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Methods for virus detection in ready-to-eat (RTE) foods

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Scientific Interpretive Summary

This SIS is prepared by MPI risk assessors to provide context to the following report for MPI risk managers and external readers

Methods for virus detection in ready-to-eat (RTE) foods ESR FW13025

Methods for detecting viruses (norovirus and Hepatitis A) which are increasingly associated with foodborne illness are lacking for foods commonly implicated in outbreaks. This paper documents the development of a suitable method, the direct Trizol method with short column–based RNA purification. Further work to validate and establish limits of detection and reproducibility will be required before the method can be used for routine analysis.

Methods for virus detection in ready to eat (RTE) foods

February 2016

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CLIENT REPORT No: FW13025

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SUMMARY

The main objective of this project was to develop and test methods for detection of norovirus genogroup I and II (NoV GI, GII) and hepatitis A virus (HAV) in a range of complex ready to eat (RTE) foods which have been implicated in either NoV or HAV outbreaks. These foods included pasta salads, coleslaw, hard and soft cheeses, sliced ham, and icing for bakery products.

Initially oil/water emulsions containing different ratios of oil to water were seeded with NoV GI, NoV GII, HAV and murine norovirus (MNV) and tested by several methods based on direct Trizol extraction, alkaline elution with PEG precipitation and neutral elution with PEG precipitation followed by either a conventional Trizol RNA purification step or a short combined Trizol silica spin column–based RNA purification step. These experiments were designed to determine if increasing quantities of oil had an effect on virus recovery as well as establish which methods were suitable for food analysis. The selected methods needed to be robust, reproducible, time and cost effective and technically feasible in the laboratory.

Two methods which performed well in the oil/water emulsions experiments and met other criteria - the direct Trizol method with short column-based purification and neutral elution with PEG precipitation and short Trizol extraction - were then used for the complex RTE food trials. Different quantities of foods were seeded with human NoV GI and GII, HAV and MNV. MNV was seeded in the virus mixture as a candidate for the process control. All four viruses were recovered from all of the foods except cottage cheese, but there was some variability in recovery across the food matrices. Overall recovery levels ranged from low (0.3%) to high (~100%) and compared favourably with published virus recovery data for RTE foods. MNV proved to be a suitable process control for the food analyses.

The direct Trizol method with short column-based RNA purification was selected as our first choice for further studies because the recoveries were generally acceptable to good with most matrices and the method was robust, reproducible, comparatively fast to carry out and cost effective. However validation of this method to establish limits of detection and reproducibility for these and other RTE foods is required before it can be used for routine analysis of implicated foods in epidemiological investigations.

The development of methods to detect pathogenic human viruses in RTE foods complements the existing ESR IANZ accredited methods for NoV and HAV detection in bivalve shellfish and fresh produce and is an important advance for public health and food safety agencies.

1. INTRODUCTION

An FAO/WHO Scientific Report (2008) report entitled 'Viruses in food: scientific advice to support risk management activities' identified the highest priority virus-food combinations as norovirus (NoV) and hepatitis A virus (HAV) in shellfish, fresh produce for raw consumption and ready to eat (RTE) prepared foods. RTE foods which have been associated with incidents of foodborne viral disease include raw or cooked foods handled and served cold or at ambient temperature. Examples of such foods include pasta and potato-based salads, bakery items, sandwiches, semi-dried tomatoes and other delicatessen foods. RTE foods identified as transmission vehicles in specific outbreaks include cake icing (Kuristky et al., 1984), ham sandwiches (Daniels et al., 2000), pasta salads (Anderson et al., 2001), semi-dried tomatoes (Donnan et al., 2012) and bread rolls (de Wit et al., 2007).

Pasta salads have been epidemiologically linked with NoV outbreaks (Anderson et al., 2001; Rutjes et al., 2006) but viral analysis of pasta salads has proved difficult and to date viruses have not been able to be detected in pasta salad samples. Researchers who have reported method development for virus detection in pasta and complex foods found that the food matrix and the virus inoculum were key factors in the success of the method (Baert et al., 2008; Stals et al., 2011). Low level NoV inocula (10^4 genome copies/10 g of food) were more difficult to detect in penne pasta than high level inocula (10^6 genome copies/10 g) and in one study, bands from non-specific PCR products were observed on electrophoresis gels (Baert et al., 2008).

For fat-containing foods including sliced meats, the direct Trizol method using guanidinium thiocyanate-phenol-chloroform extraction (using Trizol® reagent) developed by Dr Schwab at Baylor College of Medicine, Houston, Texas was used to successfully detect NoV in ham sandwiches linked to a gastroenteritis outbreak (Daniels et al., 2000; Schwab et al., 2000). In 2000, Dr Gail Greening was trained in this method during a visit to Baylor College of Medicine. She then carried out preliminary experiments using the method on ham at ESR. However, as there was no requirement for this analysis in New Zealand at that time, the method was not tested on other foods, validated, or routinely used at ESR.

Outbreaks related to consumption of NoV contaminated bakery goods have been reported (Kuritsky et al., 1984; de Wit et al., 2007) but there are no published reports of icing being analysed for viruses. In an oral presentation at the 2007 Calicivirus Meeting, Hedlund reported a NoV GII.4 outbreak linked to a marzipan rose on a cake consumed at a Swedish function (*pers comm K-O Hedlund 2007*). NoV was detected in the marzipan rose on one occasion and the contamination was believed to have originated from the food handler who prepared the marzipan rose.

There are few reports of methods for specific virus detection in cheeses and dairy products. In 1994, Gouvea et al. used guanidinium isothiocyanate, adsorption of RNA to hydroxyapatite, followed by sequential precipitation with cetyltrimethylammonium bromide and ethanol in a labour-intensive method to analyse shellfish, orange juice, lettuce, coleslaw, melon and milk for Norwalk virus (Gouvea et al., 1994). They reported good detection of viral RNA from oysters, clams and the food matrices using nested PCR but the PCR bands for milk and coleslaw were not well-defined on the electrophoresis gel. Fumian et al. (2009) developed a filtration and concentration method for NoV recovery from fresh soft cheese and Rutjes et al., (2006) also tested cheeses implicated in NoV outbreaks. Morillo et al., (2012) tested naturally contaminated soup, blue cheese, herb butter and white and Indian sauce samples from a NoV outbreak on a cruise ship using a Trizol-based method with conventional gel-based reverse transcription (RT)-PCR. They detected NoV in the blue cheese, herb butter and white sauce. However, they also comment that neither elution procedures to separate virus from the food nor appropriate process and inhibition controls were used in their study.

The European Committee for Standardisation CEN/TC275/WG6/TAG4 technical advisory group have developed standard methods for detection of NoV and HAV in foods which are due to be published as Technical Specification CEN ISO/TS 15216 (TS) in 2013. However, the TS does not include a method for RTE foods. Published research has shown that method choice depends on the nature and components in the food (Baert et al., 2008; Stals et al. 2011). Some methods can achieve high virus recovery from produce items, whereas others perform better with high fat-containing foods. Inhibitors are a problem in complex foods such as pasta salads and it is essential to monitor and control for these. Generally the more complicated the method and the more steps involved, then the greater the loss of virus, as shown in one study when greater virus loss was observed with two PEG precipitation steps than with one (Baert et al., 2008). Immunocapture is often used for recovery of many pathogens in food but is not readily achievable for norovirus due to lack of commercially available generic antibodies.

Over the last 20 years, a few methods have been developed and trialled for analysis of RTE and complex foods. These include methods developed by Gouvea et al., (1994) Schwab et al. (2000), Leggitt & Jaykus (2000), Rutjes et al. (2006), Baert et al. (2008), Fumian et al. (2009), Stals et al. (2011) and Girard et al. (2013). The basic virus recovery approaches used for different methods include direct extraction with Trizol reagent (Schwab et al. 2000), alkaline buffer elution with / without homogenization followed by 1 or 2 PEG precipitation steps (Leggitt & Jaykus, 2000; Baert et al., 2008), neutral buffer elution followed by either PEG precipitation or ultracentrifugation (Rutjes et al., 2006), alkaline buffer elution with ultrafiltration (Rutjes et al., 2006), and neutral buffer elution with negatively charged membrane filtration (Fumain et al., 2009). More recently, magnetic silica and other silica based methods have been trialled for recovery of murine norovirus (MNV) as a NoV surrogate from several foods including turkey breast and potato salad (Girard et al. 2013). Most methods included a wash step using fluorocarbon solvents such as Freon or Vertrel. Virus recovery methods were frequently combined with different RNA extraction and purification processes and then analysed by RT-PCR. Real-time quantitative RT-PCR (RT-qPCR) was only used in 3 of the above studies (Baert et al. 2008; Stals et al. 2011, Girard et al. 2013); the remainder used conventional gel-based RT-PCR and determined virus loss or recovery by end point dilution. Table 1 summarises the virus recovery results for a range of RTE foods analysed by different methods.

Rutjes et al. (2006) tested a wide range of foods implicated in norovirus outbreaks by several different methods and then selected two foods, lettuce and whipped cream, as representative foods for seeding experiments using canine calicivirus (CaCV) as a NoV surrogate to determine which methods performed best on the different food matrices (Table 1). Baert et al. (2008) also compared several methods for NoV recovery from different food matrices, including pasta salads and then evaluated 3 PCR methods, booster, semi-nested and qPCR, to compare virus recovery rates.

The MPI Food Safety virology research programme has been working to ensure methods are available for detection of the high-priority foodborne viruses, NoV and HAV, in the food groups of concern. Previous successful ESR method development projects have provided methods for shellfish and fresh produce, but no international standardised methods are currently available for virus detection in RTE or complex foods.

Due to the complex nature of most RTE foods, it is likely that development of a suitable method for virus recovery from them will be more challenging than those for shellfish and fresh produce. However, such a method would improve New Zealand's capacity for attribution of foodborne viral illness and assist in food safety management at the retail level.

Table 1: Summary of method development for virus recovery from RTE foods using virus-seeded foods

RTE food	Seeded virus	Result	Methodology	References
Sliced ham, turkey	NoV, HAV	Detected 10^1-10^2 NoV & HAV RT-PCRU/ 20-30 g	Direct Trizol	Schwab et al., 2000
Soft cheese	NoV	Recovery 6-56%	Neutral buffer with filtration	Fumian et al., 2009
Cream	Canine calicivirus (CaCV)	1 log decrease 2 log decrease 2 log decrease 3 log decrease	Neutral PBS/Vertrel/PEG precipitation/Trizol Neutral PBS/Vertrel/PEG precipitation/column Neutral PBS/Vertrel/ Ultrafiltration/Trizol Neutral PBS/Vertrel/ Ultrafiltration/column	Rutjes et al., 2006
Pasta salads	NoV	Detected 10^2-10^3 RT-PCRU /10 g Detected NoV & non-specific products	Direct Trizol short and conc Alkaline buffer with PEG precipitation & Trizol short	Baert et al., 2008
Pasta	MNV	Recoveries: 1-10% 0.1-10%	Direct Trizol short Direct Trizol conc	Stals et al., 2011
Hamburger	Poliovirus HAV	Recoveries: 10-70% 2-4%	Alkaline buffer with 2 x PEG precipitation	Leggitt & Jaykus, 2000
Turkey Potato salad	Murine norovirus (MNV)	Recoveries: 47-51% ~2.5% ~10% <1 % <2%	Magnetic silica Non-magnetic silica Silica membrane PEG +Trizol+ magnetic bead separation All methods	Girard et al., 2013

Some RTE foods of concern are high in fat which can be problematic when analysing for virus presence as it may impact on virus recovery. The first goal of this project was to examine the effects on virus recovery of increasing levels of fat or oil (0-80%) in the food matrix, and so a series of oil/water emulsions was used to evaluate the efficiency of virus recovery. As most salad dressings are acidic, an oil/ vinegar emulsion simulating a basic oil and vinegar dressing was also tested. In this project, methods that performed well in foods containing high proportions of fat were subsequently trialled and then their performance was investigated in complex foods that potentially contained inhibitors.

Objectives:

- To develop and test a method for recovery, extraction and detection of NoV and HAV in an artificial matrix, and to establish the limits of the method with respect to fat content.
- To trial the established methods in a range of RTE foods.

2. METHODS

Methods for recovery, extraction and detection of NoV and HAV were developed in an artificial matrix, and the limits of the method established with respect to fat content. The established method(s) were tested in a range of RTE foods including:

- Dairy products (e.g. grated hard cheese, soft cheese or cream)
- Compound foods (e.g. pasta salads, potato salads, coleslaw)
- Sliced meats (e.g. shaved ham, chicken)
- Icing (for bakery products)

The full method details are shown in the Appendix.

2.1 VIRUSES

Sources of NoV, HAV and MNV are described in the Appendix.

2.2 LIPIDS

Oil-water emulsions containing different quantities of lipids (oil content of 0%, 10%, 25%, 50%, 80% and also a 50% oil: 50% vinegar emulsion) were prepared. The oil / vinegar mix was included as a simulated salad dressing to determine whether acid pH (as often present in salad dressings) had any effect on virus recovery.

2.3 FOOD MATRICES

2.3.1 Pasta salads

1. Pasta salad was prepared in the laboratory. Pasta spirals were freshly cooked, divided into 25 g portions and cooled to room temperature. The pasta portions were then moistened with a dressing made from 1 part oil to 1 part white vinegar. Virus seeding and subsequent incubation with pasta salad was completed within 2 hours of pasta preparation.

2. Two different commercially prepared pasta salads. Type 1 pasta salad (chicken, chilli and lime pasta salad) contained mainly pasta and chicken with a non-creamy dressing (Figure 1). Type 2 pasta salad (Hawaiian pasta salad) contained ham, celery and other vegetables with a creamy dressing (Figure 2). Both salads contained vegetables. The vegetables and meat were removed from each salad and only the pasta was selected for processing. Samples of 25 g of pasta salad type 1 and 5 g and 25 g of pasta salad type 2 were prepared.

Following a review of the methods to be used, and a preliminary experiment with an unseeded virus-free sample, it was decided that the inclusion of a stomaching step in the procedure would be unsuitable for pasta salads as it resulted in an emulsified product that was unsuitable for further processing.

Figure 1: Commercial pasta salad type 1: Chicken and chili lime pasta salad

Figure 2: Commercial pasta salad type 2: Hawaiian pasta salad

2.3.2 Coleslaw

Coleslaw was purchased from a retail store. The main components were cabbage (60%) and coleslaw dressing containing water, sugar and vegetable oil (20%). The remaining 20% contained dextrose, salt, egg yolk, spice, dehydrated onion, carrots and various food chemicals. The fat content of the salad was not specified. Samples of 5 g were prepared.

2.3.3 Hard cheese

Edam cheese (26.4% fat) was used as an example of a hard cheese. The cheese was purchased in grated form. Samples of 5 g, 10 g and 25 g were prepared.

2.3.4 Soft cheese

Cottage cheese (4.9% fat) was used for the method comparison. Samples of 5 g, 10 g and 25 g were prepared.

2.3.5 Sliced RTE ham

Shaved unsmoked (Figure 3) and smoked ham were used to evaluate methods for virus recovery and detection. The ham was purchased from the delicatessen counter in the store. Samples of 5 g and 25 g shaved unsmoked ham and 25 g shaved smoked ham were prepared.

Figure 3: Unsmoked ham

2.3.6 Icing

Commercial soft icing (4.5 g fat) and home-made butter cream icing (50 g butter, 100 g icing sugar; 27% fat) were used for the method trial. Samples of 5 g and 10 g were prepared.

2.4 VIRUS RECOVERY FROM LIPID EMULSION

Three published virus recovery methods were selected for this initial study. The methods used were based on the:

- **direct Trizol recovery method** (Schwab et al., 2000).
- **alkaline elution method with PEG concentration step** (Baert et al., 2008) and
- **neutral elution method with one PEG concentration step** (Rutjes et al., 2006).

For each of these three methods, alternatives for the RNA extraction step were trialled.

1. Using the Trizol reagent method to lyse viruses and extract and purify viral RNA (**Trizol conc**)
2. Using a commercial product consisting of a viral lysis buffer with a silica spin column for RNA purification (**silica spin column**)
3. Combining Trizol reagent for the recovery of viral RNA and the silica spin column for RNA purification (**Trizol short**).

Therefore a total of eight methods were trialled (Figure 4). These were:

A1: Direct Trizol recovery, Trizol conc

A2: Direct Trizol recovery, Trizol short

B1: Alkaline elution, PEG precipitation, Trizol conc

B2: Alkaline elution, PEG precipitation, Trizol short

B3: Alkaline elution, PEG precipitation, silica spin column

C1: Neutral elution, PEG precipitation, PBS elution, silica spin column

C2a: Neutral elution, PEG precipitation, Trizol conc

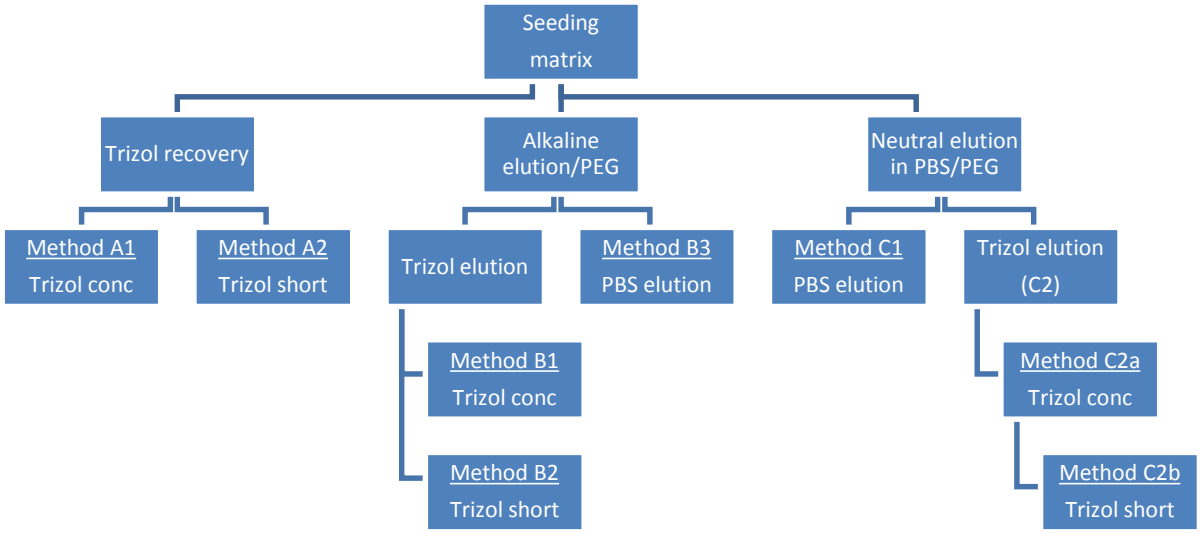
C2b: Neutral elution, PEG precipitation, Trizol short

These methods are described in detail in the Appendix.

For the first experiment, seeding and recovery of NoV GII only was carried out using all eight methods (methods A1, A2, B1, B2, B3, C1, C2a and C2b). One sample was processed for each method and the RNA recovered from each sample was analysed once by RT-qPCR. Due to the practicalities of the methodologies, the amount of sample that could be processed varied. For A1, A2, virus was seeded with 10^5 genome copies of NoV GII to 0.5 ml sample. For B1, B2, B3 virus was seeded with 10^5 genome copies of NoV GII to 10 ml sample. For C1, C2a and C2b virus was seeded with 10^5 genome copies of NoV GII to 1 ml sample. The volume of RNA recovered also varied between 50-100 µl.

In the second experiment, methods A2, B2 and C2 were compared using 0%, 10%, 25%, 50% and 80% oil. In all samples, with the exception of method A2 for 0% oil, duplicate samples of 10%, 25%, 50% and 80% oil were seeded with 10^5 genome copies of NoV GII and HAV each and 10^4 genome copies of MNV. The armored RNA (aRNA) was also added at an appropriate stage for each method. The volumes of samples seeded were 0.5 ml, 8 ml and 5 ml for method A2, method B2 and method C2 respectively. As in the first experiment, the samples were liquid and it was not possible to use the same volumes for each method due to the practicalities of the methodologies. The volume of RNA recovered was 50 µl for each method.

Figure 4: Flow chart for experiments



2.5 EXPERIMENT 3: VIRUS RECOVERY FROM FOOD EMULSIONS

Samples (5 g, 10 g or 25 g) were seeded as evenly as possible with 10^5 genome copies each of NoV GI, NoV GII and HAV and 10^4 copies of MNV, as a candidate process control. Samples were then incubated at room temperature for 15 min prior to processing. A sample was retained for analysis as an uninoculated control. Uninoculated samples were prepared and processed in each experiment.

At least one of the two most efficient methods from Experiments 1 and 2 was used to determine virus recovery efficiencies for each food matrix. The recovery methods are described in the Appendix. Several controls were used in each set of food matrix experiments. These included controls (A, B and C) comprising virus inoculum in PBS with no food matrix, an unseeded food matrix control and a food matrix control which was seeded with the same mixed virus inoculum following the first stage of recovery to control for inhibition and virus loss during the first recovery stage. All controls were taken through the complete recovery process for each method and then tested by RT-qPCR. The RT-qPCR inhibition control, aRNA, was

added where practical. Virus recovery rates were determined by comparison of seeded foods against recovery from the virus-only controls (A, B, C). Virus inocula were also tested in each experiment to check that the initial virus titres remained stable.

2.6 DETERMINATION OF VIRUS RECOVERY

The recovery (%) efficiencies for the NoV, HAV and MNV were determined for all foods using RT-qPCR (Greening & Hewitt, 2011a; Greening & Hewitt, 2011b). For NoV, HAV and MNV, the difference between Ct value of the seeded sample minus the Ct value of the control (ΔCt) was determined. The recovery (%) in the seeded sample compared to the control was then determined by raising 2 to the ΔCt power and multiplying by 100 (ie $1/2^{\Delta Ct}$ x 100%) using the equation (1/POWER(2, ΔCt))*100 in Excel (Microsoft). This equation assumes a PCR amplification efficiency of 100%, so that with each PCR cycle, the PCR copies will double. Hence a difference in 1 cycle (one Ct value) is equal to a 2-fold difference in genome copy number thus higher Ct values correspond to a lower genome copy number. For example, a difference in a Ct value of 1 between the control and sample would give a recovery efficiency of 50%.

Where used, the difference between the Ct value for the aRNA internal control in the sample was compared with that of the control (no matrix) so that any inhibition effects could be detected (Greening & Hewitt, 2011a).

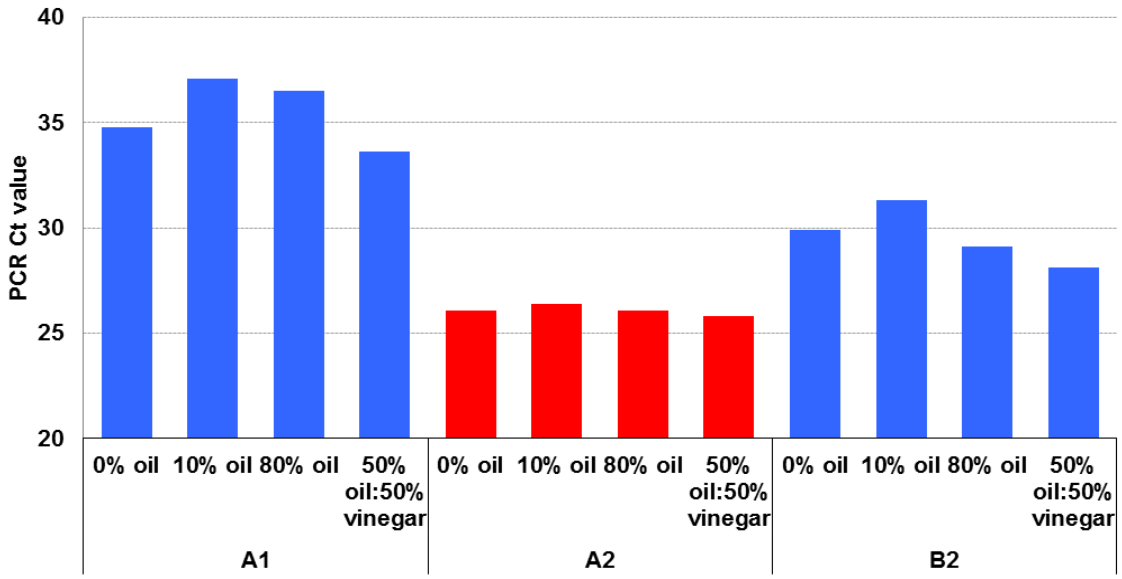
3. RESULTS

3.1 VIRUS RECOVERY FROM LIPID EMULSIONS

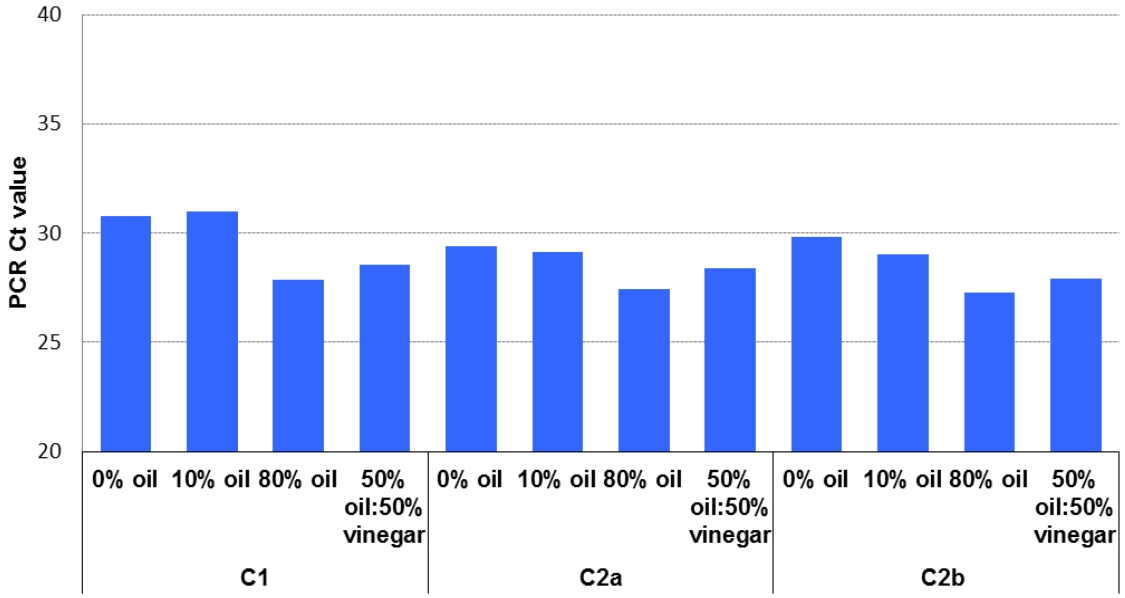
Due to the quantity of virus added and volumes used for each method, direct comparisons of the results as expressed by the PCR Ct values could only be made between methods A1, A2, B1 and B2, and between methods C1, C2a and C2b, Processing of B3 resulted in five times less virus being analysed than methods A1, A2 and B1 and B2, and 10 times less than that used in method C. For methods A1, A2, B1 and B2, 50% less virus was analysed than for methods C1, C2a and C2b. For the first experiment where eight oil/emulsion samples were analysed for NoV GII, all methods (except method B1, alkaline elution followed by Trizol conc) successfully detected NoV GII. Results for methods A1, A2, and B2 are shown in Figure 5a and methods C1, C2a and C2b are shown in Figure 5b. The direct Trizol recovery method with the Trizol short purification step (Method A2, (Direct Trizol recovery, Trizol short) produced the lowest Ct value where a lower PCR Ct value equates to detection of higher virus levels. When the quantity of virus analysed was taken into account, method A2 gave the highest recovery of NoV GII from all method combinations (data not shown) followed by method B2. There was little difference observed in recovery between C1, C2a and C2b (Figure 5b). For each of the methods, increased lipid content did not adversely affect virus recovery.

Figure 5: Norovirus GII recovery from oil/water emulsions as shown by PCR Ct value (lower PCR Ct values equate to higher virus levels)

a. Methods A1, A2 and B2



b. Methods C1, C2a and C2b



For the second experiment, NoV GII, HAV and MNV recoveries were compared using three methods. The methods were chosen based the recovery data from the first experiment, as well as factors including ease of use and turnaround time. The selected methods were method A2 (Direct Trizol recovery, Trizol short), method B2 (Alkaline elution, Trizol short) and C2b (Neutral elution, Trizol short extraction). The results (Table 2) represent the mean (%) recovery efficiencies for each virus by each method (A2, B2 and C2b) as determined by real-time RT-qPCR. The data show that the presence of oil did not appear to interfere with virus recovery.

Variation in the recovery of viruses from some oil/water emulsions was observed, with some samples showing recoveries > 100%. The PCR Ct values of biological replicates can be highly variable, and in these experiments, there was variability between the recoveries of replicate samples in the oil/water matrix. As stated earlier, a difference of 1 cycle (one Ct value) is equivalent to a 2-fold difference in genome copy number and 3.3 cycles is equivalent to a 1-log difference in titre.

The two methods that gave the highest recovery for each virus (NoV GII, HAV and MNV) were:

1. A2 : Direct Trizol recovery with Trizol short
2. C2b : Neutral elution and PEG precipitation with Trizol short

Method B2, alkaline elution with Trizol short, gave poor recovery for NoV GII, HAV and MNV. On the basis of these results, the methods selected for further experiments on a range of RTE foods were:

Method A2: Direct Trizol with the RNA purification using a silica column (Trizol short)

Method C2b: Neutral elution with PEG precipitation with the RNA purification using a silica column (Trizol short).

Table 2: Recoveries (%) of norovirus (NoV) GII, hepatitis A virus (HAV) and murine norovirus (MNV) from oil emulsions

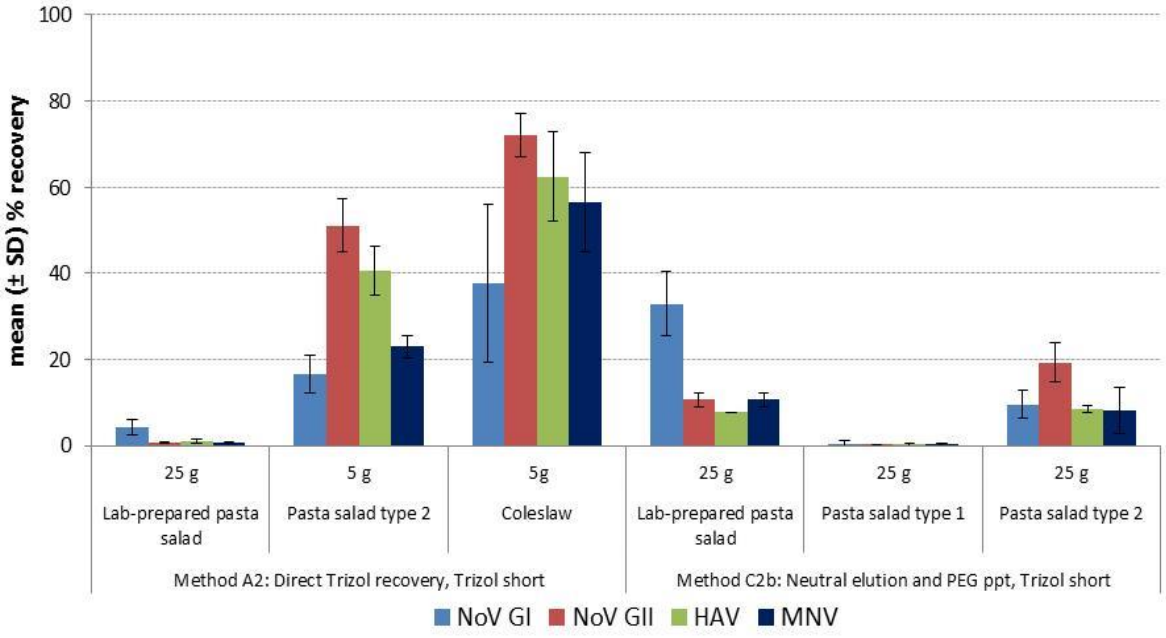
METHOD	% oil	% recovery					
		NoV GII		HAV		MNV	
		Rep a	Rep b	Rep a	Rep b	Rep a	Rep b
A2: Direct Trizol recovery, Trizol short	0	96	Not done	90	Not done	81	Not done
	10	131	84	76	134	60	75
	25	45	19	47	269	34	141
	50	115	99	224	118	90	87
	80	89	86	181	136	74	87
B2: Alkaline elution, PEG precipitation, Trizol short	0	10	17	8	13	4	6
	10	4	4	1	1	2	1
	25	3	1	1	<1	1	1
	50	8	5	4	2	4	3
	80	7	1	3	1	4	1
C2b: Neutral elution, PEG precipitation, Trizol short	0	13	15	11	20	4	3
	10	22	26	12	20	5	7
	25	33	58	26	41	9	42
	50	38	62	43	63	23	24
	80	44	67	32	38	42	27

3.2 VIRUS RECOVERY FROM FOOD MATRICES

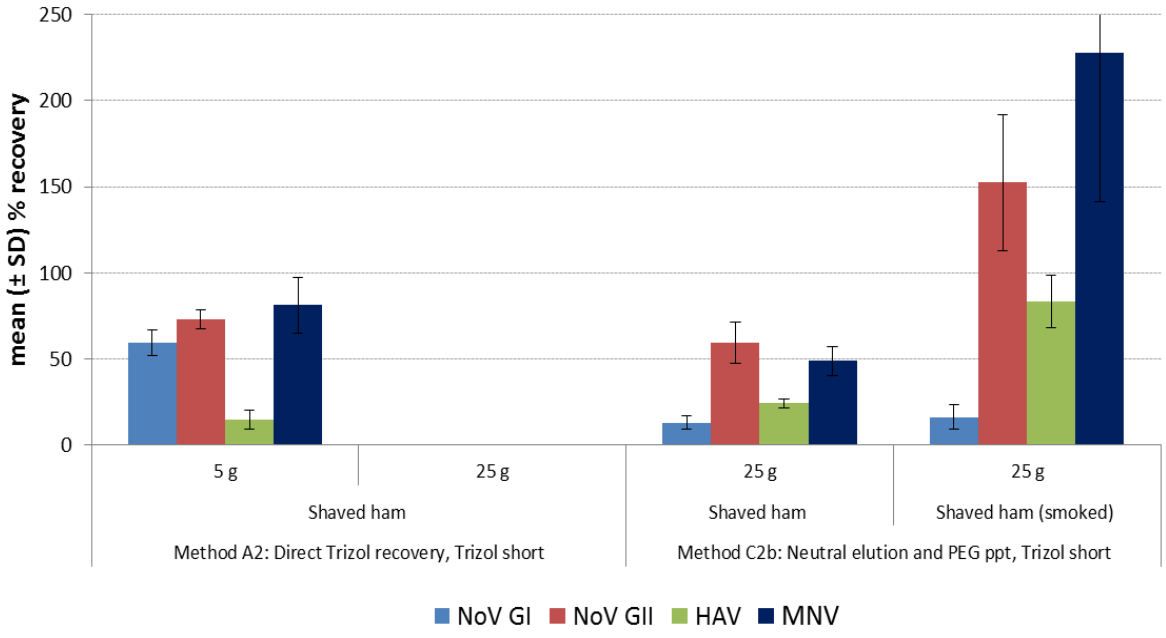
As the most efficient methods from were A2, Direct Trizol recovery with Trizol short and C2b, Neutral elution and PEG precipitation with Trizol short, at least one of these methods was used to determine recovery efficiencies for each food matrices. The mean % recovery rates for each virus from all food matrices by methods A2 and C2b is shown in Figure 6 and in Table 4 (Appendix).

Figure 6: Mean (± SD) % virus recovery from different food matrices by method A2 (direct Trizol short neutral elution / PEG precipitation with Trizol short) and method C2b (neutral elution with PEG precipitation and Trizol short)

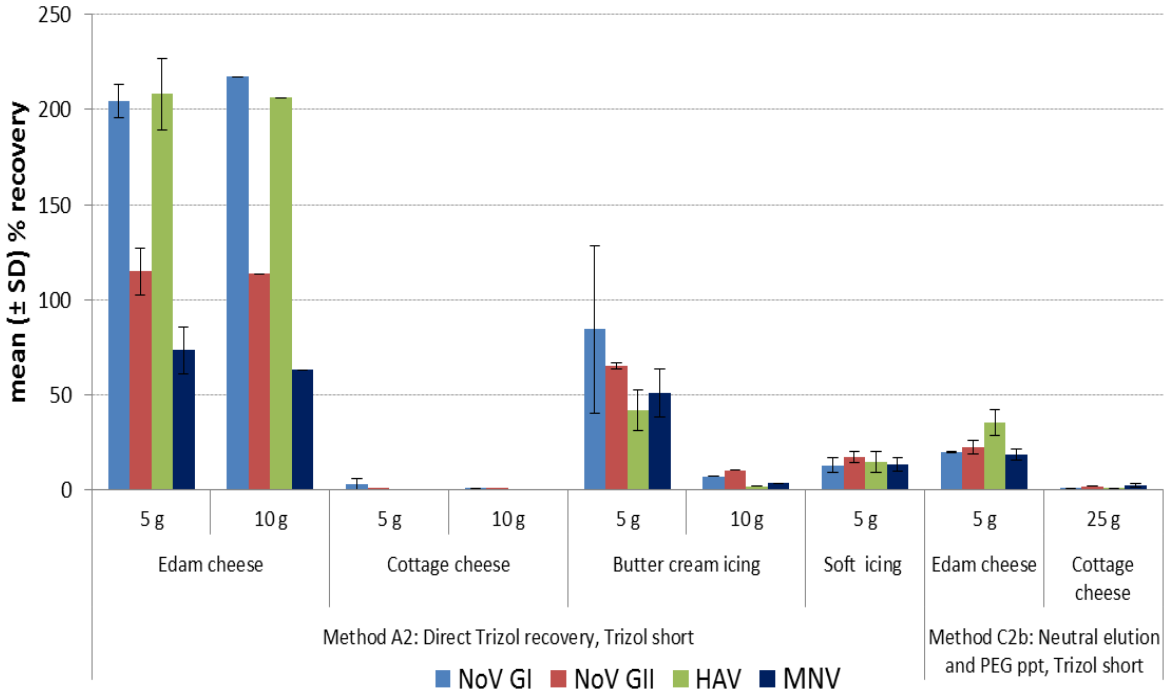
a. Pasta salads and coleslaw



b. Ham



c. Cheese and icing products



3.2.1 Virus recovery from pasta salads

A. Laboratory-prepared pasta salad

For the laboratory-prepared pasta salad (25 g), method C2b (neutral elution with PEG precipitation and Trizol short), was superior to method A2 (direct Trizol with Trizol short) for recovery of all viruses (Figure 6a). Using 25 g of pasta salad, method A2 was inefficient for recovery of any virus, with recoveries ranging from only 0.7% for NoV GII and MNV to 4.3% for NoV GI. This contrasted with method C2b which gave higher recoveries ranging from 7.7% for HAV to 32.9% for NoV GI (Table 4). Lower weights and increased amounts of Trizol were not evaluated on laboratory-prepared pasta salad.

B. Commercial pasta salad

As the results from Part A (Laboratory-prepared pasta salad) showed that method C2b (neutral elution, Trizol short) gave superior results to method A2, (direct Trizol, Trizol short), this method was evaluated first for commercial pasta salads. For method A2, as the combination of 25 g pasta and 8 ml Trizol was deemed suboptimal from the previous experiments, a higher ratio of Trizol to product (5 g pasta rather than 25 g pasta to 10 ml Trizol) was used in subsequent experiments.

Pasta salad type 1: Chicken and chilli lime pasta salad

Poor recovery (mean <1%) of all viruses and aRNA was observed from pasta salad type 1 (25 g) by the neutral elution method, C2b, which may indicate that either inhibitors were present in the system or the viruses were lost during the processing stages (Figure 6a, Table 4). This low recovery could be due to a component in either the salad or the dressing which co-extracted with the viruses or alternatively the viruses were not recovered because they became bound to a component in the salad. At one stage in the RNA extraction using Trizol a large amount of precipitate formed and could not be adequately dissolved for passage through a silica column. This probably contributed to the minimal virus recovery. Additional

experiments were carried out to resolve this problem. However it was not possible to carry out further experiments on this salad because of the subsequent unavailability of the salad.

Pasta salad type 2: Hawaiian pasta salad

An experiment to determine the stage at which virus loss was occurring was carried out using a different commercial pasta salad, Hawaiian pasta salad (type 2), due to the unavailability of pasta salad type 1. Type 2 artificially contaminated pasta salad was processed using methods A2 (direct Trizol short) and C2b (neutral elution/ PEG, Trizol short) and the recoveries compared with pasta salad seeded with virus at different stages during the processing steps. The two stages at which virus and aRNA were added to unseeded pasta salad were before and after the PEG precipitation step. Overall virus recovery from pasta salad type 2 was higher than for pasta salad type 1 by both methods. Using the neutral elution method (C2b), the % recovery from 25 g of Hawaiian pasta salad ranged from a mean of 8.5 % for HAV to 19.3% for NoV GII, and for the direct Trizol method, Trizol short (A2) the recovery from 5 g ranged from 16.6% for NoV GI to 51.1% for NoV GII (Figure 6a, Table 4).These recovery levels of up to 51% for NoV GII at 10^5 copies / 5 g of pasta are higher than those previously reported for penne pasta (Stals et al. 2011).

In summary, three types of pasta salad, laboratory-prepared basic pasta dressed with oil and vinegar, a commercial chicken chilli and lime pasta salad with a non-creamy dressing and a commercial Hawaiian pasta salad with a creamy dressing, were tested by the two selected methods with variable results. Method A2 used for 5 g sample weight gave a higher overall recovery (Table 4) but the seeded viruses were successfully detected from the Hawaiian pasta salad by both methods.

3.2.2 Virus recovery from sliced cooked RTE meats

Initial studies using the direct Trizol method (A2) on 25 g of unsmoked shaved ham were unsuccessful. No virus was recovered from seeded samples. However, when this experiment was repeated using 5 g of unsmoked ham, the method efficiently recovered all viruses. We successfully detected all viruses in the unsmoked ham using the direct Trizol method (A2) in combination with the short column-based RNA purification step. Of the food-related viruses, mean NoV GII recovery was highest at 72.8% and HAV was lowest at 14.1%.The average recovery ranged from HAV at 14.1% to 81.0% for MNV. The neutral elution method (C2b) was also successful for virus recovery from unsmoked shaved ham with a range of 12.6 % (NoV GI) to 59.0% (NoV GII), so either method is applicable for virus detection from unsmoked shaved ham. However recovery results for smoked shaved ham were variable with especially high recovery rates for NoV GII and MNV using the neutral elution/PEG method (Table 4; Figure 6b). This probably relates to the controls amplifying less efficiently than normal. They were 1 Ct higher than expected, which causes an anomaly.

3.2.3 Virus recovery from dairy products

The neutral elution/PEG method gave adequate recoveries for all viruses from hard cheese (grated Edam cheese) and a soft cheese (cottage cheese). Recoveries ranged from 18.2% for the MNV process control to 35.2% for HAV (Figure 6c, Table 4). However the results by the direct Trizol method (A2) were anomalous in that recoveries greatly exceeded the controls A, B and C for NoV GI (204.6%), GII (114.9%) and HAV (208.1%). MNV recovery was 73.3%. This relates to the controls amplifying less efficiently than normal. They were 1 Ct higher than expected which produces the anomaly.

Cottage cheese behaved very differently to the Edam cheese. Great difficulty was experienced with this cheese by both methods and recovery of all viruses was minimal. Although the matrix is low fat with mainly milk-based protein, the cheese curd did not process well in either system. It is possible that virus adsorbed into the curd and so was unable to be recovered. Consequently we consider that cottage cheese is not a suitable matrix for virus analysis.

3.2.4 Virus recovery from icing

Method A2 (direct Trizol, Trizol short) proved efficient for butter cream icing with excellent recovery ranging from 84.2% for NoV GI to 41.5% for HAV (Figure 6c, Table 4) when 5 g samples were analysed. The Trizol method was less efficient for recovery of all viruses from the commercial soft white icing, with a recovery range of 16.9% for NoV GII to 12.7% for NoV GI. However as this matrix proved difficult to work with and, as the results by the direct Trizol method were acceptable, method C2b (neutral elution /PEG) was not trialled on either icing matrix.

3.2.5 Virus recovery from coleslaw

Method A2 (direct Trizol, Trizol short) proved efficient for coleslaw with recovery ranging from 72.0% for NoV GII to 37.7% for NoV GI (Figure 6c, Table 4) when 5 g samples were analysed. As the virus recovery results by the direct Trizol method were acceptable, method C2b, neutral elution with PEG, was not evaluated for coleslaw.

4. DISCUSSION

The main objective of this project was to develop and test methods for detection of NoV GI and GII and HAV in a range of complex RTE foods which have been implicated in either NoV or HAV outbreaks. These foods included pasta salads, hard and soft cheeses, sliced ham, coleslaw, and icing for bakery products.

Initially oil/water emulsions containing different ratios of oil to water were tested by eight methods based on direct Trizol extraction, alkaline elution with PEG precipitation and neutral elution with PEG precipitation followed by either a conventional Trizol RNA purification step or a combined Trizol silica column –based RNA purification step. As far as we are aware, there are no published reports investigating virus recovery specifically from oil/water emulsions. Therefore this study was carried out to assess the influence of oil or fat on virus recovery by different methods because RTE foods contain varying levels of fat. Based on the results from these experiments, the two most efficient methods were then selected for analysis of a range of RTE foods. The two methods selected for further studies were 1: direct Trizol extraction followed by short column-based purification and 2: the neutral elution with PEG precipitation followed by the combined Trizol short silica column-based purification method.

A direct Trizol recovery with full Trizol RNA purification method (Schwab et al. 2000) was one of the first published methods developed for detection of viruses in RTE foods. This method was used to analyse ham from sandwiches epidemiologically implicated in a NoV outbreak. NoV was detected in the ham and this was the first published report describing NoV analysis of a complex food to provide evidence for an epidemiological investigation (Daniels et al., 2000). In our experiments, a modified method using direct Trizol extraction followed by short column-based purification was superior to the more labour intensive full Trizol RNA purification method. The neutral elution with PEG precipitation method was first established and reported by Rutjes and coworkers in 2006 following a comprehensive analysis of foods implicated in NoV outbreaks. These foods included dairy products, sliced meats, pasta, seafood, bakery items and vegetables. No virus was identified in any implicated foods from this study but extensive studies were carried out to develop methodologies for virus detection (Rutjes et al. 2006).

Given the complexity of the various foods and comments in published reports on method development, we expected that different methods would be required for each matrix type. Previous research studies showed that different virus recovery methods were likely to be required for each food type, as no single standard method would be optimal for each food matrix (Baert et al., 2008; Rutjes et al., 2006; Stals et al., 2011). The nature of the RTE food matrices does not allow for prewashing or other treatment and in our study, foods were used as purchased. In our experience, the direct Trizol method followed by short silica column-based RNA purification proved to be a suitable method for all matrices except cottage cheese, for which neither method trialled was successful, and also was unsuitable for the chicken, chilli and lime pasta salad when 25 g of food rather than 5 g was tested. Unfortunately we were unable to further investigate the change in virus recovery efficiency from this salad following the change in sample quantity tested as the salad was no longer available.

During the project, general problems which arose when processing complex foods included difficulties with the matrix when buffers or Trizol were added, causing the food to become unmanageable and producing inhibition in the molecular assays due to inhibitory compounds in the foods which were carried over in the RNA. Cottage cheese was especially problematic as it remained primarily as lumpy curds and neither Trizol or neutral elution buffer were useful for virus recovery.

Inhibition is an important factor in food virology. It is controlled for in the RT-PCR assays by the process control and the internal control which check results from each sample against results expected when there is no inhibition. If inhibition is detected, then both repeat testing and testing of diluted RNA are carried out. If these fail and additional sample is available, then fresh RNA can be prepared from lower sample weights and retested. In this method evaluation, the use of MNV as a process and inhibition control and armored RNA as an inhibition control for recovery and detection of the viruses worked as expected and indicated where technical issues needed to be addressed.

As each matrix will differ in composition, the virus recovery efficiency will also vary. Recovery efficiency varied depending on type of food matrix, quantity analysed, methods used and also differed for each virus. In this study, NoV GI, NoV GII and HAV were successfully detected in all matrices seeded with high titre NoV and HAV inocula, with the exception of soft cottage cheese. Whilst high recoveries of 100% may be sought, in food and environmental virology studies extraction recoveries of >10 % are considered good, between and 1 and 10% acceptable, and <1% are considered unacceptable. Our results show that the viruses analysed (NoV GI, NoV GII, HAV and MNV) had similar recovery ranges to each other under one set of conditions. For example, considering the categories of recoveries (good, acceptable and poor), the recoveries for all four viruses were similar under the same conditions.

We consider our recovery rates are high compared with published reports for RTE foods which range between 0.1% and 70%, depending on the virus used for seeding and the food matrix (Baert et al., 2008; Stals et al., 2011). HAV has been reported as being difficult to recover from foods, with poor recoveries recorded (Leggitt & Jaykus, 2000). In this study, the recovery of HAV was similar to the other viruses. We tested three pathogenic viruses, NoV GI, NoV GII and HAV, with MNV as a candidate process control, whereas other researchers have used NoV surrogate viruses such as MNV and CaCV or only NoV rather than a range of viruses. Several groups have not included a process control. In our study, MNV was shown to be suitable as a process control. The process control is important in all foods analysed from outbreaks to determine whether inhibition or virus loss have occurred and so help to reduce the risk of false negative results.

A major drawback for routine viral analysis of foods is that the methods can be slow and laborious at the initial processing stage and so are not easily adaptable for high throughput analysis (i.e. more than 20-30 samples per batch). Whilst evaluating different methods for virus detection in RTE foods, as well as sensitivity and specificity of the methods, consideration was also given to the time and cost aspects of methods trialled. Our aim was to set up a rapid, efficient, cost-effective, robust method which was not too labour-intensive or time consuming, gave reproducible results, used readily available commercial reagents with a long shelf-life and was able to analyse multiple samples in a batch. A further consideration was that the technical steps were similar to those currently used in the laboratory so it would be easy for staff to carry out the analysis as required.

Methods using PEG precipitation are generally slower and more labour intensive because of the precipitation step and may also increase virus loss (Baert et al. 2008).The direct Trizol method with Trizol short (Method A2) was relatively fast and simple to perform, and of all the methods trialled, was the most suitable method for processing several samples in a batch. Although a number of samples are able to be processed at one time using this method, the number of replicate samples, multiple controls and standards included in each run can limit the number of samples analysed per PCR assay to under 20 samples (based on a 96 well plate run) and much less for a Rotorgene run (based on 36 tube capacity). The Trizol short column-based method can also be automated for the RNA purification stage.

However there are still issues with methods detection of viruses in RTE foods. Most experiments, including ours, were carried out using high virus inocula but as the infective dose for NoV is believed to be as low as 20 particles there is the possibility that these methods are

not sufficiently sensitive to detect an infectious dose. For example, if testing only 5 g of a 200 g serving of pasta salad containing 20 particles, the detection limit would need to be 1 particle per 10 g of pasta. Therefore there is a definite risk of false negative results for food contaminated with low levels of virus. In addition, molecular methods do not determine infectivity of NoV or HAV. The detection of these viruses in foods therefore indicates a risk as they should not be present and there are no information on the survival time for NoV in foods.

In conclusion, efficient detection methods for NoV and HAV in selected RTE foods have been set up at ESR. Validation experiments are now required to test a wider range of samples in each food type, establish limits of detection and ensure that these methods are robust and reproducible. Once this has been achieved, these methods can be used to assist in NoV or HAV outbreak epidemiological investigations where there is strong evidence that RTE foods such as pasta salads, sliced cold meats, hard cheeses and bakery products are implicated as an infection source. The first method of choice for viral analysis of these foods would be the direct Trizol method with the short column based purification step. This method has proved capable of detecting NoV and HAV from the majority of food products tested and meets our criteria with regard to time, cost and technical aspects.

The development of methods to detect pathogenic human viruses in RTE foods complements the existing ESR IANZ accredited methods for virus detection in bivalve shellfish and fresh produce and is an important advance for public health and food safety agencies.

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APPENDIX

A.1 VIRUSES

NoV GI and GII suspensions were prepared from high titre NoV-positive faecal specimens collected from gastroenteritis cases and referred to ESR for testing (Greening et al., 2012). HAV HM-175 and fetal rhesus monkey kidney (FRhK-4) cells were kindly provided by Dr. M. D. Sobsey (University of North Carolina, Chapel Hill). Murine norovirus (MNV strain 1) was kindly provided by Prof Virgin/Dr C. Wobus (Washington University School of Medicine, MO, USA) and RAW 264.7 cells from Department of Microbiology and Immunology, University of Otago.

Stock preparations of 10^7 genome copies/ml NoV GI, 10^7 genome copies/ml NoV GII, 10^7 genome copies/ml HAV and 10^6/mL MNV were used to prepare the combined virus inoculum for seeding of samples. For 1 ml of inoculum, 200 µl NoV GI, 200 µl NoV GII, 200 µl HAV and 200 µl MNV stock were combined with 200 µl sterile distilled water. This gave final concentrations of 2 x 10^6 genome copies /ml for NoV GI, NoV GII and HAV, and 2 x 10^5 genome copies/ ml for MNV.

A.2 VIRUS RECOVERY PROCEDURES

For all methods (Table 3), the same amount of virus inoculum was added to the food sample, to either the same volume of Trizol or PBS as appropriate.

Table 3: Summary of methods

Method group	Method subgroup	Initial step	Intermediate step	RNA purification /extraction step
A	1:	Direct Trizol recovery	NA	Trizol conc
	2:	Direct Trizol recovery	NA	Trizol short
B	1:	Alkaline elution	PEG precipitation	Trizol conc
	2:	Alkaline elution	PEG precipitation	Trizol short
	3:	Alkaline elution	PEG precipitation	Silica spin column
C	1:	Neutral elution	PEG precipitation PBS elution	Silica spin column
	2a:	Neutral elution	PEG precipitation	Trizol conc
	2b:	Neutral elution	PEG precipitation	Trizol short

NA Not applicable

Method A1. Direct Trizol recovery- Trizol conc

1. Add Trizol reagent. The volume to add is one of the following:
   a. 5 ml to 0.5 ml oil emulsion
   b. 8 ml to 25 g food
   c. 10 ml to 5 g food
2. Mix sample and allow to stand for 5 min (for oil emulsion) to 15 min (for other foods) at room temperature.
3. Centrifuge at 12,000 x g for 20 min, 4°C (not necessary for the oil emulsion samples)
4. Remove the upper lipid (fat) layer if present. Transfer the remaining solution to a clean tube and discard the tube with the pellet
5. *Phase separation*. Add 0.2 ml chloroform per 1 ml Trizol reagent added. Mix well by shaking for 15 seconds and incubate at RT for 3 min.

6. Centrifuge at 12,000 x g for 15 min, 4°C. Transfer the aqueous layer (RNA) into a clean container.
7. To the recovered aqueous phase, add 0.5 ml isopropanol per 1 ml Trizol reagent, and mix for 30 sec.
8. Incubate at RT for 10 min.
9. Centrifuge for either at 8,000 x g for 20 min (or 12,000 x g for 10 min) at 4^{o}C
10. Discard supernatant.
11. Add 1 ml 75% (v/v) ethanol per 1ml Trizol and centrifuge at 7,000 x g for 5 mins at 4^{o}C
12. Discard supernatant.
13. Air dry the RNA pellet.
14. Add 50-100 µl RNAse free water to the pellet to resuspend in 100 µl RNAse free water is added to the oil / water emulsion experiments.
15. Store at -80^{o}C if not used in a RT immediately.

Method A2. Direct Trizol recovery-Trizol short

1. Perform steps 1-6 as for Method A1 (Direct Trizol recovery-Trizol conc).
2. *Ethanol precipitation followed by column purification.* Add an equal volume of 70% (v/v) ethanol to the RNA solution to give a final volume of 35% (v/v) ethanol. Mix well by vortexing.
3. Invert the tube to disperse any visible precipitate that may form.
4. Transfer sample to a silica column* in a collection tube (*used from the Roche High Pure Viral Nucleic Kit).
5. Centrifuge 6,200 x g for 15 sec at RT.
6. Discard flowthrough and place the spin column in a collection tube.
7. If necessary, repeat by adding aliquots of sample and repeat centrifugation until all sample has passed through the filter.
8. Add 0.5 ml inhibition wash buffer and centrifuge 6,200 x g for 1 min.
9. Add 0.45 ml wash buffer and centrifuge 6,200 x g for 1 min. Repeat wash step.
10. Remove column and discard waste and centrifuge 12,000 x g for 1 min at RT to dry the membrane.
11. Add a maximum of 2 x 50 µl RNAse free water to the filter and incubate 5 min.
12. Centrifuge 16,200 x g 1 min and retain the filtrate (nucleic acid).
13. Store at -80^{o}C if not used in a RT immediately.

Method B1. Alkaline elution step and PEG concentration followed by Trizol conc

1. Add 0.05 M glycine-0.15M NaCl pH9 (adjusted with 10M NaOH) to matrix and virus controls.
   Volume to add:
   a. 32 ml to 8 ml oil emulsion
2. Centrifuge at 10,000 x g for 15 min, 4°C.
3. Remove the upper lipid (fat) layer if present. Transfer the remaining solution to a clean tube and discard the tube with the pellet and recover the supernatant.
4. Adjust pH the supernatant to 7.2–7.4.
5. Add PEG 6000 and NaCl to give a final concentration of 10% PEG–0.3M (2%) NaCl and dissolve.
6. Shake overnight at 120 rpm, 4°C.
7. Centrifuge at 10,000 x g for 30 min, 4°C.
8. Remove and discard supernatant. Retain pellet. aRNA may be added at this stage.
9. Add 1-4 ml Trizol.
10. Perform steps 2-15 in Method A1 as previously described.

Method B2. Alkaline elution step and PEG concentration followed by Trizol short

1. Perform steps 1- 9 as described in Method B1 Alkaline elution and PEG precipitation method.
2. Perform steps 2-6 as described in Method A1.
3. Perform steps 2-13 as described in Method A2 (*ethanol precipitation followed by silica column purification*)

Method B3. Alkaline elution step and PEG concentration, PBS elution of pellet followed by silica column RNA extraction

1. Perform steps 1- 8 as described in Method B1 Alkaline elution method and PEG precipitation method.
2. Add 4 ml PBS to the pellet and dissolve pellet for at least 5 min.
3. Add 4 ml chloroform and centrifuge at 10,000 x g for 15 min at 4^{o}C.
4. Recover supernatant.
5. Extract RNA from 0.2 ml sample using the Roche High Pure Viral Nucleic Acid Kit according to manufacturer's instructions.

Method C1. Neutral elution followed by PEG precipitation followed by silica column RNA extraction

1. Add PBS; pH 7.2 to food and mix well (200 rpm for 5 min, orbital shaker). The volume of PBS to add is one of the following:
   a. 4.5 ml to 0.5 ml oil emulsion (initial NoV GII only experiment)
   b. 5 ml to 5 ml oil emulsion
   c. 25 ml to 25 g food
2. Add chloroform and shake, and rotate at 200 rpm for 5 min. The volume of chloroform to add is one of the following:
   a. 5 ml to 0.5 ml oil emulsion (initial NoV GII only experiment)
   b. 5 ml to 5 ml oil emulsion
   c. 25 ml to 25 g food
3. Centrifuge at 5,000 x g for 10 min at 4^{o}C.
4. Remove the supernatant and add to a clean container. Measure the volume and adjust to pH to 7-7.2 if necessary.
5. Add PEG 6000 and NaCl to give a final concentration of 10% PEG–0.3M (2%) NaCl and dissolve.
6. Centrifuge at 10,000 g for 30 min, 4°C.
7. Remove and discard supernatant. Retain pellet.
8. Resuspend the pellet in:
   a. 200 ul for oil emulsion experiments
9. Extract RNA from whole sample using the Roche High Pure Viral Nucleic Acid Kit according to manufacturers' instructions.

Method C2a. Neutral elution followed by PEG precipitation and Trizol conc

1. Perform steps 1-7 as described in Method C1.
2. Add 1 ml Trizol to the pellet (can add aRNA at this stage).
3. Perform steps 2-15 as described in Method A1.

Method C2b. Neutral elution followed by PEG precipitation and Trizol short

1. Perform steps 1-7 as described in Method C1.
2. Add 1 ml Trizol to the pellet (can add aRNA at this stage).
3. Perform steps 2-6 as described in Method A1.

4. Perform steps 2-13 as described in Method A2 (*ethanol precipitation followed by silica column purification*).

A.3 REAL-TIME RT-QPCR METHODS

Norovirus (two-step RT-qPCR)

Viral RNA (5 µl) was transcribed with reverse primers (NoV GI, NoV GII and aRNA) (Kageyama et al., 2003, Wolf et al., 2010) at 50°C for 30 min using Superscript™ III reverse transcriptase (Invitrogen) to produce 10 µl cDNA. The RT was followed by real-time qPCR amplification using Platinum® Quantitative PCR Supermix-UDG (Invitrogen). Each 25 µl PCR reaction (GI or GI & aRNA or GII or GII & aRNA) contained 5 µl of cDNA, 12.5 µl of 2X qPCR Supermix-UDG (Invitrogen), 0.4 µM of each NoV primer, 0.2 µM NoV probe, and where required 0.1 µM aRNA primer and 0.2 µM aRNA probe) as previously described (Greening & Hewitt, 2008). The PCR conditions were 95°C for 5 min, followed by a two-step cycling protocol, comprising denaturation at 95°C for 15 sec and annealing/extension for 60 sec at 57°C for NoV GI and 56°C for NoV GII for 45 cycles. Real-time RT-PCR assays were carried out using Rotor-Gene™ 3000 (GII) or 6000 (GI) real-time rotary analyzers (Corbett Research Ltd., Sydney, Australia). Raw data was analysed using Rotor-Gene™ software and the Ct values calculated.

Known concentrations NoV and aRNA were extracted as positive RNA extraction controls. Viral RNA (NoV GI and GII) standards (1000, 100 and 10 RTPCRU) extracted from NoV positive faecal specimens were also included for each NoV assay as RT-qPCR controls. In addition, 10^4 copies DNA plasmid was used as a positive control, DNase /RNase-free water used as a negative extraction control, and as a reagent blank in each RT-qPCR.

Procedures to prevent false positive or false negative results were carried out, including the use of separate areas for PCR reagent preparation, viral NA extractions/RT, and PCR assays.

Murine norovirus (two-step RT-qPCR)

Viral RNA (2.5 µl) was transcribed with MNV reverse primers at 50°C for 30 min using Superscript™ III reverse transcriptase (Invitrogen) to produce 5 µl cDNA. The RT was followed by real-time qPCR amplification using Platinum® Quantitative PCR Supermix-UDG (Invitrogen).Each 25 µl PCR reaction contained 5 µl of cDNA, 12.5 µl of 2X qPCR Supermix-UDG (Invitrogen), 0.4 µM of each MNV primer and 0.2 µM MNV probe (Hewitt *et al.*, 2009). The PCR conditions were 95°C for 5 min, followed by a two-step cycling protocol, comprising denaturation at 95°C for 15 sec and annealing/extension for 60 sec at 56°C for 45 cycles. Real-time RT-qPCR assays were carried out using Rotor-Gene™ 6000 real-time rotary analyzers (Corbett Research Ltd).

A known concentration of MNV (same as inoculum) was extracted as a positive RNA extraction control. In addition, 10^4 copies DNA plasmid were used as a positive control, DNase /RNase-free water used as a negative extraction control, and as a reagent blank in each RT-qPCR.

Procedures to prevent false positive or false negative results were followed as described above.

Hepatitis A virus (two-step RT-qPCR)

Viral RNA (2.5 µl) was transcribed with HAV 240 reverse primer at 50°C for 30 min using Superscript™ III reverse transcriptase (Invitrogen). The RT was followed by real-time qPCR amplification using Platinum® Quantitative PCR Supermix-UDG (Invitrogen). Each 25 µl PCR reaction contained 5 µl of cDNA, 12.5 µl of 2X qPCR Supermix-UDG (Invitrogen), 0.4 µM of each HAV primer and 0.2 µM HAV probe (Costafreda et al., 2006). The PCR conditions were 95°C for 5 min, followed by a two-step cycling protocol, comprising denaturation at 95°C for

15 sec and annealing/extension for 60 sec at 60°C for 45 cycles. Real-time RT-qPCR assays were carried out using Rotor-Gene™ 3000 real-time rotary analyzers (Corbett Research Ltd) as described above. Raw data was analysed using Rotor-Gene™ software and the Ct values calculated.

DNA plasmid (10^4 copies) was used as a positive control in addition to DNase/RNase-free water as a negative extraction control, and as a reagent blank in each RT-qPCR. Procedures to prevent false positive or false negative results were followed as described above.

Table 4: Summary of RTE foods methods trial results

Method A2, Direct Trizol recovery with Trizol short, and Method C2b, neutral elution and PEG precipitation with Trizol short were selected for the trial

Food matrix	Quantity tested	Direct Trizol recovery with Trizol short				Quantity tested	Neutral elution and PEG precipitation with Trizol short			
		Mean % (± SD) recovery					Mean % (± SD) recovery			
		NoV GI	NoV GII	HAV	MNV		NoV GI	NoV GII	HAV	MNV
Lab made pasta salad	25 g	4.3 ± 1.8	0.7 ± 0.3	1.0 ± 0.6	0.7 ± 0.3	25 g	32.9 ± 7.4	10.6 ± 11.5	7.7 ± 0.1	10.6 ± 1.5
Commercial pasta - type 1	25 g	not done	not done	not done	not done	25 g	0.5 ± 0.6	0.2 ± 0.1	0.3 ± 0.3	0.2 ± 0.0
Commercial pasta - type 2	5 g	16.6 ± 4.3	51.1 ± 6.1	40.7 ± 5.8	23.0 ± 2.7	25 g	9.6 ± 3.3	19.3 ± 4.6	8.5 ± 0.7	8.1 ± 5.4
Coleslaw	5 g	37.7 ± 18.2	72.0 ± 5.1	62.4 ± 10.4	56.6 ± 11.5	not done	not done	not done	not done	not done
Shaved ham (normal)	5 g	59.2 ± 7.7	72.8 ± 5.3	14.2 ± 5.5	81.0 ± 16.1	25 g	12.6 ± 3.9	59.0 ± 11.9	23.9 ± 2.7	48.5 ± 8.4
Shaved ham (smoked)	not done	not done	not done	not done	not done	25 g	15.9 ± 7.1	>100 ± 39.4	83.3 ±15.3	>100 ± 86.3
Edam cheese (pre-grated)	5 g,10 g	>100 ± 8.8	>100 ± 12.3	>100 ± 18.7	73.3 ± 12.3	25 g	19.3 ± 0.3	22.1 ± 3.5	35.2 ± 6.7	18.2 ± 2.9
Cottage cheese	5 g,10 g	2.7 ± 3.0	0.0 ± 0.0	<0.01	<0.01	25 g	0.1 ± 0.1	1.6 ± 0.2	0.1 ± 0.0	2.0 ± 0.8
Butter cream icing	5 g,10 g	84.2 ± 43.9	65.0 ± 1.9	41.5 ± 10.7	51.0 ± 12.7	not done	not done	not done	not done	not done
Soft white icing	5 g	12.7 ± 3.9	16.9 ± 2.8	14.2 ± 5.5	13.0 ± 3.4	not done	not done	not done	not done	not done

>100 % recovery was observed. Murine norovirus (MNV) was not recovered from shaved ham (25 g) using the direct Trizol method (results not shown).

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