International Journal of Food Microbiology 311 (2019) 108349

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Development of an extraction method to detect enteric viruses in dressed vegetables

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ARTICLE INFO

Keywords:
Dressed vegetables
HAV
NoV
Detection

ABSTRACT

Food-borne viral infections are caused mainly by noroviruses (NoV) and the hepatitis A virus (HAV), which respectively cause gastroenteritis and hepatitis.

Various foods have been implicated in viral outbreaks, including vegetables that are consumed in a variety of forms, often with salad dressing. NF EN ISO procedures (15216-1:2017) propose standard methods for quantifying NoV and HAV in high-risk food categories, such as vegetables, based on viral elution and PEG concentration methods, but these methods are not suitable for composite meals like salads dressed with oily, fatty or emulsified food ingredients. The development of sensitive and reliable techniques for the detection of viruses in these products is therefore needed to ensure the safety of these products. The aim of this study was to develop an RT-qPCR based method for the detection and quantification of NoV and HAV in various vegetables with different dressings. Three methods for recovering NoV and HAV from artificially contaminated dressed vegetables were evaluated. The selected method was based on the use of Trizol reagent and, according to the type of dressing, the limit of detection ranged from 10^4 to 10^6 genome copies/g for NoV and from 10^2 to 10^3 PFU/g for HAV. The described method can be applied for detecting NoV and HAV in food containing salad dressing for routine diagnosis needs.

1. Introduction

Among the enteric viruses implicated in foodborne outbreaks, human noroviruses (NoV) and hepatitis A virus (HAV) are the two leading causes of viral food-borne illness, with NoV now estimated as the most prevalent agent of food-borne disease (Gould et al., 2013; Hall, 2012).

HAV and NoV are small non-enveloped viruses and have a positive-sense, single-stranded RNA genome. HAV are classified in the Hepatovirus genus of the *Picornaviridae* family and NoV belonging to genogroups I (NoV GI) and II (NoV GII) are classified in the *Caliciviridae* family. HAV and NoV are mainly transmitted *via* the faecal-oral route, either through person-to-person contact or upon ingestion of contaminated water or food (Kotwal and Cannon, 2014; Matthews et al., 2012). Food can become contaminated in the field during the growth phase, as well as during processing, storage, distribution or final preparation.

Various foods have been implicated in viral outbreaks, including vegetables that are consumed in a variety of forms, being a major component of almost all meals. These food types have the potential of being associated with large outbreaks, as has occurred in Europe and in the United States with leafy greens, carrots or semi-dried tomatoes (Donnan et al., 2012; Ethelberg et al., 2010; Herman et al., 2015; Kamińska et al., 2014; Müller et al., 2016; Wadl et al., 2010). The various vegetables involved in viral outbreaks are often consumed with salad dressing.

The general strategy for the detection of enteric viruses in food samples consists of three steps: virus extraction, purification of viral RNA and quantitative molecular detection of the purified RNA. NF EN ISO procedures (15216-1:2017) describe standard methods for quantifying NoV and HAV in high-risk food categories such as vegetables, but they have not been validated for composite meals such as dressed salads due to the difficulty in recovering NoV and HAV from a turbid food emulsion (Baert et al., 2008; Gallot et al., 2011; Girard et al., 2013) and the presence of substances that can inhibit PCR amplification (Fraisse et al., 2017; Lee et al., 2012; Maunula et al., 2013; Suffredini et al., 2014).

A sensitive and reliable method for the detection of NoV and HAV in

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https://doi.org/10.1016/j.ijfoodmicro.2019.108349
Received 28 February 2019; Received in revised form 8 August 2019; Accepted 5 September 2019
Available online 06 September 2019

oily, fatty or emulsified food needs to be developed to ensure the safety of these products. The aim of this study was (i) to develop an RT-qPCR based method for the detection of NoV and HAV in ready-to-eat vegetables with dressing using MNV-1 as process control virus and (ii) to validate the method from artificially contaminated vegetables by measuring the mean recovery rates and the limit of detection (LOD) useful to apply it for routine diagnosis in the future.

2. Materials and methods

2.1. Viruses and cells

HAV strain HM175/18f, clone B (VR-1402) was obtained from the American Type Culture Collection (ATCC). This clone replicates rapidly and has cytopathic effects in cell culture (Lemon et al., 1991). HAV stock containing 5.45×10^6 plaque-forming units/mL (PFU/mL) was produced by propagation in foetal rhesus monkey kidney (FRhK-4) cells (ATCC, CRL-1688) (Cromeans et al., 1987) and titrated using a plaque assay (Dubois et al., 2006).

Stool samples of NoV GI (E8050) and NoV GII (E7022) from infected humans were provided by the "Centre National de Référence Virus des gastro-entérites", Dijon, France. The faecal samples were suspended in 10 mM phosphate buffered saline (PBS), pH 7.4 to obtain a final 10% suspension (w/v), and then vortexed and centrifuged at 4000 ×*g* for 20 min at 4 °C. Aliquots of 100 µL were kept frozen at −80 °C for later use. The genomic titres of the clarified faecal suspensions were determined by RT-qPCR using a standard curve obtained with the 10-fold diluted *in vitro* RNA transcripts as previously described (Hennechart-Collette et al., 2014). Based on this approach, the clarified suspension stocks of NoV GI and NoV GII had titres of approximately 1.2×10^8 and 8.5×10^7 genome copies/mL, respectively.

A process control virus, the murine norovirus MNV-1 (CW1 strain) was provided by Dr. H. Virgin from Washington University (Saint Louis, MO, USA) to the ANSES Fougères Laboratory (Fougères, France) and was propagated in a mouse leukemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line (Cannon et al., 2006). RAW 264.7 was grown at 37 °C in an atmosphere containing 5% CO_2 in DMEM supplemented with GlutaMAX™, 1% non-essential amino acids and 10% foetal bovine serum (Life Technologies, Saint Aubin, France). The production stock of MNV-1 had a titre of approximately 2.15×10^7 of the 50% tissue culture infective dose ($TCID_{50}$)/mL.

2.2. Food samples and salad dressings

For spiking experiments, three ready-to-eat vegetables (lettuce, grated carrots and a mixture of raw grated vegetables (carrots, celery and cabbage)) and three types of salad dressing (dressing A, dressing B and dressing C) with different quantities of fat were purchased from a local market. Details of the composition of the salad dressings are described below.

Dressing A (74 g of fat for 100 mL): an olive oil vinaigrette with lemon and balsamic vinegar (extra virgin olive oil (37%), sunflower oil (37%), balsamic vinegar (25%), natural lemon extract (1%), sulphites).

Dressing B (26 g of fat for 100 mL): a whole grain mustard vinaigrette, containing water, 25% rapeseed oil, whole grain Dijon mustard, (water, mustard seeds, alcohol vinegar, salt, preservative: potassium metabisulfite, acidifier: citric acid), wine vinegar, dextrose, 4% whole grain mustard, alcohol vinegar, salt, modified corn starch, thickening, colouring, flavouring.

Dressing C (32 g of fat for 100 mL): a light vinaigrette (balsamic vinegar, dried tomatoes) containing 20% extra virgin olive oil, water, white and red wine vinegars, half-reduced tomato puree, 12.6% balsamic vinegar (wine vinegar, grape must syrup, food colouring: E150d, preservative: potassium metabisulfite), rapeseed oil, garlic, salt, pepper, 1% dried tomatoes.

For each vegetable sample, 20% of its weight in dressing was mixed with the sample. Depending on the method used, 25 g or 2.5 g of vegetables with 20% of dressing corresponded respectively to 3.7 g or 0.37 g of fat for dressing A, at 1.3 g or 0.13 g of fat for dressing B and at 1.6 g or 0.16 g of fat for dressing C.

2.3. Artificial contamination of dressed vegetables

To compare different elution-concentration methods, all food samples with 20% salad dressing were separated into 25 g placed in a 400 mL polypropylene bag containing a filter compartment and 2.5 g placed in a 50 mL centrifuge tube (Falcon). Food samples were spiked by adding 100 µL of 10-fold dilutions of the MNV-1 stock prepared in DEPC-treated water (Fisher Bioblock Scientific, Illkirch, France) to food samples just before adding elution buffer.

To assess the LOD of the selected method, the inoculation of dressed vegetables (20% of dressing) was performed with serial dilutions of NoV GI, NoV GII and HAV to obtain four inoculation levels ranging from 4.70×10^6 to 4.70×10^3 genome copies/g of NoV GI, from 3.40×10^6 to 3.40×10^3 genome copies of NoV GII/g and from 2.20×10^4 to 2.20×10^1 PFU/g of HAV. Each sample with dressing was co-inoculated with 8.6×10^3 $TCID_{50}$ of MNV-1/g to control the analytical processus.

One unspiked sample was used as a negative control. Each experiment was performed in triplicate.

2.4. Sample processing for recovery of viruses

Three methods for recovering viruses from dressed vegetables were evaluated. Fig. 1 gives an overview of these three methods, each of which was tested on lettuce, grated carrots and a mixture of raw grated vegetables and with three different salad dressings. Details of the

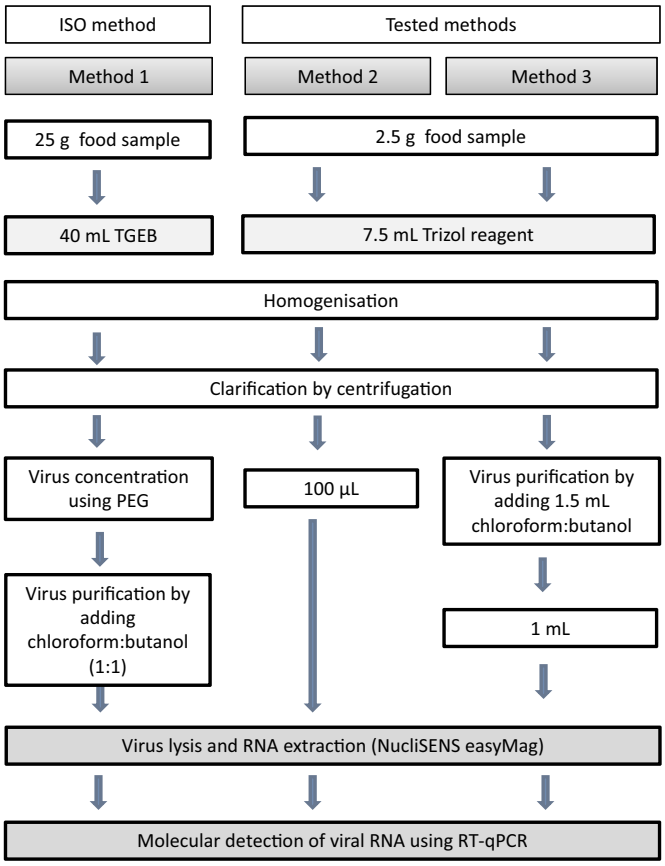

Fig. 1. Flowchart of Methods 1, 2 and 3 assessed for recovery and detection of MNV-1 in dressed vegetable samples. TGBE, Tris-glycine-beef extract solution.

extraction methods are described below.

Method 1

Method 1 is the method described for vegetables in the NF EN ISO procedure (ISO 15216-1) to detect enteric viruses. Briefly, each inoculated sample (25 g) was placed in a 400 mL polypropylene bag containing a filter compartment and was soaked in 40 mL of elution buffer (100 mM Tris-HCl, 50 mM glycine, 1% beef extract, pH 9.5). The rinse fluid was removed *via* the filter compartment of the bag and was centrifuged at 10,000 ×*g* for 30 min at 4 °C to pellet the food debris. The pH of the decanted supernatant was adjusted to 7.2 +/− 0.2 with the addition of 5 N HCl while the fluid was swirled constantly. The neutralised supernatant was supplemented with 10% (*w/v*) polyethylene glycol (PEG) 8000 (Sigma-Aldrich, Saint-Quentin-Fallavier, France), and 0.3 M NaCl, and was then incubated 1 h. Viruses were concentrated by centrifugation of the solution at 10,000 ×*g* for 30 min at 4 °C. The supernatant was discarded and an additional centrifugation was performed at 10,000 ×*g* for 5 min at 4 °C to pack the pellet. The pellet was suspended in 1 mL of PBS and vortexed with 1 mL of 1:1 chloroform:butanol (v/v). The suspension was then incubated for 5 min at room temperature, and centrifuged at 8000 ×*g* for 15 min at 4 °C. The upper aqueous phase containing viruses was directly processed using the nucleic acid extraction procedure.

Method 2 and Method 3

Method 2 and Method 3 are based on the use of Trizol reagent. The use of Trizol reagent has already been described for ready-to-eat foods and delicatessen foods to detect enteric viruses (Baert et al., 2008; Schwab et al., 2000; Stals et al., 2011) and this method was adapted to dressed vegetables.

Each spiked food sample (2.5 g) was homogenised in 7.5 mL of Trizol reagent by inverting the tube several times. After an incubation of 15 min at room temperature with constant shaking at approximately 60 rpm, the food sample was centrifuged at 8500 ×*g* for 15 min at 4 °C, the supernatant was transferred to another tube and vortexed. For Method 2, 100 µL of the suspension was then directly processed using the nucleic acid extraction procedure. For Method 3, 1.5 mL of chloroform:butanol was added and the suspension was then incubated for 5 min at room temperature, and centrifuged at 8000 ×*g* for 15 min at 4 °C. Then, 1 mL of the upper aqueous phase containing viruses was directly processed using the nucleic acid extraction procedure.

For all three methods, each step of the experiment, from the spiking to the RNA extraction, was performed three times and the RNA extracts (pure RNA and 10-fold diluted RNA) were analysed in duplicate with the RT-qPCR assays. Uninoculated food samples were used as negative controls during the entire sample processing and viral detection procedures.

2.5. Viral RNA extraction

NucliSENS® easyMAG™ lysis buffer (BioMérieux, Marcy l'Etoile, France) was added to the virus suspension (up to 3 mL) and total nucleic acid extraction was carried using the NucliSENS® easyMAG™ platform with the "off-board Specific A" protocol according to the manufacturer's instructions. Nucleic acids were eluted in 70 µL of elution buffer and stored at −80 °C.

2.6. Primers and probes

Primers and probes used to quantify HAV, NoV GI and NoV GII have been described previously (Costafreda et al., 2006; da Silva et al., 2007; Kageyama et al., 2003; Loisy et al., 2005; Pinto et al., 2009; Svraka et al., 2007) and are recommended in the NF EN ISO 15216-1 for detecting NoV GI and NoV GII in foodstuffs. The sequences of the primer pairs and the TaqMan probes are given below. For HAV, the sense primer (HAV68) was 5′-TCACCGCCGTTTGCCTAG-3′, the anti-sense primer (HAV241) was 5′-GGAGAGCCCTGGAAGAAAG-3′ and the TaqMan probe (HAV150-) was 5′-FAM-CCTGAACCTGCAGGAATTAA-MGB-3′. For NoV GI, the sense primer (QNIF4) was 5′-CGCTGGATGC GNTTCCAT-3′, the anti-sense primer (NV1LCR) was 5′-CCTTAGACGC CATCATCATTTAC-3′ and the TaqMan probe (NVGG1p) was 5′-FAM-TGGACAGGAGAYCGCRATCT-BHQ1–3′. For NoV GII, the sense primer (QNIF2) was 5′-273 ATGTTCAGRTGGATGAGRTTCTCWGA-3′, the anti-sense primer (COG2R) was 5′-TCGACGCCATCTTCATTCACA-3′ and the TaqMan probe (QNIFS) was 5′-ROX-AGCACGTGGGAGGGCGATCG-BHQ2–3′. The primers and the TaqMan® probe targeting the ORF1 polyprotein of MNV-1, which were designed using Beacon Designer software (Bio-Rad, Marnes-la-Coquette, France), have been described previously (Martin-Latil et al., 2012). The sequences of the primer pairs and the TaqMan probe were as follows: the sense primer (MNV-3193-F) was 5′-CCGCCATGGTCCTGGAGAATG-3′, the anti-sense primer (MNV-3308R) was 5′-GCACAACGGCACTACCAATCTTG-3′ and the TaqMan probe (MNV-3227-T) was 5′-ROX-CGTCGTCGCCTCGGTCCTTGTCAA-BHQ2-3′. All primers and probes were purchased from Applied Biosystems (Courtaboeuf, France) or Eurofins (Les Ulis, France).

2.7. RT-qPCR conditions

One-step RT-qPCR amplifications were performed in duplicate on the CFX96™ real-time PCR detection system (Bio-Rad). Reactions were performed in a 25 µL reaction mixture containing 1× of RNA UltraSense™ master mix and 1.25 µL of RNA UltraSense™ enzyme mix, which are components of the RNA UltraSense™ One-Step Quantitative RT-PCR System (Life Technologies), 2 U RNase inhibitor (Life Technologies), 1× of bovine serum albumin (Life Technologies), 500 nM (HAV, NoV GI, NoV GII and MNV-1) of forward primer, 900 nM (HAV, NoV GI, NoV GII and MNV-1) of anti-sense primer, 250 nM of probe for all viral targets and 5 µL of RNA extract. Positive controls containing RNA extracted from virus suspensions and a negative control containing all the reagents except the RNA template were included with each set of reaction mixtures. The one-step RT-qPCR programme involved a 60 min reverse-transcription of RNA at 55 °C, followed by a 5 min denaturation step at 95 °C, and finally 40 cycles of 15 s at 95 °C, 1 min at 60 °C and 1 min at 65 °C. Fluorescence was recorded by the apparatus at the end of the elongation steps (1 min at 65 °C) for each amplification cycle. All samples were characterised by a corresponding Ct value. Negative samples gave no Ct value. A standard curve for each viral target was generated with RNA extracts resulting from serial dilutions of the viral stock suspension in distilled water. The slopes (S) of the regression lines were used to calculate the amplification efficiency (E) of the RT-qPCR reactions, according to the formula $E = 10^{|-1/s|} - 1$ to determine the performance of the RT-qPCR assays. RNA extracts were analysed in duplicate with the RT-qPCR assay.

2.8. Data analysis

For Method 1, viral recovery rates from spiked samples were calculated with the following formula and expressed as percentages: (Quantity of virus recovered after spiking experiments/Quantity of viral inoculum) × 100.

For Method 2 and Method 3, recovery rates from spiked samples were calculated with the following formula: (Quantity of virus recovered after spiking experiments for 1 mL × volume of elution buffer/Quantity of viral inoculum) × 100.

One microliter of HAV (5.8×10^1 genome copies/µL), NoV GI (6.6×10^3 genome copies/µL) or NoV GII (8.4×10^5 genome copies/

μL) RNA transcript was used as an external amplification control (EAC) to monitor RT-PCR inhibition in dressed vegetable samples. This approach has been described in the NF EN ISO 15216-1 where an external control (EC) RNA (an RNA species carrying the target sequence of interest) is added to an aliquot of RNA sample. Comparison of these results with the results of EAC RNA in the absence of sample RNA (*i.e.* in water) provides the degree of RT-PCR inhibition in each tested sample. HAV, NoV GI and NoV GII inhibition rates were calculated using the following formula: 1 - (quantity of external control RNA recovered in sample/quantity of external control RNA recovered in ultrapure water) × 100.

2.9. Statistical analysis

All statistical analyses were performed using the Statgraphics Centurion XV.II software. The influence of extraction method on the recovery rates of MNV-1, used as a process control virus, from three contaminated vegetables (grated carrots, mixture of raw grated vegetables and lettuce,) with three different salad dressings (dressing A, dressing B and dressing C) was first assessed using a one-way analysis of variance (ANOVA). The result of the ANOVA is a *p*-value associated with the hypothesis that the mean recovery rates of all groups were the same. Because the extraction yields were statistically different according to the extraction method used (ANOVA, $p < 0.01$), a multiple comparison procedure (Fisher's least-significant-differences (LSD)) was applied to determine which extraction method could provide the highest recovery rates. Given that there are three group means, there are also three pairs to compare. Graphs plotting the mean and its standard error for each group illustrate the multiple comparison procedure. When confidence intervals of means do not overlap, the difference between two groups of a factor is significant.

The influence of additional factors on the recovery rates of pathogenic viruses (HAV, NoV GI or NoV GII) calculated from pure RNA extracts were tested with the selected method using a one-way ANOVA. Two factors were tested on recovery rates: (i) the quantity of pathogenic virus and (ii) the type of dressing.

3. Results

3.1. Comparison of three methods to recover MNV-1 from artificially contaminated dressed vegetables

To select a method for detecting MNV-1 in vegetables with added salad dressing, three methods (Method 1, Method 2 and Method 3) were evaluated on vegetables artificially contaminated with 8.6×10^3 $TCID_{50}$ of MNV-1/g. The mean recovery rates obtained for MNV-1 are reported in Table 1.

The mean recovery rate of the MNV-1 with pure and 10-fold diluted RNA extracts ranged from 0.44% to 6.44% for Method 1, from 15.61% to 95.49% for Method 2 and from 29.89% to 90.82% for Method 3 regardless the dressing. Method 2 and Method 3 gave the highest average recovery rates.

Testing the 10-fold diluted RNA extracts showed that recovery rates for MNV-1 were improved by a factor that ranged from 0.89 to 6.59 using Method 1, Method 2 and Method 3. These results point to enzyme inhibition (Table 1).

To identify whether the extraction method influenced the recovery rates of MNV-1, statistical analysis was performed by using a one-way ANOVA, which detected significant differences among the three methods (*p*-value < 0.001). The multiple comparison test showed that Methods 2 and 3 had significantly higher recovery rates than Method 1 (Fig. 2) and that there were no significant differences between Method 2 and Method 3, which were therefore comparable in terms of virus recovery. The highest average recovery rates were obtained using Methods 2 and 3, but Method 2 was preferred because it does not require any organic solvent (chloroform, butanol).

With the selected method (Method 2), the differences between experiments were not significant for the recovery rates of MNV-1 (one-

Table 1
Comparison of mean recovery rates of MNV-1 from artificially contaminated dressed vegetable samples processed using three methods. Samples of dressed vegetables (25 g or 2.5 g) were spiked with 8.6×10^3 $TCID_{50}$ of MNV-1/g. For each sample type and for each type of dressing, three experiments were performed and pure and 10-fold diluted RNA extracts were tested twice. Results are expressed as mean virus recovery rates (%) ± standard deviations (SD). The ratio (F) between the mean extraction yields obtained with pure RNA extracts and those obtained with 10-fold diluted RNA extracts was calculated to determine whether the dilution of RNA extracts enhanced mean extraction yields.

			Methods					
			Method 1		Method 2		Method 3	
		RNA extracts	Recovery rates (% ± SD)	Factor (F) (diluted/ pure)	Recovery rates (% ± SD)	Factor (F) (diluted/ pure)	Recovery rates (% ± SD)	Factor (F) (diluted/ pure)
Dressing A	Grated carrots	Pure	2.13 ± 2.82	1.97	24.44 ± 18.21	2.78	33.68 ± 32.16	2.39
		10-fold diluted	4.20 ± 2.25		68.15 ± 17.67		80.43 ± 162.64	
	Grated mixture	Pure	3.15 ± 3.62	2.04	15.61 ± 5.14	5.62	55.54 ± 60.93	1.38
		10-fold diluted	6.44 ± 4.92		87.73 ± 39.17		76.54 ± 70.57	
	Lettuce	Pure	2.02 ± 2.74	2.16	30.81 ± 23.63	2.18	38.09 ± 51.51	2.06
		10-fold diluted	4.38 ± 3.05		67.42 ± 35.12		78.75 ± 31.22	
Dressing B	Grated carrots	Pure	1.46 ± 1.18	1.01	61.95 ± 12.12	1.09	59.14 ± 10.56	1.07
		10-fold diluted	1.48 ± 1.16		67.89 ± 12.12		63.47 ± 8.42	
	Grated mixture	Pure	0.64 ± 0.30	1.06	57.04 ± 18.65	1.31	54.95 ± 29.32	1.09
		10-fold diluted	0.68 ± 0.30		75.00 ± 21.76		60.02 ± 22.65	
	Lettuce	Pure	0.72 ± 0.63	1.11	78.13 ± 16.10	1.02	86.15 ± 30.17	1.05
		10-fold diluted	0.80 ± 0.62		80.26 ± 18.30		90.82 ± 26.08	
Dressing C	Grated carrots	Pure	1.08 ± 0.24	3.75	19.66 ± 20.03	3.26	29.89 ± 8.40	1.79
		10-fold diluted	4.05 ± 2.79		64.18 ± 8.14		53.57 ± 4.50	
	Grated mixture	Pure	0.44 ± 0.63	6.59	65.16 ± 31.64	1.46	86.12 ± 16.86	0.89
		10-fold diluted	2.90 ± 3.93		95.49 ± 20.02		77.44 ± 15.04	
	Lettuce	Pure	1.78 ± 1.29	2.16	29.86 ± 33.13	2.89	48.48 ± 31.02	1.76
		10-folddiluted	3.85 ± 0.34		86.43 ± 21.43		85.57 ± 12.98	

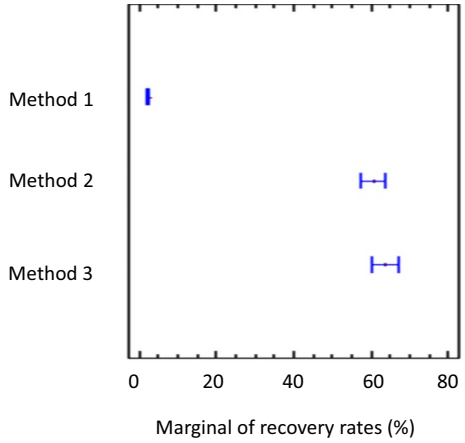

Fig. 2. Comparison of mean recovery rates of MNV-1 from spiked dressed vegetables processed according to the extraction method.

way ANOVA; p-value = 0.5932). The dilution of RNA extracts enhanced recovery rates of MNV-1 by a factor ranging from 1.02 to 5.62. The effect of the dilution for RNA extracts was statistically confirmed (one-way ANOVA; p-value < 0.001) showing a significant amplification inhibition. Furthermore, statistical analysis showed that recovery rates obtained with Method 2 was not influenced by the type of vegetables (one-way ANOVA; p-value = 0.0537), whereas was influenced by the type of dressing (one-way ANOVA; p-value = 0.0391). A multiple comparison test showed that vegetables with dressing A were significantly different to dressing B and vegetables with dressing A and B were not significantly different to dressing C (Fig. 3).

3.2. Validation of the selected method for the detection of HAV and NoV in grated carrots with two types of dressing

To validate Method 2, grated carrot samples with 20% of dressing A or B were tested, because the selected method was not influenced by the type of vegetable, but by the type of dressing.

3.2.1. Mean virus recovery rates from grated carrots with dressing A and dressing B

The recovery rates of HAV, NoV and MNV-1 from spiked grated carrots were determined. Table 2 gives the mean recovery rates calculated with pure RNA extracts for HAV and NoV according to the inoculum levels and for the control process virus (MNV-1).

All the experiments with grated carrots with dressing A and dressing B spiked with HAV, NoV GI or NoV GI showed that the process control virus was consistently detected in RNA extracts. The average of MNV-1 recoveries for every level of HAV, NoV GI or NoV GII inoculation ranged from 6.90% to 83.00% in grated carrots with dressing A or dressing B, with the highest recoveries for dressing B.

The average of HAV, NoV GI and NoV GII recoveries ranged respectively from 16.29% to 58.21%, from 54.79% to 58.70% and from 36.17% to 32.32%, with dressing A or dressing B.

As expected, no viral RNA was detected in the uninoculated samples. The statistical analysis showed that the recovery rates for HAV, NoV GI and NoV GII were not statistically different whatever the inoculation levels (one-way ANOVAs, HAV, p-value = 0.6978; NoV, p-

Table 2
Recovery rates obtained for HAV, NoV GI, NoV GII and for the process control virus (MNV-1) from grated carrots with dressing A and dressing B and the PCR inhibition assay in RNA extracts. Results are expressed as mean viral extraction yields (%) ± standard deviations (SD). For each inoculation level, three experiments were performed and pure RNA extracts were tested twice, resulting in six mean viral extraction yields for each sample type. The number of positive Ct determinations (n = 6) are given for HAV, NoV GI and NoV GII. For each sample type, the lowest concentration at which all six Ct determinations are positive is shown in bold: it corresponds to the LOD. PCR inhibition assay on RNA extracts ± standard deviations (SD) were calculated for HAV, NoV GI and NoV GII using RT-qPCR.

		Grated carrots	
	Virus quantity/g	Dressing A	Dressing B
VHA	2.20×10^4 PFU	6/6	6/6
	2.20×10^3 PFU	**6/6**	6/6
	2.20×10^2 PFU	3/6	**6/6**
	2.20×10^1 PFU	1/6	3/6
	Recovery rates (% ± SD)	16.29 ± 10.67	58.21 ± 21.94
	PCR inhibition (% ± SD)	69.20 ± 29.20	18.20 ± 29.90
MNV-1	8.6×10^3 $TCID_{50}$	24/24	24/24
	Recovery rates (% ± SD)	22.84 ± 28.35	83.00 ± 33.76
NoV GI	4.70×10^6 genome copies	6/6	**6/6**
	4.70×10^5 genome copies	**6/6**	4/6
	4.70×10^4 genome copies	3/6	2/6
	4.70×10^3 genome copies	0/6	0/6
	Recovery rates (% ± SD)	54.79 ± 4.79	58.70 ± 17.78
	PCR inhibition (% ± SD)	61.60 ± 40.60	38.40 ± 34.90
MNV-1	8.6×10^3 $TCID_{50}$	24/24	24/24
	Recovery rates (% ± SD)	6.90 ± 4.89	74.71 ± 15.90
NoV GII	3.40×10^6 genome copies	6/6	6/6
	3.40×10^5 genome copies	**6/6**	6/6
	3.40×10^4 genome copies	3/6	**6/6**
	3.40×10^3 genome copies	0/6	1/6
	Recovery rates (% ± SD)	36.17 ± 13.80	32.32 ± 14.80
	PCR inhibition (% ± SD)	51.90 ± 40.50	33.40 ± 36.70
MNV-1	8.6×10^3 $TCID_{50}$	24/24	24/24
	Recovery rates (% ± SD)	14.17 ± 13.05	67.42 ± 15.07

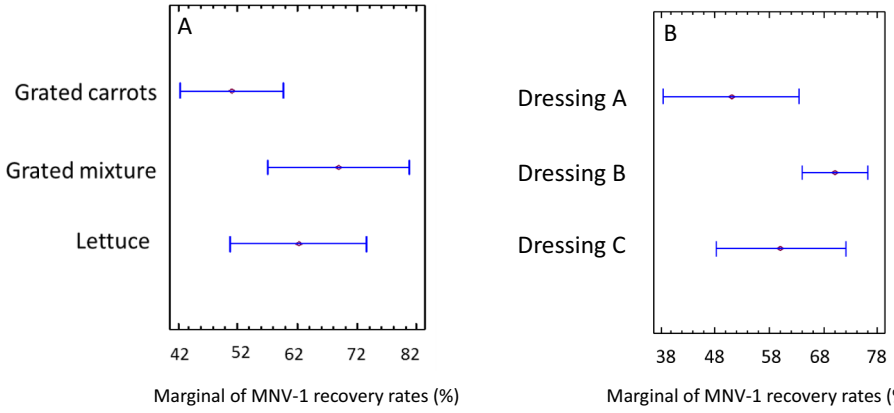

Fig. 3. Mean MNV-1 recovery rates under various conditions with (selected) Method 2. The influence of two experimental factors of MNV-1 extraction is illustrated by a multiple comparison test. A: type of food (p-value = 0.0537) and B: type of dressing (p-value = 0. 0391).

value = 0.1080 for NoV GI and NoV GII, p-value = 0.7071).

Moreover, statistical analysis revealed that the type of dressing did not influence NoV recoveries from grated carrots (one-way ANOVAs; p-value = 0.6601 for NoV GI and p-value = 0.4558 for NoV GII), but influenced HAV recoveries (one-way ANOVA; p-value < 0.001). Similar to MNV-1, recovery rates for HAV were higher with dressing B than with dressing A.

The limits of detection (LOD) for HAV and NoV were assessed from artificially contaminated dressed carrots. The lowest spiking concentration that gave all six positive Ct values in an experiment set was considered as the LOD_{100}. The LOD_{100} of NoV GI and NoV GII were respectively 4.7×10^5 genome copies/g and 3.4×10^5 genome copies/g of grated carrots with dressing A. With dressing B, the LOD_{100} of NoV GI and NoV GII were 4.7×10^6 genome copies/g and 3.4×10^4 genome copies/g of grated carrots, respectively. For HAV, the LOD_{100} was 2.2×10^3 PFU/g of grated carrots with dressing A and 2.2×10^2 PFU/g with dressing B.

3.2.2. Recovery rates of the external amplification control (EAC)

The implementation of an EAC corresponding to each viral target was used to examine RT-qPCR inhibition. The rates of inhibition in pure and diluted RNA extracts from grated carrots with dressing A and dressing B were determined and varied respectively from 51.90% to 69.20% and from 18.20% to 38.40% (Table 2). Moreover, the rates of inhibition varied significantly with the type of dressing sauce (ANOVA; *p*-value = 0.0005). Statistical analysis showed that the inhibition rates obtained in RNA extracts with dressing A were higher than with dressing B.

4. Discussion

Food poisoning outbreaks may be associated with a wide variety of food, including dressed vegetables, which have been implicated in NoV and HAV outbreaks. In contaminated dressed salads, viruses can persist for few days. Takahashi and al showed that the infectivity of MNV-1 decreased by 2.6 log PFU/ml in 5 days in the vinaigrette dressing stored at 4 °C, whereas in mayonnaise or thousand island dressing, the infectivity of MNV-1 didn't significantly decrease in the same period (Takahashi et al., 2016).

A concentration method based on PEG has been employed for long for virus detection from salad vegetables, soft fruits or in oysters (Dubois et al., 2002, 2004) and was described in the NF EN ISO 15216-1:, 2017 for detecting NoV and HAV in high-risk food categories such as vegetables. The virus recovery rates are suitable for raw vegetables with this standard method (Coudray et al., 2013; Summa et al., 2012), but our results showed that the PEG concentration method is not optimal for complex foods. The virus recovery rate for MNV-1 obtained using NF EN ISO 15216-1 based Method 1 was in agreement with data reported in other studies. The recovery rates of NoV using the PEG concentration method varies from 0.02% to 2.11% for meals mixed with mayonnaise and oily dressing (Pan et al., 2012; Saito et al., 2015). The composition of food products can affect virus extraction (Blaise-Boisseau et al., 2010; Butot et al., 2007; Summa et al., 2012) and different virus recovery methods are likely to be required for each food type (Baert et al., 2008; Dubois et al., 2006; Fumian et al., 2009; Hennechart-Collette et al., 2017; Martin-Latil et al., 2014; Stals et al., 2011).

In this study, the highest average recovery rates were obtained using Methods 2 and 3, which both involve the use of Trizol reagent. Virus recovery with Method 2 and Method 3 were similar, but Method 2, which does not require any organic solvent, was preferred because organic solvents could interfere with molecular amplification. The recovery rate of MNV-1 with the selected method showed a 25-fold increase in comparison with the recovery rate using the PEG concentration method (Method 1). Higher samples sizes were not tested because an increase of the amount of fat could rise consequently the PCR inhibition. Moreover, it should be necessary to use higher amounts of Trizol which is a chemical reagent.

A number of virus detection methods in complex food have been described and various methods have been developed by using direct extraction with Trizol reagent (Baert et al., 2008; Morillo et al., 2012; Schwab et al., 2000; Stals et al., 2011). Trizol reagent extraction followed by conventional RT-qPCR assay is a suitable methodology for the identification of NoV in Indian sauces, herbal butter, deli ham and potato salad (Boxman et al., 2007; Girard et al., 2013; Morillo et al., 2012; Rutjes et al., 2006). In comparison with the direct virus extraction method used on pasta salads (Stals et al., 2011), 5 times higher recovery rates of norovirus from grated carrots with dressings were obtained with the method 2.

Unlike the direct virus extraction method developed by Stals et al. (2011), virus recovery rates obtained with Method 2 were not influenced by the virus inoculation level or by the type of vegetable (lettuce, grated carrots or a mixture of raw grated vegetables (carrots, celery and cabbage)). Indeed, virus extraction yields can vary according to food type. The differential behavior of the spiked viruses depends on the dressing used because the viral recovery is highly dependent on several factors, such as food type, viral extraction procedure, and the virus itself (Hennechart-Collette et al., 2015; Mormann et al., 2010; Scherer et al., 2010).

The LOD_{100} values of NoV with dressing ranged respectively from 10^5 to 10^6genome copies/g for NoV GI and from 10^4 to 10^5 genome copies/g for NoV GII which are in agreement with data reported in other studies in food. The reported LOD_{100} values of NoV GI and NoV GII are respectively 10^5 genome copies and 10^3 genome copies in milk products, 10^4 and 10^3 genome copies in water and 10^3 genome copies of NoV GII in pasta salads, 10^2 genome copies of NoV GI and GII in fruit salads and vegetable salads (Baert et al., 2008; Cheng et al., 2018; Hennechart-Collette et al., 2014, 2017). Dressing vegetables are complex vegetables because of the oily, fatty or emulsified food ingredients which can explain the highest LOD_{100} obtained for NoV and HAV in dressing vegetables in comparison with the LOD recently reported for lettuce (< 1 genome copies per g for NoV and 3 genome copies per g for HAV) (Lowther et al., 2019).

To conclude, method developed in this study successfully detected viruses in oily vegetables according to the ISO recommendation in terms of controls (process control and EAC). Indeed, rates of inhibition in RNA extracted from food samples were lower than 75%, and MNV-1 extraction yields were higher than 1% which validate the controls according to the recommendations in the NF EN ISO 15216-1. It could be further evaluated to analyze naturally contaminated food samples in case of outbreaks. Finally, supplementing the ISO procedure, the method described herein can be applied to detect NoV and HAV in dressed products for routine diagnosis needs.

Acknowledgments

We thank P. Pothier (CNR des virus des gastroentérites, Dijon, France) for providing contaminated stools.

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