbiology* 84 **(3)** Bartsch, S, M, Lopman, B, A, Ozawa, S, Hall, A, J and Lee B, Y (2016). Global Economic Burden of Norovirus Gastroenteritis. *PLOS ONE* 11(4)