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Collaborative validation of a rapid method for efficient virus concentration in bottled water

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ABSTRACT

Enteric viruses, including norovirus (NoV) and hepatitis A virus (HAV), have emerged as a major cause of waterborne outbreaks worldwide. Due to their low infectious doses and low concentrations in water samples, an efficient and rapid virus concentration method is required for routine control. Three newly developed methods, A, B and C, for virus concentration in bottled water were compared against the reference method D: (A) Convective Interaction Media (CIM) monolithic chromatography; filtration of viruses followed by (B) direct lysis of viruses on membrane; (C) concentration of viruses by ultracentrifugation; and (D) concentration of viruses by ultrafiltration, for each methods' (A, B and C) efficacy to recover 10-fold dilutions of HAV and feline calicivirus (FCV) spiked in bottles of 1.5 L of mineral water. Within the tested characteristics, all the new methods showed better performance than method D. Methods A, B and C shared a limit of detection (LOD_{50}) of nine 50%-tissue culture infectious dose ($TCID_{50}$) of FCV/1.5 L, but differed with regard to the LOD_{50}'s of HAV with 45, 361 and 3607 $TCID_{50}$/1.5 L, respectively, and the percentage of recoveries of HAV/FCV with 34/6, 32/25 and 0.3/0.5, respectively. Method B resulted in significantly ($p<0.0001$) lower C_t-values for both HAV and FCV relative to the reference method D than any of the other methods. The most efficient method (B) was evaluated through a collaborative trial by five laboratories for the detection of HAV, FCV and NoV genogroup I and II (GI and GII), which resulted in the corresponding average LOD_{50}'s and percentage of recoveries: 211 $TCID_{50}$/1.5 L and 51% for HAV; 66 $TCID_{50}$/1.5 L and 34% for FCV; 9 reverse transcriptase-PCR Units (RT-PCR U)/1.5 L and 61% for NoV GI and 286 RT-PCR U/1.5 L and 35% for NoV GII. The results indicate that method B could be considered robust enough for routine control and useful for harmonized data generation.

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1. Introduction

The increasing global demand for bottled water has resulted in the total consumption of 154 billion litres (41 billion gallons) in 2004 (Gleick, 2008). Thus, there is a major public health interest in risks associated with consumption of water. Detection of NoV RNA has been reported in some brands of bottled water (Beuret, 2003; Whatley et al., 1968), while the risk of intentional contamination of the drinking water with enteric viruses has been considered (Rose, 2002; Whatley et al., 1968). Enteric viruses, spread by the faecal–oral route and responsible for gastroenteritis and hepatitis, are a major cause of water-related diseases (Bosch et al., 2008), in particular, norovirus (NoV) (Anderson et al., 2003; Boccia et al., 2002; Kukkula et al., 1997; Kukkula et al., 1999) and hepatitis A virus (HAV) (De Serres et al., 1999; Whatley et al., 1968) have been documented as causative agents in several outbreaks. In addition, although viral outbreaks related to bottled water have not yet been documented, it has been reported that bacterial counts in bottled water often exceeded those in tap water (Lalumandier and Ayers, 2000). Because human excreta present in these source waters have the potential to harbor hundreds of pathogenic microorganisms of public health concern (Leclerc et al., 2002), it has been recommended to test regularly, but not routinely, for viral pathogens (Leclerc and Moreau, 2002). Finally, the call text of the EU research program that funded the present work (part of *BIOTRACER* project) had specifically asked for appropriate methodologies to be used in the case of suspicion of bio-attack on the water supply (including bottled water). The data generated by such tests on the presence and spread of virus in the bottled water production chain can be fed into a virtual contamination scenario to develop a decision-support model for control authorities.

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The infective dose of enteric viruses is usually low; and particularly for NoV, as few as 18 particles are enough to cause illness (Teunis et al., 2008). Combined with the presumption that enteric viruses can be found in low numbers in contaminated water, it is important to develop a non-complex and efficient screening method that is able to detect low concentration of viruses in drinking or bottled water (Brassard et al., 2005).

Virus testing of presumably contaminated water is usually a two-step procedure. First, the virus particles are concentrated from a large volume of water using positive or negative charged membranes (Brassard et al., 2005; Dubois et al., 2007; Mendez et al., 2004; Queiroz et al., 2001; Whatley et al., 1968). Second, the adsorbed viruses are eluted from the membrane using an appropriate buffer, and the viruses are further concentrated by e.g. ultracentrifugation (Kurdziel et al., 2001) or ultrafiltration (Dubois et al., 2007; Gilgen et al., 1997; Haramoto et al., 2004; Katayama et al., 2002; Rutjes et al., 2006). Recent papers describe the development and evaluation of new methods carried out in Europe (Di Pasquale et al., 2010a; Kovac et al., 2009; Perelle et al., 2009). The first method used Convective Interaction Media (CIM) monolithic chromatographic columns for viral concentration as an alternative to positively charged membranes (Kovac et al., 2009). CIM is a chromatographic support made of a single block of porous material with interconnected channels that are based on convective flow. It can enable rapid mass transfer of the sample molecules between the mobile and the stationary phase (Strancar et al., 2002). The larger channels with a diameter of about 1500 nm can allow the circulation of viruses, for example. The properties of the system can speed the separation process and reduce the back pressure and non-specific binding. CIM may increase the productivity of chromatographic processes by one order of magnitude over traditional chromatographic columns packed with porous particles (Strancar et al., 2002). The second method describes a direct extraction of the viruses from the positively charged membrane using a robotic RNA purification protocol based on the NucliSens easyMAG platform (Perelle et al., 2009). This automated instrument uses the same extraction chemistry as the semi-automated NucliSens mini-MAG. The reagents in NucliSens are based on the widely used Boom method (Boom et al., 1990), combined with magnetic silica particles. Hence, guanidine thiocyanate (GITC) is utilised to denature viral coat proteins, and released nucleic acid is bound to silica which is then purified through successive washing stages before final elution in a small volume. In the third study, the authors found that virus solutions were more effectively concentrated by ultracentrifugation than by ultrafiltration. However, they defined that the critical point of both procedures is the capacity of the buffer solution to elute viruses trapped in the membrane during the first step of the concentration process (Di Pasquale et al., 2010a).

The present study is the first harmonized attempt to compare the efficiency of the above methods against an ultrafiltration-based method, which is slightly modified from Gilgen et al. (1997). Then, the most promising protocol to efficiently concentrate enteric viruses from bottled water followed by extraction of RNA was evaluated for its robustness in a collaborative trial among five European laboratories. The validation criteria were limit of detection, percentage of recovery, reproducibility, rapidity and simplicity.

2. Materials and methods

2.1. Viruses and cells

Reference stocks of HAV (ATCC HM175/18f) containing 2×10^7 $TCID_{50}$/mL and FCV (strain F9 — ATCC VR-782) containing 1×10^7 $TCID_{50}$/mL were correspondingly propagated in Frp/3 cells derived from FRhK-4 (De Medici et al., 2001; Venuti et al., 1985) and Crandell–Reese feline kidney (CRFK) cells (ATCC CCL-94) (Crandell et al., 1973; Doultree et al., 1999) and titrated by $TCID_{50}$ assays (Crandell et al., 1973; De Medici et al., 2001; Reed and Muench, 1938) at the Instituto Superiore di Sanità (ISS), Rome, Italy, and distributed to partner laboratories. Two stool samples each containing NoV GI.4 or NoV GII.4 both quantified to 1×10^7 reverse-transcriptase - polymerase chain reaction (RT-PCR) units (U)/mL, determined by endpoint real-time RT-PCR titration (da Silva et al., 2007; Loisy et al., 2005; Svraka et al., 2007) were supplied to the participating laboratories by ISS (NoV GI) and Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia (NoV GII). Suspensions of 10-fold serial diluted stocks of HAV, FCV, NoV GI.4 and NoV GII.4 were made in phosphate-buffered saline (PBS; 145 mM NaCl, 7.7 mM Na_2HPO_4, and 2.3 mM NaH_2PO_4, pH 7.4) at the recipient laboratories prior to being used in the experiments during the comparative study (HAV and FCV) and collaborative trial (HAV, FCV, NoV GI.4 and NoV GII.4).

2.2. Spiking of bottled water

Bottled mineral water, 1.5 L, "Monte Cimone", kindly provided and distributed to the laboratories by *BIOTRACER* partner COOP, Bologna, Italy, was used throughout this study. For the comparative study, five bottles of mineral water were spiked with 100 µL of the diluted suspensions of HAV and FCV stocks resulting in final quantities ranging from 10 to 10^5 $TCID_{50}$ of HAV and FCV. For the collaborative trial, six bottles of water were spiked with 100 µL of diluted suspensions of all four viruses to final quantities ranging from 1 to 10^5 $TCID_{50}$ HAV and FCV and 10 to 10^6 RT-PCR U of NoV GI and NoV GII. A sample of non-inoculated water was used as negative process control in each experiment carried out for both the comparative study and the collaborative trial.

2.3. RNA extraction procedures

In the comparative study, viruses were concentrated and the RNA extracted from 1.5 L of spiked bottled mineral water using the methods A, B, C and D described below and illustrated in Fig. 1, whereas method B was further applied in the collaborative trial. In all cases, the extracted nucleic acids were eluted in 100 µL of elution buffer.

2.3.1. Method A

The first step for virus concentration from samples of bottled water consisted of anion-exchange chromatography using positively charged CIM quaternary amine (QA) 8-mL tube monolithic column (BIA Separations, d.o.o., Ljubljana, Slovenia) connected to an HPLC pump and a UV–Vis detector (Knauer, Berlin, Germany) equipped with a preparative flow cell. Samples were pumped through the tube column and viruses were eluted from the matrix by washing with at least 10 column volumes of 50 mM of phosphate buffer containing 1 M NaCl, pH 7.0. The eluted sample of typically 15 mL was further concentrated by ultracentrifugation (Beckman L8-80 M, Fullerton, USA) at 85,750×g and 4 °C for 1 h and the resulting pellet was resuspended in 560 µL of AVL buffer (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany) followed by RNA isolation according to the manufacturer's instructions (Kovac et al., 2009).

2.3.2. Method B

Viruses were concentrated from samples of bottled mineral water by membrane filtration under vacuum using a Zetapor (Cuno Filtration SAS 3 M, Cergy Pontoise, France) 47-mm positive-charged membrane of pore size 0.45 µm. The flow rate used during filtration was around 1.5 L/17 min. Then, filters were processed as described previously by Perelle et al. (2009). The filter membrane was directly incubated 20 min at ambient temperature in a 60-mm diameter Petri dish containing 3 mL of NucliSens easyMAG lysis buffer (BioMérieux, Marcy l'Etoile, France). The entire lysate was collected and subjected to the NucliSens easyMAG platform (BioMérieux) for total nucleic acid

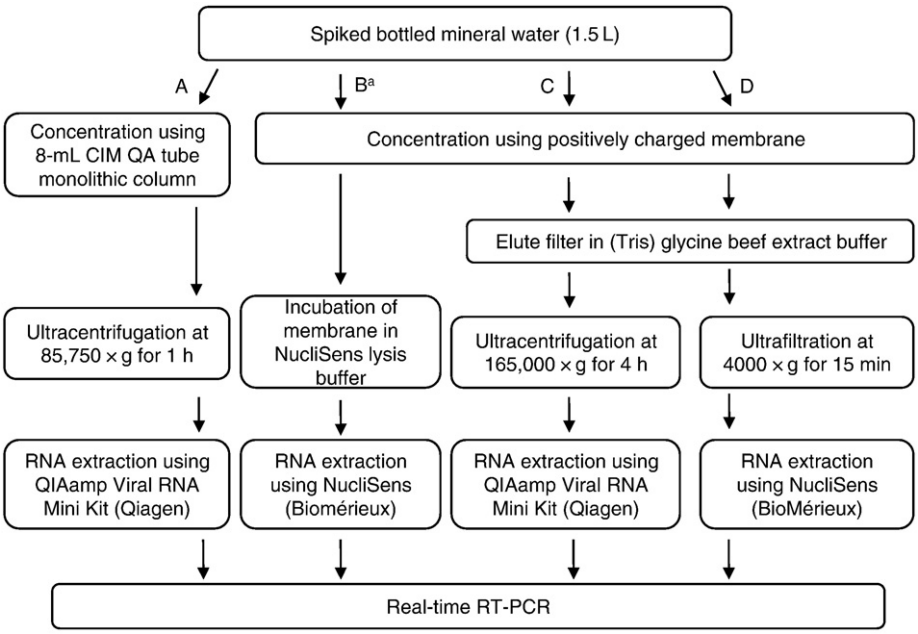


Fig. 1. Flow diagram of methods A (Kovac et al., 2009), B (Perelle et al., 2009), C (Di Pasquale et al., 2010a) and D (Gilgen et al., 1997) used in the comparative study showing the main steps of viral concentration and RNA extraction of viruses in bottled mineral water.
[a]Method B was further evaluated in the collaborative trial.

purification by the 'off board Specific A' protocol according to the manufacturer's instructions. In the collaborative trial, laboratory L1, L3, L4 and L5 used the semi-automatic NucliSens miniMAG (BioMérieux) protocol according to the manufacturer's instructions as an alternative to the fully automatic easyMAG platform that was used in partner laboratory L2.

2.3.3. Method C

Viruses were concentrated from samples of bottled mineral water by filtration using 47-mm electropositive-charged membrane with a pore size of 0.45 µm (Sartolon Polyamid, 25006-47-N, Sartorius, Goettingen, Germany). The membrane was subsequently transferred to a 50-mL centrifuge tube, and 3 mL of 50 mM glycine 1% beef extract pH 9.4 (GBE) buffer were added. The centrifuge tubes were vortexed and shaken at ambient temperature at 500 rpm for 20 min. The membrane was removed and the sample was transferred into an ultracentrifuge tube and ultracentrifuged (TLA 100.4 Beckman Coulter, Cassina de Pecci (MI), Italy) at 165,000 × g for 4 h at 4 °C in order to concentrate the virus particles. Finally, the pellet was resuspended in 100 µL of phosphate-buffered-saline (PBS) and viral RNA was extracted by QIAamp viral RNA mini kit (Di Pasquale et al., 2010a).

2.3.4. Method D

As the reference method, a modified version of the protocol described by Gilgen et al. (1997) was used. Initially, viruses were concentrated using a positively charged membrane as described for method B. After filtering the water sample, the filter was transferred to a 50-mL centrifuge tube containing 10 mL of 100 mM Tris 50 mM glycine 1% beef extract pH 9.5 (TGBE) buffer and shaken at ambient temperature at 500 rpm for 20 min. The solution was adjusted to pH 8.0 with HCl and the entire volume was transferred to centrifugal filter unit (Amicon Ultra-15, Ultracell-100 membrane, Millipore) and centrifuged for 13 min at 4000 × g until a volume of 500 µL or below was reached. If needed, the concentrate was adjusted to 500 µL with PBS and retained for RNA extraction using the NucliSens MiniMAG (BioMérieux) protocol according to the manufacturer's instructions.

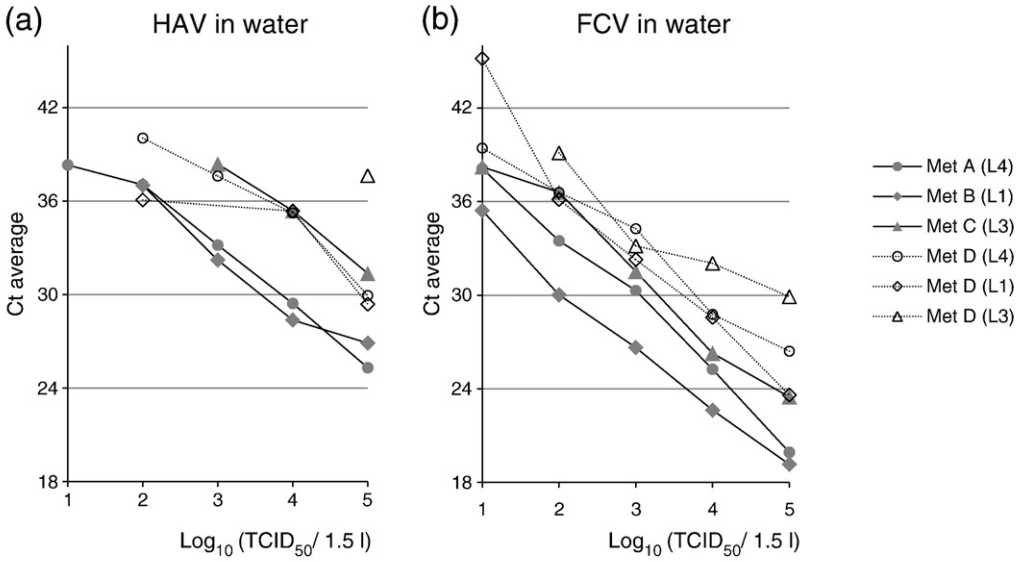
2.4. Viral detection by real-time RT-PCR

Detection of HAV and FCV RNA extracted from viral stocks and spiked water samples in the comparative study as well as the collaborative trial was performed using a two-step TaqMan RT-PCR as reported by Di Pasquale et al. (2010b). For the detection of NoV GI and NoV GII RNA extracted from stool and water samples in the collaborative trial, a one-step TaqMan RT-PCR format with the forward primer/reverse primer/probe, QNIF4/NV1LCR/NVGG1p (da Silva et al., 2007; Svraka et al., 2007) for NoV GI and QNIF4/COG2R/QNIFS (Kageyama et al., 2003; Loisy et al., 2005) for NoV GII were used. All probes were labelled in 3′ with FAM and in 5′ with TAMRA.

For HAV and FCV detection, 10 µL of the extracted viral RNA was added to 15 µL of RT mixture, containing 1 × StrataScript Buffer (Stratagene), 3 mM $MgCl_2$ (Fermentas), 0.2 mM of each dNTP (Eppendorf) and 0.5 µM of the reverse primer for either HAV or FCV and incubated for 60 min at 42 °C to produce cDNA followed by heating for 2 min at 95 °C to terminate the reaction. Then, 2.5 µL of the cDNA was added to 22.5 µL of real-time PCR mixture, containing 12.5 µL of 2 × TaqMan MasterMix (Applied BioSystem), 0.9 µM of both reverse and forward primer and 0.2 µM of probe. The real-time PCR was carried out on an ABI 7000 (L1), ABI 7700 (L3 and L5) and ABI 7900 using a 96- or 384-well plate (L2 and L4), applying the following reaction conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, and 60 °C for 1 min (Di Pasquale et al., 2010b).

For the detection of NoV GI and NoV GII, the RNA Ultrasense One-step qRT-PCR system (Invitrogen) was applied in a total of 25 µL per reaction mixture constituting 5 µL of extracted viral RNA and 20 µL of RT-PCR reaction mixture containing 1 × Thermoscript reaction mix, 500 mM forward primer, 900 mM reverse primer, 250 mM Probe, 1 × Rox reference dye and 1 × UltraSense enzyme mix. The RT-PCR was carried out using the following reaction conditions: RT at 55 °C for 1 h, preheating at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C for 15 s, amplification at 60 °C for 1 min and annealing/extension at 65 °C for 1 min.

Two negative controls were included in each run, while RNA extracted from HAV, FCV, NoV GI and NoV GII stocks served as positive amplification controls. The cycle threshold (C_t) was the cycle at which fluorescence became distinguishable from the background and a



(c)

Method characteristics	Laboratory performance of method A, B, C and D											
	HAV						FCV					
	A	B	C	D			A	B	C	D		
	L4	L1	L3	L4	L1	L3	L4	L1	L3	L4	L1	L3
LOD_{50}[a]	45	361	3607	692	6919	85960	9	9	9	29	45	86
Percentage of recovery[b]	34 ± 5	32 ± 10	0.3 ± 0.1	3 ± 3	1 ± 1	<0.1	6 ± 6	25 ± 8	0.5 ± 0.4	0.6 ± 0.3	0.8 ± 0.6	<0.1 ± 0.0

Fig. 2. Data obtained by evaluating methods A, B and C against the reference method D for the efficiency to recover HAV (a) and FCV (b) RNA from 1.5 L of bottled mineral water spiked with 10-fold serial dilutions of viral stocks. C_t-values obtained by using method D (non-filled symbols) was compared against method A (filled circle) by L4, method B (filled square) by L1 and method C (filled triangle) by L3.
[a]LOD_{50}, Limit of detection ($TCID_{50}$/1.5 L for HAV and FCV or RT-PCR U/1.5 L for NoV GI and NoV GII) at which 50% of replicates are positive. [b]The average percentage of recovery obtained for the different loads of virus were calculated for each participating laboratory.

sample was considered positive when it gave rise to a sigmoid curve crossing the threshold line placed above the background signals of the non-virus containing samples.

2.5. Data analysis

In order to evaluate the efficiency of methods A, B and C, the performance of each method to recover RNA from 10-fold serial dilutions of HAV and FCV spiked in 1.5 L of bottled mineral water were tested against the reference method D in three laboratories, L1, L3 and L4. Based on the results obtained from the comparative study, method B was chosen to be further evaluated in five partner laboratories (L1, L2, L3, L4 and L5) for its reproducibility, robustness and efficiency to recover RNA from bottled mineral water spiked with 10-fold serial dilutions of HAV, FCV, NoV GI and NoV GII (see Section 2.2).

2.5.1. Comparative study

All steps from the water spiking to the RNA extraction were performed in three repetitions in the comparative study, and each of the resulting RNA extracts were tested in duplicate for viral recovery using the viral-specific RT-PCRs described in Section 2.4. Standard curves based on RNA extracts of the same levels of viruses as were used for the water spiking experiments were carried out in parallel to these (data not shown). The dataset returned from the water spiking experiments consisted of 216 PCR determinations in total; where each laboratory (L1, L3 and L4) provided 72 C_t-values per virus (HAV and FCV), recovered from six spiking levels (including negative control), extracted using the methods A and D (L3), B and D (L1), and C and D (L4). The results are shown in Fig. 2, where mean C_t-values are plotted against viral load/1.5 L of water. For each method and each virus, the level where 50% of the replicates was positive or negative (LOD_{50}) was determined (Fig. 2c) using the Spearman–Kärber method. The laboratory-specific and mean percentage of equivalent viral recovery (Fig. 2c) from spiked water samples were calculated using the regression lines of the standard curves created for the respective viruses in each laboratory. In addition, the method characteristics based on C_t-values were evaluated statistically (Table 1). Data with a C_t-value more than 5 U lower than its replicates or than the expected mean value were considered as outliers and removed from the dataset before analysis. In total, three observations were removed. The data was statistically analysed by using SAS. The experimental design was an incomplete block design, since the experiment was carried out in three different laboratories (blocks) and since all four methods were not tested in each laboratory (incomplete). Since each laboratory evaluated one of the methods A, B, and C against the reference method D, a variance analysis for method D alone was carried out to test the block effect. As the block effect was significant, the data obtained by using methods A, B and C were compared with the corresponding data

Table 1
Results from 3 variance analyses based on the C_t-values obtained in the comparative study. Methods A, B and C were each evaluated against the reference method D for the efficiency to recover 10-fold serial dilutions of HAV and FCV spiked in 1.5 L of bottled water. The p values for each variable, *Method*, *Virus* and *Concentration*, are estimated. Numbers in brackets are insignificant variables and were removed successively from the model.

Source of variation	A vs D	B vs D	C vs D	C vs D[a]
Virus	0.129	<0.0001	<0.0001	<0.0001
Concentration	<0.0001	<0.0001	<0.0001	<0.0001
Method	0.095	<0.0001	0.214	<0.0001
Concentration × *Virus*	0.026	(0.160)	(0.465)	(0.465)
Method × *Virus*	(0.398)	(0.950)	(0.948)	(0.948)
Concentration × *Method*	0.012	(0.249)	0.017	(0.060)

[a] Calculated values if the dataset, *Method* = D, *Virus* = HAV, *Concentration* = 5 $TCID_{50}$/1.5 L water, is considered as outlier and removed from the model.

obtained by the relevant laboratory when using method D. In all variance analyses, a full model with interactions was set up and reduced as insignificant variables were identified. The main variables were *Laboratory*, *Virus*, *Concentration*, and *Method*. In the analysis of the block effect (method D alone) the variable, *Method* and its interactions, were not included. All variables were categorical except for *Concentration,* which together with its interactions was a regressor, representing the different slopes in the regression lines.

2.5.2. Collaborative trial

All steps from the water spiking to the RNA extraction were performed in two repetitions for the collaborative trial, and each of the resulting RNA extracts were tested in duplicate for viral recovery using the viral-specific RT-PCRs described in Section 2.4. Standard curves (Fig. 3) related to either the NucliSens easyMAG (used in L1) or the NucliSens miniMAG (used in L2, L3, L4 and L5) were plotted by testing 100 µL of 10-fold serial dilutions of viral stocks resulting in a final range of 10^{-1}–10^{5} $TCID_{50}$ HAV, 10^{-2}–10^{4} $TCID_{50}$ FCV or 1–10^{6} RT-PCR U of both NoV GI and NoV GII. The slopes of the regression lines were used to calculate the laboratory-specific (not shown) and mean (Fig. 3e) amplification efficiency (E_a) of the real-time RT-PCRs, according to the formula:

$$E_a = \left(10^{-1/\text{slope}}\right) - 1 \quad (1)$$

(Tichopad et al., 2003).

The dataset obtained from the experiments with spiked water samples consisted of 560 PCR determinations in total; 28 C_t-values per virus (HAV, FCV, NoV GI.4 and NoV GII.4) recovered from seven spiking levels (including negative control), and extracted using method B in each of the participating laboratories; L1, L2, L3, L4 and L5.

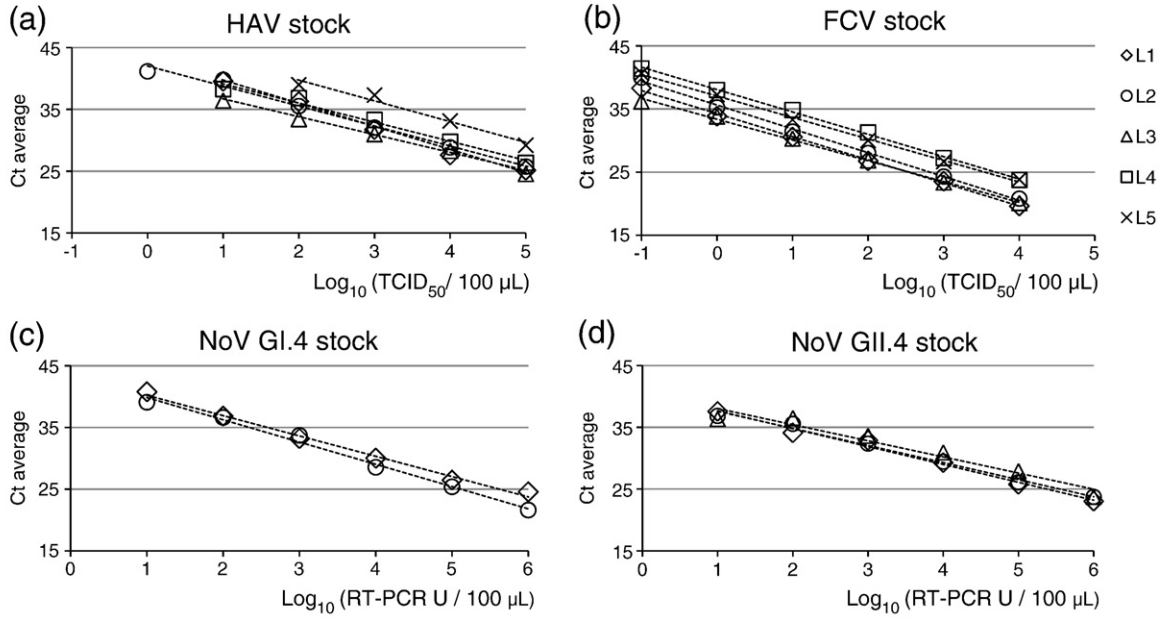
The number of positive detections of four RT-PCR determinations per viral load for each virus obtained by method B is illustrated in Table 2 and the C_t-values were used to plot the curves representing mean C_t-values against viral load/1.5 L of water in Fig. 4. From these data, we determined the qualitative limit of detection (LOD_{50}) of the assay expressed as $TCID_{50}$ for HAV and FCV or RT-PCR U for NoV GI.4 and NoV GII.4 (Table 2).

The regression lines of the standard curves (Fig. 3) created for each virus in each laboratory were used to calculate the laboratory-specific (not shown) and mean (Table 2) percentage of equivalent viral recovery from spiked water samples. In order to compare the amplification efficiency of viral RNA extracted from stocks (Fig. 3e) and spiked water samples, we calculated the laboratory-specific (not shown) and mean (Fig. 4e) parameters of the regression lines obtained on RNA extracted from water samples as well.

3. Results

3.1. Comparative study

The results from the comparative study are reported in Fig. 2, where curves representing C_t-values against viral loading show the endpoint detection of HAV and FCV extracted by the different



(e)

Parameters	Data[a] from standard curves			
	HAV[b]	FCV[b]	NoV GI[c]	NoV GII[d]
Slope	-3.26 ± 0.32	-3.54 ± 0.19	-3.45 ± 0.22	-2.76 ± 0.14
E_a[e]	1.04 ± 0.14	0.92 ± 0.07	0.95 ± 0.08	1.31 ± 0.10
Y intercept when X = 0.0	40.64 ± 2.77	35.68 ± 0.93	43.47 ± 0.03	40.53 ± 1.15
R^2[f]	0.99 ± 0.01	1.00 ± 0.00	0.99 ± 0.00	0.97 ± 0.02

Fig. 3. Standard curves and corresponding data of the real-time RT-PCR detection performed on extracted RNA from serial 10-fold dilutions of viral stocks. Viral RNA was extracted by either the NucliSens fully automatic easyMAG (L1) or the semi-automatic miniMAG (L2, L3, L4, and L5) on two distinct occasions, and virus detection was carried out in duplicates by real-time RT-PCR. Results are given in C_t-values against viral loadings expressed in $\log_{10}$ $TCID_{50}$ for (a) HAV and (b) FCV and $\log_{10}$ RT-PCR U for (c) NoV GI.4 and (d) NoV GII.4. Parameters for the amplification efficiencies are shown in the Table (e).

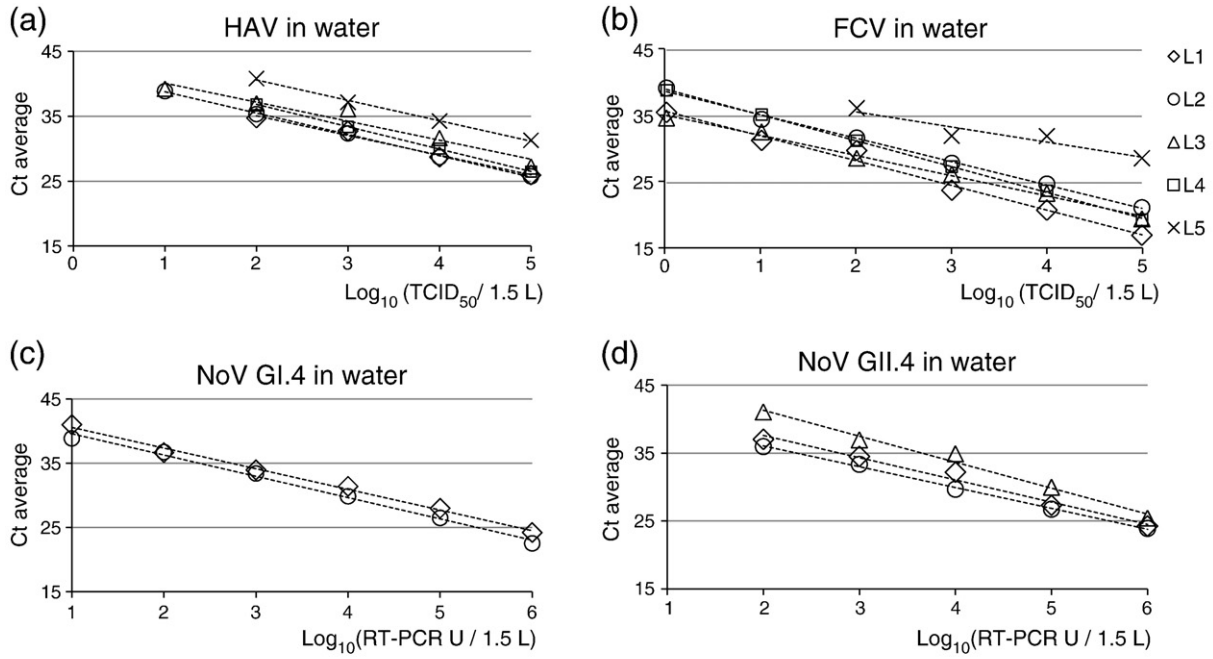
[a]Mean values and standard deviations of standard curve parameters obtained in each laboratory were calculated for each virus stock. [b]Five laboratories returned data for HAV and FCV, while [c]two and [d]three laboratories returned data for NoV GI.4 and NoV GII.4, respectively. [e]The amplification efficiency and [f]the correlation coefficient.

Table 2
Data obtained in the collaborative study showing the viral recovery expressed by endpoint detection, limit of detection (LOD_{50}) and percentage of recovery of viruses in 1.5 L of bottled mineral water. Bottles of mineral water were spiked with 10-fold serial dilutions of HAV, FCV, NoV GI and NoV GII. Viral RNA was extracted on two distinct occasions using method B and detection of viruses were carried out in duplicates, resulting in four determinations for each type and load of virus.

Viral loads[a]	No. of positive analysis obtained from each participating laboratory (L) on each virus type														
	HAV					FCV					NoV GI		NoV GII		
	L1	L2	L3	L4	L5	L1	L2	L3	L4	L5	L1	L2	L1	L2	L3
1×10^6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4	4	4	4	4
1×10^5	4	4	4	4	4	4	4	4	4	2	4	4	3	4	4
1×10^4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4
1×10^3	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4
1×10^2	2	4	4	4	2	3	4	4	4	3	4	4	3	4	2
1×10^1	0	4	1	0	0	4	4	4	4	0	4	4	0	0	0
1×10^0	0	0	0	0	0	4	4	3	4	0	ND	ND	ND	ND	ND
LOD_{50}[b]	448	9	62	86	448	1	1	3	1	323	9	9	324	86	448
Mean LOD_{50}[c]	211 ± 218					66 ± 144					9 ± 0		286 ± 184		
Percentage of recovery[d]	63 ± 8	70 ± 35	8 ± 6	70 ± 17	43 ± 13	72 ± 28	13 ± 4	20 ± 10	66 ± 12	1 ± 1	61 ± 13	61 ± 8	24 ± 16	64 ± 25	17 ± 29
Mean Percentage of recovery	51 ± 26					34 ± 32					61 ± 0		35 ± 25		

ND, not done.
[a] Viral loads are expressed in $TCID_{50}$ for HAV and FCV and RT-PCR U for NoV GI and NoV GII.
[b] LOD_{50}, Limit of detection at which 50% of replicates are positive.
[c] Mean values and standard deviations of the LOD50's were calculated for each virus.
[d] The mean percentage of recoveries obtained for each virus by each laboratory were calculated.

methods. All of the methods examined were able to detect as few as 10 $TCID_{50}$ of FCV/1.5 L of water, while methods A, B and D could detect 100 $TCID_{50}$ of HAV/1.5 L of water in at least one of six replicates. However, differences were observed between the methods when LOD_{50}'s and virus recoveries were calculated, see Fig. 2c, and the C_t-values were statistically analysed.



(e)

Parameters	Data[a] from viral recoveries in water			
	HAV[b]	FCV[b]	NoV GI[c]	NoV GII[d]
Slope	-3.17 ± 0.20	-3.28 ± 0.65	-3.27 ± 0.07	-3.39 ± 0.39
E_a[e]	1.07 ± 0.09	1.10 ± 0.39	1.02 ± 0.03	0.99 ± 0.15
Y intercept when X = 0.0	43.36 ± 2.21	37.79 ± 2.21	43.36 ± 0.63	45.09 ± 3.48
R^2[f]	0.98 ± 0.03	0.97 ± 0.04	0.99 ± 0.00	0.99 ± 0.01

Fig. 4. Results obtained in the collaborative trial; correlation between the recovery of spiked virus and mean C_t-value within the linear range. In each laboratory (L), virus from artificially contaminated bottled mineral water samples were extracted on two distinct occasions using method B, and detection was carried out in duplicates by real-time RT-PCR. Results are given in C_t-values against viral loading expressed in $\log_{10}$ $TCID_{50}$ for (a) HAV and (b) FCV and $\log_{10}$ RT-PCR U for (c) NoV GI.4 and (d) NoV GII.4. Parameters for the amplification are shown in the Table (e).
[a]Mean values and standard deviations of amplification parameters were calculated for each recovered virus. [b]Five laboratories returned data for HAV and FCV while [c]two and [d]three laboratories returned data for NoV GI.4 and NoV GII.4, respectively. [e]The amplification efficiency and [f] the correlation coefficient.

The analysis of the block effect (method D alone) showed significant ($p<0.0001$) differences among laboratories using the reference method D, as higher C_t-values were measured by L3 than L1 and L4. However, all six slopes from the regression lines applied in the model showed statistical equivalence, as all interactions with "Concentration" were insignificant (Table 1).

The analysis of the relative effect of each of the evaluated methods (A, B, and C) compared with the reference method D, showed that method B had significantly ($p<0.0001$) lower C_t-values (5.2 in average) for both HAV and FCV relative to method D than any of the other methods (Table 1). From the analysis of method B versus method D (L1) it was moreover found that the slopes of the regression lines for the C_t-values was independent of virus (HAV and FCV) and method (B and D), since all interactions with "Concentration" were insignificant. These findings are attractive features when comparing and summing up results. Analysis of A versus D and C versus D resulted in several significant interactions, and on average, a smaller difference on the C_t-value between the test method (A and C) and the reference method D.

Based on the following method performance criteria; LOD_{50}'s, percentage of viral RNA recoveries, lowest C_t-values in the linear range compared with the reference method D, simplicity and equipment demands (see Discussion section), method B was chosen for the collaborative trial on spiked bottled mineral water.

3.2. Collaborative trial

3.2.1. RNA extraction and virus detection

The standard curves (Fig. 3) issued from the laboratories participating in the collaborative trial were obtained by testing 10-fold serial dilution of the RNA extracts of the corresponding viruses, HAV, FCV, NoV GI and NoV GII (see Section 2.5.2). Results, expressed in C_t-values against viral load expressed in $\log_{10}$ $TCID_{50}$ for HAV and FCV and RT-PCR U for NoV GI and NoV GII (Fig. 3), showed that the laboratories obtained equivalent standard curves for HAV, FCV, NoV GI and NoV GII regardless if the NucliSens easyMAG (L1) or the NucliSens miniMAG (L2, L3, L4 and L5) were used. This equivalence is also mirrored in the small standard deviations of the parameters given in Fig. 3e, where, e.g. the average amplification efficiencies (E_a) on the viral RNA extracted using the NucliSens easyMAG/NucliSens miniMAG procedure in the participating laboratories were 1.04 ± 0.14 for HAV, 0.92 ± 0.07 for FCV, 0.95 ± 0.08 for NoV GI and 1.31 ± 0.10 for NoV GII.

3.2.2. Viral recovery from spiked water samples

Regarding the experiments of viral recovery from bottled mineral water using method B, two laboratories returned results for the detection of all four viruses, another laboratory returned results for the detection of HAV, FCV and NoV GII, and finally two laboratories returned results just for the detection of HAV and FCV (Table 2 and Fig. 4). The lack in return of NoV GI and GII results by three of the laboratories and two of the laboratories, respectively, was because the laboratories had difficulties to implement the one-step RT-PCR assay during the project period. The mean LOD_{50}'s, were found to be 211 and 66 $TCID_{50}$ for HAV and FCV, respectively (five laboratories), 9 RT-PCR U for NoV GI (two laboratories) and 286 RT-PCR U for NoV GII (three laboratories), see Table 2. The mean percentage of the recovery of each virus from 1.5 L of water is shown in Table 2, spanning from 34% for FCV to 61% for NoV GI.

4. Discussion

The results obtained from the comparative study indicated that the tested methods A (Kovac et al., 2009), B (Perelle et al., 2009) and C (Di Pasquale et al., 2010a) were, to a varying degree, more efficient to recover HAV and FCV than the chosen reference method D (Gilgen et al., 1997). Methods A, B and C could all detect as low as 9 $TCID_{50}$ of FCV/1.5 L water. Nevertheless, compared with methods A and B, the ultracentrifugation method (C) showed consistently higher C_t-values for the detected levels of both FCV and HAV. This resulted in a lower percentage of recoveries (5%–30%) for FCV and HAV and an approximately 1 to 2 $\log_{10}$ higher detection limit of HAV than methods B and A, respectively (Fig. 2c). In addition, method C required multiple sample preparation steps prior to RNA extraction (Fig. 1) and an ultracentrifuge that might not be available in all laboratories. The use of CIM (method A) to concentrate virus-spiked bottled mineral water instead of positive-charged membrane (methods B, C and D) showed promising results compared with the reference method D. Method A provided comparable results in terms of LOD_{50} (9 $TCID_{50}$/1.5 L of water) for FCV and percentage of recoveries for HAV (approx. 30%) to direct extraction of the viruses from the membrane using a robotic RNA purification protocol (method B). However, while method A reached almost one $\log_{10}$ lower limit of detection for HAV than method B, method B stood out by reaching approximately 20% higher recovery for FCV and by overall showing significantly ($p<0.0001$) the lowest C_t-values for both HAV and FCV relative to method D, than any of the other methods (A, C) (Table 1). Moreover, during this evaluation, we demonstrated that in method B the NucliSens easyMAG can be replaced by the less expensive NucliSens miniMAG without compromising the efficiency of RNA extraction. Method B requires only a few handling steps, due to the direct viral extraction from the membrane followed by application of (semi-) automatic RNA extraction equipment. These features can be helpful for routine analysis in water virology laboratories, as it can minimize the risk of cross contamination among samples, and it may be easier to implement. For these reasons, method B was further evaluated in five laboratories for its robustness and ease of use to recover viral RNA from spiked bottled mineral water.

The data obtained from the collaborative trial showed an overall easy implementation, robust extraction efficiency and good viral RNA recovery (Table 2 and Fig. 4). The pattern of C_t-values obtained by detecting spiked dilutions of all four viruses (Fig. 4) was comparable among the participating laboratories, as illustrated in the small standard variation of the parameters calculated from the standard curves (Fig. 4e). In addition, an overall comparison of the parameters calculated for the amplification of viral RNA extracted from virus stocks (Fig. 3e) and spiked bottled mineral water (Fig. 4e) showed overall similar amplification efficiencies demonstrating clean RNA extracts.

While the study showed an inter-laboratory average LOD_{50} of 211 $TCID_{50}$ for HAV and 286 RT-PCR U for NoV GII, we underestimated the performance of method B to recover low amounts of FCV and NoV GI by only spiking in levels as low as 1 $TCID_{50}$ or 10 RT-PCR U, respectively. These levels were detected by most of the laboratories which returned their data. Hence, the average LOD_{50} of method B was at least as low as 66 $TCID_{50}$ of FCV and 9 RT-PCR U of NoV GI.

As reported by Perelle et al. (2009), during the development of method B, an LOD_{50} of one plaque forming unit HAV/1.5 L of water was obtained using a one-step RT-PCR detection assay. In this study, only one out of five laboratories obtained a LOD_{50} below 10 $TCID_{50}$ HAV/1.5 L (Table 2) when using a two-step RT-PCR format. The difference in detection limit observed in the present study and the one of Perelle et al. (2009) could be due to the application of different HAV stocks, assay formats that were used to determine the infective titter (plaque assay versus $TCID_{50}$ assay) and/or HAV RT-PCR detection formats (one-step versus two-step).

A noteworthy variation in the LOD_{50}'s and percentage of recoveries of particularly HAV, FCV and NoV GII (Table 2) were obtained among the laboratories, despite the simplicity of method B (Fig. 1) and the fact that a harmonized batch of bottled water, stocks of viruses, and reagents were used. This important observation indicates that part of the variation is caused by the use of inadequate standardized virus

stocks, different PCR platforms, or the human factor (laboratory personnel). The observation emphasizes the need for standardized control viruses and RNA to be used in validation studies.

We believe that a reason for a more efficient viral RNA recovery obtained by methods A and B than C or D is the elimination of the viral elution of the filter by (T) GBE buffer, as this step has been reported to be critical in concentration procedures (Di Pasquale et al., 2010a), where it seems to cause a considerable loss of viruses.

Surprisingly, the two-step TaqMan RT-PCR detection format used for HAV and FCV was easily implemented in all the partner laboratories, while the simpler and supposedly easier to use one-step TaqMan RT-PCR format used for NoV GI and NoV GII caused problems in some laboratories at the time of this study. Hence, some laboratories failed to return results for NoV GI and NoV GII, which caused a data gap for the method evaluation. Moreover, this study allowed only limited training during the method implementation, which is particularly reflected in the results of the less experienced laboratory L5 during the endpoint detection of HAV and FCV from water samples (Table 2 and Fig. 4).

In the present study, the inoculum input for HAV and FCV was expressed as infective viruses (determined by $TCID_{50}$/1.5 L water) while the input for NoV GI and GII was expressed as viral genomes (determined by RT-PCRU/1.5 L). There is a known lack of correlation between the amount of infective viruses and viral genomes in stocks of viruses for HAV (Hewitt and Greening, 2006) and FCV (Gassilloud et al., 2003), where the presence of non-encapsidated RNA or defect virus particles will result in higher genomic titter than infective titter. This lack of correlation should be taken into account in the interpretation of the results, as the genomic titter of the HAV and FCV stocks applied in this study may be higher than the infective titter. When comparing the endpoint detection of the viral stocks (Fig. 3) with the artificially spiked water (Fig. 4), a consistent loss of at least 1 $\log_{10}$U for FCV and NoV GII in water was observed in all the laboratories that returned their data. This lack of equivalent endpoint detection in the extracts of the artificially spiked water was less evident for HAV (where three out of five laboratories experienced a similar loss) and completely absent for NoV GI (where both laboratories reached the same endpoint detection of both extracts). If we assume the presence of free RNA in the HAV, FCV and NoV GII stocks that would be kept intact and detected in the RNA extract of the virus stocks but degraded after being spiked in the water sample, this would explain part of the loss in endpoint detection of FCV and NoV GII in the spiked water samples.

In conclusion, any method considered as standard needs to demonstrate reproducibility and robustness in different laboratories. In this study, five laboratories showed, to some extent, that method B could fulfil these criteria for one type of bottled water. A logical follow-up would be to study the performance of method B on a range of bottled water in order to evaluate if different compositions may affect the assay performance. In addition, it is important that a potential future standard method can detect virus in samples of naturally contaminated water, which is the case for method B, as it was used to identify HAV in drinking water suspected in an HAV outbreak in Montenegro during the summer 2008 (Werber et al., 2009).

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