A Novel High-Throughput Method for Molecular Detection of Human Pathogenic Viruses Using a Nanofluidic Real-Time PCR System
Coralie Coudray-Meunier, Audrey Fraisse, Sandra Martin-Latil, Sabine Delannoy, Patrick Fach, Sylvie Perelle

To cite this version:
Coralie Coudray-Meunier, Audrey Fraisse, Sandra Martin-Latil, Sabine Delannoy, Patrick Fach, et al.. A Novel High-Throughput Method for Molecular Detection of Human Pathogenic Viruses Using a Nanofluidic Real-Time PCR System. PLoS ONE, 2016, 11 (1), pp.e0147832. 10.1371/journal.pone.0147832. anses-02013787

HAL Id: anses-02013787
https://anses.hal.science/anses-02013787
Submitted on 11 Feb 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
A Novel High-Throughput Method for Molecular Detection of Human Pathogenic Viruses Using a Nanofluidic Real-Time PCR System

Coralie Coudray-Meunier, Audrey Fraisse, Sandra Martin-Latil, Sabine Delannoy, Patrick Fach, Sylvie Perelle

1 Université Paris-Est, ANSES, Food Safety Laboratory, Enteric viruses Unit, 14 rue Pierre et Marie Curie, 94701 Maisons-Alfort Cedex, France
2 Université Paris-Est, ANSES, Food Safety Laboratory, Idenotypath, 14 rue Pierre et Marie Curie, 94701 Maisons-Alfort Cedex, France

* sylvie.perelle@anses.fr

Abstract

Human enteric viruses are recognized as the main causes of food- and waterborne diseases worldwide. Sensitive and quantitative detection of human enteric viruses is typically achieved through quantitative RT-PCR (RT-qPCR). A nanofluidic real-time PCR system was used to develop novel high-throughput methods for qualitative molecular detection (RT-qPCR array) and quantification of human pathogenic viruses by digital RT-PCR (RT-dPCR). The performance of high-throughput PCR methods was investigated for detecting 19 human pathogenic viruses and two main process controls used in food virology. The conventional real-time PCR system was compared to the RT-dPCR and RT-qPCR array. Based on the number of genome copies calculated by spectrophotometry, sensitivity was found to be slightly better with RT-qPCR than with RT-dPCR for 14 viruses by a factor range of from 0.3 to 1.6 log10. Conversely, sensitivity was better with RT-dPCR than with RT-qPCR for seven viruses by a factor range of from 0.10 to 1.40 log10. Interestingly, the number of genome copies determined by RT-dPCR was always from 1 to 2 log10 lower than the expected copy number calculated by RT-qPCR standard curve. The sensitivity of the RT-qPCR and RT-qPCR array assays was found to be similar for two viruses, and better with RT-qPCR than with RT-qPCR array for eighteen viruses by a factor range of from 0.7 to 3.0 log10. Conversely, sensitivity was only 0.30 log10 better with the RT-qPCR array than with conventional RT-qPCR assays for norovirus GIV detection. Finally, the RT-qPCR array and RT-dPCR assays were successfully used together to screen clinical samples and quantify pathogenic viruses. Additionally, this method made it possible to identify co-infection in clinical samples. In conclusion, given the rapidity and potential for large numbers of viral targets, this nanofluidic RT-qPCR assay should have a major impact on human pathogenic virus surveillance and outbreak investigations and is likely to be of benefit to public health.
**Introduction**

Human enteric viruses constitute a serious public health concern, since they are capable of causing a variety of acute illnesses, including the most commonly reported acute gastrointestinal illness. They are mainly transmitted via the fecal-oral route either by person-to-person contact or by ingestion of contaminated water and food, particularly shellfish, soft fruits and vegetables. Enteric viruses are shed in enormous quantities in feces (10^9 to 10^10/g) and have an infectious dose on the order of tens to hundreds of virions. Enteric viruses are host-specific and are not capable of replicating in the environment, but they survive for long periods of time on food or food contact surfaces or in water (ground, surface, and drinking water) [1]. These characteristics enable enteric viruses to play a significant role in food- and waterborne outbreaks. Aside from noroviruses, which have been recognized as the largest cause of outbreaks, the viruses most often implicated in outbreaks include hepatitis viruses (hepatitis A virus and hepatitis E virus), rotavirus, adenovirus (40, 41), astrovirus, enterovirus [2, 3, 4, 5, 6, 7]. Additional viruses of lesser epidemiologic importance include human bocavirus, cosavirus, parovirus, sapovirus, tick-borne encephalitis virus (TBEV), Aichi virus, and coronavirus [8, 9, 10, 11].

Tools for rapid detection of viral pathogens are important for analyzing clinical, environmental and food samples. Detection of these enteric viruses based on their infectivity is complicated by the absence of a reliable cell culture method and the low levels of contamination of food and environmental samples [12,13]. To date, real time RT-PCR has been one of the most promising detection methods due to its sensitivity, specificity, and speed. Recently, the ISO/TS 15216–1 and 15216–2 standards covering real time RT-PCR for both quantitative determination and qualitative detection of NoV and HAV in foodstuffs were published [14, 15, 16].

The aim of this study was to develop real time RT-PCR assays for detection of a total of 19 human enteric viruses (including 3 genogroups of norovirus and 4 coronaviruses) and two control process viruses (mengovirus and murine norovirus) generally used for monitoring the recovery of viral foodstuff extraction methods. Limits of detection of the viral genomes were determined with the conventional RT-qPCR system and with the Fluidigm’s BioMark System by using the qualitative nanofluidic real-time RT-PCR array and the quantitative digital RT-PCR array. The advantages of these new detection techniques were determined by detecting and quantifying pathogenic viruses in clinical samples.

**Methods**

**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 [17]. HAV stock was produced by propagation in foetal rhesus monkey kidney (FRhK-4) cells (ATCC, CRL-1688) [18] and titrated by plaque assay [19]. The titer of viral production was established in HAV RNA genomic copies with an RT-qPCR standard curve obtained with the ten-fold diluted in vitro RNA transcripts. Based on this approach, HAV stocks had titres of 9.33 x 10^8 genome copies / mL.

Dr. H. Virgin from Washington University in the USA provided ANSES’s Fougères Laboratory in France with MNV-1 (CW1 strain) which was then propagated in mouse leukemic monocyte macrophage (RAW 264.7, ATCC TIB-71) cell line [20]. MNV-1 stock was produced as previously described [21]. The extracted RNA was confirmed with RT-qPCR and quantified by measuring absorbance at 260 / 280 nm with a NanoDrop ND-1000. Based on this approach, the production stock of MNV-1 had titres of approximately 1.36 x 10^12 genome copies / mL.
Mengovirus (strain MC0) was obtained from clarified supernatant provided by Dr. Albert Bosch from the "Enteric Virus Group" of the University of Barcelona. Mengovirus stock was produced by propagation in HeLa cells (ATCC, CCL-2™) [22]. The extracted RNA was confirmed with RT-qPCR and quantified by measuring absorbance at 260 / 280 nm with a NanoDrop ND-1000. Based on this approach, the production stock of MNV-1 had titres of approximately 6.68 x 10^{11} genome copies / mL.

Rotavirus strain Wa (human rotavirus) was obtained from the Pasteur Institute (Paris, France) and was propagated in MA-104 rhesus monkey epithelial cell line (ATCC CRL-2378) [23]. The extracted RNA was confirmed with RT-qPCR and quantified by measuring absorbance at 260 / 280 nm with a NanoDrop ND-1000. Based on this approach, the production stock of Wa had titres of approximately 3.21 x 10^{11} genome copies / mL.

Four viral strains were obtained from infected cell culture supernatants: two enterovirus strains (human B6 coxsackievirus (Schmitt strain: ATCC VR-1037AS/MK™) and human echovirus 19 (Burke strain)), one Adenovirus 40 strain (ATCC VR-931) and one Astrovirus GI strain.

Viruses and stools
The study was conducted in accordance with the ethics principles of the Declaration of Helsinki. Hepatitis A virus infection is a notifiable disease in France. The current system of mandatory reporting was approved by the Commission Nationale de l’Informatique et des Libertés (deliberation n° 02–082, November 19 2002). Patients receive oral and written information on the finality of the notification and on the modalities of information recording. This information is available on line on the web site of the Institut de Veille Sanitaire (IVS) at http://www.invs.sante.fr/content/download/6498/42945/version/2/file/fiche_info_patient.pdf for HAV samples and on the web site of the NRC at www.cnr-ve.org for enteric virus samples. All clinical and biological parameters are treated anonymously. The virological surveillance of strain diversity is performed on stored samples obtained for hepatitis A diagnosis (no need for any additional blood draw). Diagnostic laboratories are asked to contribute to HAV and enteric virus strains surveillance by sending samples to the National Reference Centre (NRC). The study was not specifically approved by an ethics committee. Human samples were collected before the study and they are anonymously collected and analyzed.

The following human stool samples were provided by the National Reference Center (NRC) for Enteric Viruses (Dijon, France): adenovirus 41 (stool n°E5669), astrovirus GI (E4883), norovirus GI (E5486; E5569; E8050), norovirus GI1 (E6929; E6618; E7859; E7022; E6992), rotavirus (RV G12P8 = E7622; RV G1P8 = E8097), Aichi virus (E6841) and norovirus GI13 + norovirus GIV.

The following human stool samples were provided by the National Reference Center (NRC) for HAV/HEV (Villejuif, France): HAV G1A (stools no. 780627147; 1181216151), HAV G1B (1280210015; 1280514230), HEV G3c (1280511146), HEV G3f (1280418084; 1280530128) and HEV G4 (1280615097; 1280522166).

The faecal samples were suspended in 1X Phosphate Buffered Saline (PBS), pH 7, to obtain a final 10% suspension (w/v), vortexed and centrifuged at 3000 g for 30 min at 4°C. Aliquots of 100 μL supernatant were kept frozen at -80°C for later use.

The extracted genomic RNA/DNA of adenovirus, astrovirus and rotavirus were confirmed with RT-qPCR and quantified by measuring absorbance at 260 / 280 nm with a NanoDrop ND-1000.

The extracted genomic RNA of norovirus GI, GII and GIV, sapovirus, Aichi virus, HAV and HEV were confirmed and quantified with RT-qPCR by using in vitro RNA standard curves (see “DNA and RNA standards”).
DNA and RNA standards

Sequences from reference strains were inserted into recombinant plasmids (Table 1). The HEV, HAV, NoV GI and NoV GII cDNA were cloned in pGEM-T Easy vector (Promega, Charbonnières-les-Bains, France) and propagated in E. coli One Shot® TOP10F™ (Life technologies, Saint Aubin, France). High-quality DNA plasmids containing HAV or NoV regions were purified using the Qiagen Plasmid midi kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s protocol. Then, NoV GI plasmid was digested with NCOI (Life technologies), and HEV DNA, HAV DNA and NoV GII DNA plasmids were digested with SpeI (Life technologies) and transcripts were obtained by using a MEGAscript™ kit (Life technologies) according to the manufacturer’s protocol. Synthesized RNA were treated with Turbo™ DNase (Life technologies) according to the manufacturer’s protocol in order to remove the DNA template following transcription, and purified by using the MEGAClear™ kit (Life technologies). The synthesized DNA and RNA were confirmed with (RT)-qPCR and quantified by measuring absorbance at 260 / 280 nm with a NanoDrop ND-1000 (Thermoscientific, Courtaboeuf, France) and the free software available on the “http://endmemo.com/bio/dnacopynum.php” website. Aliquots of 10μL containing 10⁹ genome copies / μL were kept frozen at -20°C for later use and used as standards.

Sapovirus, TBEV, norovirus GIV, Aichi virus, 229E, HKU1, cosavirus, OC43 and NL63 cDNA were cloned into the pBluescriptIISK+ vector by Genecust (Dudelange, Luxembourg). All recombinant plasmids were purified by Genecust and used to produce RNA transcripts. Sapovirus, TBEV, norovirus GIV, 229E, cosavirus and NL63 DNA plasmids (0.5 μg) were digested with EcoRV (Life technologies) and Aichi virus, HKU1 and OC43 DNA plasmids were digested with SpeI (Life technologies).

Digested plasmids were transcribed by using the MEGAscript™ kit (Life technologies) according to the manufacturer’s protocol. Synthesized RNA was treated with Turbo™ DNase (Life technologies) according to the manufacturer’s protocol in order to remove the DNA template following transcription, and purified by using the MEGAClear kit (Life technologies) according to manufacturer’s instructions. The synthesized RNA was confirmed with RT-qPCR and quantified by measuring absorbance at 260 / 280 nm with a Nanodrop ND-100 (Thermoscientific, France) and the free software available on the “http://endmemo.com/bio/dnacopynum.php” website. RNA stocks were diluted to contain 10⁹ copies / μL. Aliquots of 10 μL were kept frozen at -20°C for later use as standards.

Bocavirus and parvovirus cDNA were cloned into the pBluescriptIISK+ vector by Genecust (Dudelange, Luxembourg). Both recombinant plasmids were purified by Genecust. DNA plasmids (0.5 μg) were digested with SpeI (Life technologies) to be linearized. The synthesized DNA was confirmed with qPCR and quantified by measuring absorbance at 260 / 280 nm with a Nanodrop ND-100 (Thermoscientific, France) and the free software available on the “http://endmemo.com/bio/dnacopynum.php” website. DNA stocks were diluted to contain 10⁹ copies / μL. Aliquots of 10 μL were kept frozen at -20°C for later use as standards.

Nucleic acid extraction

Adenovirus 40, adenovirus 41, astrovirus, rotavirus, coxsackievirus B6, MNV-1, mengovirus, HAV (HM175/18f, HAV GIA and HAV GIB), HEV (HEV G3c, HEV G3G et HEV G4), NoV (GI, GII, GII+GIV), sapovirus, echovirus 19, Aichi virus, and astrovirus DNA or RNA were extracted using the NucliSens® easyMAG™ platform (Biomérieux Marcy l’Etoile, France) for total nucleic acid extraction by the “off board Specific A” protocol according to manufacturer’s instructions. Nucleic acids were eluted in 100 μL of elution buffer and stored at -80°C.
Adenovirus 41, astrovirus, rotavirus, coxsackievirus B6, MNV-1 and mengovirus DNA or RNA were confirmed with (RT)-qPCR and quantified by measuring absorbance at 260 / 280 nm with a NanoDrop ND-1000 (Thermoscientific, Courtaboeuf, France) and used as standards.

Extracted RNA from HAV (HM175/18f, HAV GIA and HAV GIB), HEV (HEV G3c, HEV G3G and HEV G4), NoV (GI, GII, GII+GIV), sapovirus, echovirus 19, Aichi virus, adenovirus 40 and astrovirus were quantified with (RT)-qPCR using standards (in vitro transcribed RNA, plasmidic DNA, extracted genomic RNA).

Primers and probes
The primers and probes used to detect all the viruses of this study are described in Table 2. Those used to detect NoV GI, NoV GII, HAV and mengovirus are described in ISO/ TS 15216–1 / 15216–2 (2013). All the primers and probes were purchased from Life Technologies or Eurofins MWG Operon (Les Ulis, France).

RT-qPCR with the CFX96™ real time PCR detection system
One-step RT-qPCR amplifications were performed on a CFX96™ real time PCR detection system from Bio-Rad (Marnes-la-Coquette, France). Reactions were performed in a 15 μL reaction mixture containing 1X of RNA UltraSense™ master mix and 0.63 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), 5 μg of bovine serum albumin (Life Technologies), 500 nM of forward primer, 900 nM of reverse primer, 250 nM of probe, and 5 μL of RNA extract. A negative control containing all the reagents except the RNA template was included with each set of reaction mixtures.

The one-step RT-qPCR program involved 60 min reverse transcription of RNA at 55°C, followed by a 15 min denaturation step at 95°C, 45 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 minute 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 target was generated with

Table 1. GenBank accession number for viral sequences used to obtain recombinant plasmids.

| Virus                  | Reference sequence | Position of the genomic sequence cloned | Plasmid used          |
|------------------------|--------------------|-----------------------------------------|-----------------------|
| Hepatitis A virus      | M59808.1           | 39–518                                  | pGEM-T Easy vector    |
| Hepatitis E virus      | AB097812           | 5301–5371                               | pGEM-T Easy vector    |
| Norovirus GI           | M87661             | 5257–5413                               | pGEM-T Easy vector    |
| Norovirus GII          | X86557             | 4981–5135                               | pGEM-T Easy vector    |
| Norovirus GIV          | JQ613567           | 4961–5140                               | pBluescriptIISK       |
| Sapovirus              | NC_006269          | 5051–5200                               | pBluescriptIISK       |
| Aichi virus            | AB040749           | 241–350                                 | pBluescriptIISK       |
| TBEV                   | U27495             | 11031–11141                             | pBluescriptIISK       |
| Parvovirus B19         | AB550331           | 2221–2420                               | pBluescriptIISK       |
| Cosavirus              | NC_012800          | 701–860                                 | pBluescriptIISK       |
| Bocavirus              | NC_012042          | 2511–2700                               | pBluescriptIISK       |
| 229E                   | AF304460           | 25351–25530                             | pBluescriptIISK       |
| HKU1                   | HM034837.1         | 28751–28940                             | pBluescriptIISK       |
| NL63                   | JX504050           | 26191–26380                             | pBluescriptIISK       |
| OC43                   | JN129835.1         | 28791–28940                             | pBluescriptIISK       |

doi:10.1371/journal.pone.0147832.t001
Table 2. Primers and probes used in this study. (F: Forward; R: Reverse; P: Probe)

| Virus            | Specificity | Sequence (5’—3’) | Location Size | Target | Reference |
|------------------|-------------|------------------|---------------|--------|-----------|
|                  |             | (+-adapted for this study) |               |        |           |
| **Hepatitis A virus** | All genotypes | F—TCA CCG CCG TTT GCC TAG | 68–85 | 5’UTR | [22] |
|                  |             | R—GGA GAG CCC TGG AGA AAA G | 241–223 | | |
|                  |             | P—FAM—CCT GAA CCT GCA GGA ATT AA—MGB | 169–150 | | |
| **Hepatitis E virus** | All genotypes | F—CGG TGG TTT CTT GGG TGA C | 5260–5278 | ORF2/ORF3 | [35] |
|                  |             | R—AGG GGT TGG TTT GAT GAA TAT AG | 5330–5308 | M14707 | [22] |
|                  |             | P—FAM—GGG TGG ATT CTC AGG CCT TCG C—BHQ1 | 5280–5301 | 71bp | |
| **Rotavirus** | Serotype A | F—ACC ATC TWC ACR TRA CCC TC | 963–982 | NSP3 (segment 7) | [36], adapted |
|                  |             | R—GCT CAC ATA ACG CCC C | 1049–1034 | X81436 | [37] |
|                  |             | P—FAM—ACA ATA GTT AAA AGC TAA CAC TGT CAA—BHQ1 | 990–1016 | 87bp | |
| **Norovirus** | Genogroup I | F—CGG TGG ATG CNG TCG TTC CAT | 5291–5308 | 5’ end of ORF2 | [38] |
|                  |             | R—GCT TAG AGC CCA TCA TCA TTT AC | 5376–5334 | M73218 | [35] |
|                  |             | P—FAM—TGG ACA GGA GAC CAG RAT CT—BHQ1 | 5321–5340 | 86bp | |
| **Norovirus** | Genogroup II | F—ATG TTC AGG TGG ATG AGR TTC TCG W GA | 5012–5037 | 5’ end of ORF2 | [40] |
|                  |             | R—TGG AGC CCA TCT TCA TTC ACA | 5080–5100 | X86557 | [41] |
|                  |             | P—FAM—AGC TGG GAG GGC GAT CG—BHQ1 | 5042–5061 | 89bp | |
| **Norovirus** | Genogroup IV | F—GGA TGC GRT TGT GCT ACT TTC TCG W GA | 4986–5003 | ORF1-ORF2 | This study |
|                  |             | R—TCT TCA TTC ACA AAR TCG GGA G | 5055–5034 | JQ613567 | This study |
|                  |             | P—FAM—TGG GAG GGG GAT CGC GAT CT—BHQ1 | 5012–5031 | 70bp | |
| **Sapovirus** | GG 1, 2, 4, 5 | F—GAC CAG GCT CTC GCY ACC TAC | 5074–5094 | Polymerase / capsid junction | [43] |
|                  |             | R—GCT TCC ATY TCA AAC ACT AWT TTG | 5177–5154 | NC_006269 | [43] |
|                  |             | P—FAM—CCC ACT GGG TCA RGT ACT GTA C—BHQ1 | 5135–5114 | 104bp | This study |
| **Aichi virus** | / | F—CCA GCC TGA CCG TAT ATC ACA AGG | 268–287 | 5’ UTR | [44] |
|                  |             | R—CAG TAC TGC CAG TAT AGC AGC TT | 329–307 | AB040749 | [44] |
|                  |             | P—FAM—CTG TGT GAA GGC C—MB | 288–300 | 62bp | |
| **Astrovirus** | / | F—TCT YAT AGA CCG YAT TAT TGG | 2209–2229 | ORF 1a | [45] |
|                  |             | R—TCA AAT TCT ACA TCA TCA CCA A | 2322–2301 | NC_001943 | [45] |
|                  |             | P—FAM—CCC CAD CCA TCA TCT TCA TCA C—BHQ1 | 2295–2272 | 114bp | |
| **Adenovirus** | 40 and 41 (serotype F) | F—CTC GAC ATG ACT TTT GAG GT | 20256–20275 | Hexon protein | [45], adapted |
|                  |             | R—GTA GAC GGC CTC GAT GAC | 20375–20358 | NC_001454 | [45], adapted |
|                  |             | P—FAM—AGG ATG AGC CCA CAC TTC TYA TGB—BHQ1 | 20290–20302 | 120bp | |
| **Coronavirus** (human) | 229 (α-coronaV) | F—CAT ACT ATC AAC CCA TTT AAC AAG | 25374–25397 | glycoprotein | [46] |
|                  |             | R—GAC GGC AAC TGT CAT GTA TT | 25510–25491 | AF304460 | [46] |

(Continued)
Table 2. (Continued)

| Virus                                      | Specificity | Sequence (5’—3’) | Location Size | Target                               | Reference            |
|--------------------------------------------|-------------|------------------|---------------|--------------------------------------|----------------------|
| HKU1 (β-coronaV)                           |             | P—FAM-ATG AAC CTG AAG ACC TGA AGC CA TCT ATG-BHQ1 | 25480–25451   | 137bp                                | [47]                 |
|                                            |             | P—TCC TAC TAY TCA AGA AGC TAT CC                | 28775–28797   | phosphoprotein                       | [46]                 |
|                                            |             | R—AAT GAA CGA TTA TTG GGT GCA C                  | 28921–28900   | HM034837.1                           | [46]                 |
|                                            |             | P—FAM-TYC GCC TGG TAC GAT TTT GCC TCA-BHQ1      | 28808–28831   | 147bp                                | [47]                 |
| NL63 (α-coronaV)                           |             | P—GTT CTG ATA AGG CAC CAT ATA GG                | 26215–26237   | phosphoprotein                       | [46]                 |
|                                            |             | R—TTT AGG AGG CAA ATC AAC ATG                   | 26357–26337   | JX504050                             | [46]                 |
|                                            |             | P—FAM-CGC ATA CGA CAA CCG TCT TGA ACA-BHQ1     | 28626–28630   | 143bp                                | [47]                 |
| OC43 (β-coronaV)                           |             | P—CAT ACY CTG CTG GTC ACA ATA AT A              | 28812–28835   | glycoprotein                         | [46]                 |
|                                            |             | R—ACC TTA GCA ACA GTC ATA TAA GC                | 28921–28999   | JN129835.1                           | [46]                 |
|                                            |             | P—FAM—TG CAA AGA ATA GCC ART ACC TAG T—BHQ1    | 28899–28965   | 110bp                                | [47], adapted        |
| Tick-borne encephalitis virus              | European    | P—GGG CGG TTC TTG TTC TCC                       | 11054–11071   | 3’NCR                                | [48], adapted        |
|                                            | And Far-    | R—ACW CAT CAC CTC CTT GTG AGA CT                | 11121–11099   | U27495                               | [48], adapted        |
|                                            | Eastern      | P—FAM—TGA GCC ACC ATC ACC CAG ACA CA—BHQ1      | 11073–11095   | 68bp                                 | [48], adapted        |
|                                            | subtypes     | F—CCC CGG GAC CAG TCC AGG                       | 2241–2258     | NS                                   | [49]                 |
|                                            |             | R—CCC TCT ACA CCR TCC CAC AC                   | 2393–2374     | AB550331                             | [49]                 |
|                                            |             | P—FAM—ATC ATY TGT CGG AAG CYC GTT TCC CTG CG—BHQ1 | 2262–2290    | 153bp                                | [49]                 |
| Parvovirus B19                             |             | F—GCC CCT GAA TGC GGC                          | 334–348       | Polyprotein                          | [50]                 |
|                                            |             | R—GAT TGT CAC CAT AAG CAG C                     | 481–464       | AJ295199                             | [51], adapted        |
|                                            |             | P—FAM—CGG ACC CTA CTT TGG GTT TCC GTG—BHQ1     | 416–441       | 148pb                                | [51]                 |
| Entero-virus                               |             | F—GGC TCA CTT TGC GTC GTC GTC                 | 735–758       | 5’-UTR                               | [52], adapted        |
|                                            |             | R—CCA YTG TGG TCG TCC CCC                       | 827–808       | NC_012800                            | [52], adapted        |
|                                            |             | P—FAM—CTC ACA GGC CRR AAG CCC GTG C—BHQ1       | 783–807       | 93bp                                 | [52], adapted        |
| Cosavirus                                  |             | F—TTG TAG YGA TGC TGT RTG TGT GTG             | 7251–2550     | Gene NP-1                            | [53]                 |
|                                            |             | R—CCA YTG TGG TGG TCC TCC                      | 827–808       | NC_012800                            | [53]                 |
|                                            |             | P—FAM—AAC CAC CAT CCA GGA GCA TCT G—BHQ1      | 2646–2622     | 145bp                                | [53]                 |
| Bocavirus hBoV2                            |             | F—TCA GAC CAA GCG AGC AAG AC                   | 2531–2550     | Gene NP-1                            | [53]                 |
|                                            |             | R—CTC TAG CAA GYC TAG TAG AAT GCC              | 2675–2652     | NC_012042                            | [53]                 |
|                                            |             | P—FAM—AAC CAC CAT CCA GGA GCA TCT G—BHQ1      | 2646–2622     | 145bp                                | [53]                 |
| MNV                                        | MNV-1       | F—CGT CAA TGG TCC TGG AGA ATG—3’               | 3193–3213     | Polyprotein                          | [54]                 |
|                                            |             | R—GCA CAA CGC CAC TAC CAA TCT TG—3’           | 3330–3308     | DQ285629                             | [54]                 |
|                                            |             | P—FAM—CGT CGG CTC GCT GCC TGT CAA—BHQ1        | 3227–3250     | 138bp                                | [54]                 |

(Continued)
synthesized RNA (HAV, HEV, NoV GI, NoV GII, NoV GIV, sapovirus, cosavirus, Aichi virus, human coronavirus (HKU1, 229E, NL63, OC43), TBEV), RNA extracts (rotavirus, astrovirus, enterovirus, MNV and mengovirus), synthesized DNA (parvovirus, bocavirus) or DNA extract (adenovirus) resulting from serial dilution in ultrapure 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 \(E = 10^{\frac{S}{\Delta S} - 1}\), to determine the RT-qPCR assay performance.

**RT-dPCR with the BioMark System**

Digital PCR works by partitioning a single sample into hundreds of individual PCR reactions. RT-dPCR amplifications were performed on a Fluidigm BioMark System by using qdPCR 37k IFC digital array microfluidic chips (Les Ulis, France). Utilizing nanoscale valves and channels, the Biomark Integrated Fluidic Circuit (IFC) controller partitions each of the 48 samples pre-mixed with PCR reagents into a panel of 770 PCR reaction chambers (i.e. 36,960 individual qPCR reactions on a digital array). By counting the number of positive reactions, the number of target molecules in each sample can be accurately estimated based on the Poisson distribution.

Reactions were performed in a 10 μL reaction mixture containing 1X of RNA UltraSense™ master mix, 1X of ROX reference dye and 0.44 μL of RNA Ultrasense™ enzyme mix, which are components of the RNA UltraSense™ One-Step Quantitative RT-PCR System (Life Technologies), 1X of 20X GE Sample Loading Reagent (Fluidigm), 2 U RNase inhibitor (Life Technologies), 500 nM of forward primer, 900 nM of reverse primer, 250 nM of probe, and 5.8 μL of RNA extract. A negative control containing all the reagents except the RNA template was included with each set of reaction mixtures. 6 μL out of ten reaction mix was charged onto the chip with the IFC controller MX, but 0.65 μL were effectively partitioned into the 770 chambers of one panel, including 0.38 μL of RNA extract.

The one-step RT-dPCR program involved 60 min reverse transcription of RNA at 55°C, followed by a 15 min denaturation step at 95°C, and lastly 45 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 minute at 65°C) for each amplification cycle.

The Digital PCR Analysis software (Fluidigm) was used to count the number of positive chambers out of the total number of chambers per panel.

The Poisson distribution was used to estimate the average number of template copies per chamber in a panel [24, 25]. All samples were characterised by a corresponding absolute quantity. No positive chambers were observed in negative samples.

**RT-qPCR with the BioMark System**

The 48.48 dynamic arrays were automatically loaded using an integrated fluidic circuit (IFC) controller (Fluidigm Corporation), and real-time reactions were performed and analyzed using
a BioMark real-time PCR system and analysis software (Fluidigm Corporation), respectively. As a quality control, negative control samples were included on every array for each viral genome.

RT reactions were performed in a 25 μL reaction mixture containing 1X of First-Strand Buffer, 10mM of DTT and 1 μL of SuperScript® III RT enzyme, which are components of SuperScript® III Reverse Transcriptase (Life technologies), 2 U RNase inhibitor (Life technologies), 2μM of Random hexamer (Life technologies), 200 μM of dNTP (Life technologies), and 10 μL of nucleic acids. A negative control containing all the reagents except the RNA template was included with each set of reaction mixtures. The RT program involved 5 min at 25°C, followed by 60 min at 55°C, and lastly 15 min at 70°C. Aliquots were kept frozen at -80°C for later use.

Preamplification reactions were performed in a 10 μL reaction mixture containing 1X of SuperMix, a reagent of Perfecta Preamp SuperMix (Quanta), 0.2μl of 0.2X primer pool (1X = 500nM Forward and 900nM Reverse), and 5 μL of cDNA. A negative control containing all the reagents except the cDNA template was included with each set of reaction mixtures. The preamplification program involved 10 min at 95°C, followed by 14 cycles of 15 s at 95°C and 4 min at 6°C. Immediately after the pre-amplification PCR, products were diluted (1:4) and stored at -80°C prior to use in qPCR.

For the qPCR array, 48 x 6 μL reaction mixture containing 1X of RNA UltraSense™ master mix, 1X of ROX reference dye and 0.27 μL of RNA UltraSense™ enzyme mix, which are components of the RNA UltraSense™ One-Step Quantitative RT-PCR System (Life Technologies), 1X of 20X GE Sample Loading Reagent (Fluidigm) and 2.7 μL of DNA extract were charged on the right part of the "48.48 Dynamic Array IFC" plate (BioMark). Negative controls containing all the reagents except the DNA template were included with each set of reaction mixtures. In addition, 48 x 5 μl of a mix of 500 nM of forward primer, 900 nM of reverse primer and 250 nM of probe were deposited on the left part of the plate.

Nine nl of reaction volume mix were charged onto each of the 2304 chambers on the chip with the IFC controller MX.

The qPCR program involved a 15 min denaturation step at 95°C followed by 45 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 minute at 65°C) for each amplification cycle. Negative samples gave no Ct value.

Results

Conventional RT-qPCR and nanofluidic PCR (RT-dPCR, RT-qPCR array)

The sensitivity of conventional qPCR assays targeting 21 viral genomes was compared to the quantitative digital RT-PCR array and to the qualitative nanofluidic real-time PCR array performed on Fluidigm’s BioMark System.

Quantitative detection by conventional and digital real time RT-PCR assays

Digital RT-PCR’s potential for sensitive and accurate quantification was assessed on 10-fold dilution series of 21 viral genomes (Table 3). The sensitivity was slightly better with RT-qPCR than with RT-dPCR for ten viruses by a factor ranging from 0.3 to 0.9 log10 and for four viruses by a factor ranging from 1.3 to 1.6 log10. Conversely, sensitivity was better with RT-dPCR than with RT-qPCR for seven viruses by a factor ranging from 0.1 to 1.4 log10.
### Table 3. Comparison of RT-qPCR, RT-dPCR and RT-PCR array assays.

Characteristics of standard curves based on the RT-qPCR assays and limit of detection (LOD) of viral targets by RT-qPCR, by RT-dPCR and RT-PCR array assays. The differences between relative quantification (by RT-qPCR) and absolute quantification (by RT-dPCR) were indicated.

| Virus         | Sample Type of sample | RT-qPCR (CFX96) | RT-dPCR (FLUIDIGM) | RT-PCR Array (FLUIDIGM) |
|---------------|-----------------------|----------------|------------------|------------------------|
|               | Genome Type of sample | Slope  E  R² | Range of detection (Ct value) LOD  | log₁₀ₐ(log₁₀(qPCR))-log₁₀ₐ(log₁₀(dPCR)) Difference between quantification type  | LOD  | log₁₀ₐ(log₁₀(qPCR))-log₁₀ₐ(log₁₀(dPCR)) |
| Bocavirus     | DNA ss Plasmidic DNA  | -3.20 ± 0.20 | 106% 0.966 | 16.90–37.36 0.20 | 0.80 | -0.60 1.00 | -0.70 |
| Parovirus B19 | DNA ss Plasmidic DNA  | -3.05 ± 0.14 | 113% 0.980 | 15.22–36.35 0.10 | 4.00 | -1.60 -0.31 | 1.00 |
| Sapovirus     | RNA+ ss Transcript RNA | -3.55 ± 0.29 | 91% 0.963 | 20.21–36.01 1.00E | 4.00E | +01 -0.60 0.89 | 1.00E |
| Hepatitis E   | virus RNA+ ss Transcript RNA | -3.49 ± 0.23 | 93% 0.972 | 19.91–38.15 1.00E | 4.00E | +01 -0.60 1.25 | 1.00E |
| TBEV          | RNA+ ss Transcript RNA | -3.10 ± 0.21 | 110% 0.970 | 21.66–37.54 1.00E | 7.90E | +01 -0.90 1.03 | 1.00E |
| Aichi virus   | RNA+ ss Transcript RNA | -3.34 ± 0.46 | 99% 0.933 | 27.18–36.53 1.00E | 7.89E | +02 0.10 1.32 | 1.00E |
| 229E          | RNA+ ss Transcript RNA | -3.05 ± 0.37 | 113% 0.934 | 25.97–36.89 2.00E | 3.95E | +02 -1.30 1.64 | 1.00E |
| HKU1          | RNA+ ss Transcript RNA | -3.41 ± 0.22 | 97% 0.982 | 25.84–36.34 2.00E | 7.90E | +01 -0.60 1.57 | 1.00E |
| Cosavirus     | RNA+ ss Transcript RNA | -3.16 ± 0.27 | 107% 0.978 | 24.26–31.41 1.00E | 7.90E | +01 1.10 1.35 | 1.00E |
| Norovirus     | RNA+ ss Transcript RNA | -3.37 ± 0.28 | 98% 0.973 | 26.97–38.43 1.00E | 7.90E | +01 0.10 1.44 | 1.00E |
| Norovirus GGI | RNA+ ss Transcript RNA | -3.53 ± 0.35 | 92% 0.952 | 22.77–37.92 2.00E | 7.90E | +03 1.40 1.51 | 1.00E |
| Norovirus GGV | RNA+ ss Transcript RNA | -3.44 ± 0.32 | 95% 0.954 | 23.01–38.53 1.00E | 7.90E | +01 0.10 1.64 | 1.00E |
| OC43          | RNA+ ss Transcript RNA | -3.39 ± 0.31 | 97% 0.952 | 22.84–37.70 1.00E | 3.95E | +02 -1.60 1.67 | 1.00E |
| NL63          | RNA+ ss Transcript RNA | -3.31 ± 0.83 | 100% 0.851 | 28.59–36.63 2.00E | 3.95E | +02 -0.30 1.97 | 1.00E |
| Hepatitis A   | virus RNA+ ss Transcript RNA | -3.27 ± 0.46 | 102% 0.916 | 26.03–39.80 1.00E | 3.95E | +02 -0.60 2.14 | 1.00E |
| Adenovirus 41 | DNA ds Stool | -3.00 ± 0.16 | 115% 0.986 | 23.43–35.08 1.00E | 7.90E | +01 0.10 1.47 | 1.00E |
| Astrovirus    | RNA+ ss Stool | -3.65 ± 0.54 | 88% 0.943 | 32.88–42.23 2.00E | 7.89E | +03 -1.60 3.40 | 1.00E |
| Rotavirus Wa  | RNA + ds Cell production | -3.51 ± 0.79 | 93% 0.812 | 22.05–36.45 1.00E | 7.89E | +03 -0.90 1.56 | 1.00E |
| MNV-1         | RNA+ ss Cell production | -3.01 ± 0.32 | 115% 0.947 | 28.33–38.14 1.00E | 3.95E | +02 -0.60 1.95 | 1.00E |
| Enterovirus   | RNA+ ss Cell production | -3.36 ± 0.59 | 98% 0.894 | 25.28–38.79 1.00E | 3.95E | +02 0.40 1.97 | 1.00E |
| Mengovirus    | RNA+ ss Cell production | -3.21 ± 0.20 | 105% 0.983 | 26.77–38.00 1.00E | 7.89E | +02 -0.90 2.05 | 1.00E |

*a LOD expressed as number of genome copies/μl of nucleic acids.

*b formula = [log₁₀(OD)-log₁₀([digital])].

The expected numbers of genome copies calculated via the standard curve by RT-qPCR were close to the direct measurement of the target concentrations by RT-dPCR only by testing DNA from plasmids. By testing RNA transcripts, the numbers of genome copies as determined by direct RT-dPCR measurement of the target concentrations were 0.9 to 2.1 log₁₀ lower than
the expected copy numbers calculated via the standard curve by RT-qPCR. Similarly, by testing genomes from viruses in stools and RNA from virus production in cells, the limit of detection (LOD) as determined by RT-dPCR was respectively 1.5 to 3.4 log_{10} and 1.6 to 2.1 log_{10} lower than the expected copy numbers calculated via the standard curve by RT-qPCR.

Sensitive and accurate detection by RT-qPCR array

The potential of the RT-PCR array for sensitive detection was assessed on a dilution series of 21 viral genomes (Table 3). The limits of detection obtained with RT-qPCR array assays ranged from 1 to 10^3 genome copies / μl of RNA / DNA extracts for 11 viruses and from 10^4 to 10^5 genome copies / μl of RNA extracts for the others. RT-qPCR array assays commonly showed a slightly lower sensitivity than conventional RT-qPCR. The sensitivity of both RT-qPCR and RT-qPCR array assays was found to be similar for two viruses (enterovirus, adenovirus 41), and was slightly better with the RT-qPCR than with the RT-qPCR array for 18 viruses by a factor ranging from 0.7 to 3.0 log_{10}. Conversely, sensitivity was only 0.3 log_{10} higher with the RT-qPCR array than with conventional RT-qPCR assays for norovirus GIV detection.

Viral screening by RT-qPCR array and quantitative detection of clinical samples by RT-dPCR

The nanofluid-based (RT)-PCR assays developed were applied to characterize 25 samples (4 culture supernatants and 21 clinical samples previously characterized by NRC) for detection of hepatitis (HAV, HEV) and enteric virus genomes. First, the samples were tested on the RT-PCR array to perform a qualitative screening of the 19 viral genomes. Then the viral-positive samples were specifically quantified by RT-dPCR and by conventional RT-qPCR. Results are shown on Table 4.

RT-qPCR array assays detected the previously determined viruses in 100% of the samples. Furthermore, positivity for more than one virus was found in two clinical samples. A stool previously identified as positive for HAV IB was found positive for HAV and Aichi virus and a stool identified as positive for Aichi virus was found positive for Aichi virus, adenovirus and astrovirus. The stool previously identified as co-infected by NoV GII.13 and NoV GIV was confirmed positive for both viruses.

Following the viral screening of 25 samples, the 29 detected viral genomes were successfully quantified by both RT-qPCR and RT-dPCR. The number of genome copies determined for 28 viruses was lower by RT-dPCR with a difference of quantification comprised between 0 and 1 log_{10} for 7 out of the 29 samples (24%), between 1 and 2 log_{10} for 17 out of the 29 samples (59%) and higher than 2 log_{10} for 4 out of the 29 samples (14%). So the numbers of genome copies calculated by absolute quantification (RT-dPCR) were lower than the expected numbers of genome copies calculated by using standard curve of RT-qPCR except in the sample co-infected with NoV GII and NoV GIV. In the latter sample, the NoV GII quantification was 0.4 log_{10} higher by RT-dPCR than by the RT-qPCR assays (1 out of the 29 samples, i.e. 3%).

Discussion

Enteric viruses are able to persist for long periods in the environment and can be transmitted with a low infectious dose by human contact, water, food and fomites [26]. They pose a significant public health concern. They are associated with gastroenteritis in humans, but also with hepatitis and other diseases including respiratory infections, conjunctivitis, aseptic meningitis, encephalitis, myocarditis, and paralysis which have high mortality rates, particularly in immunocompromised individuals [27]. Commonly studied groups of enteric viruses include noroviruses and hepatitis viruses, but new tools for detecting the full range of pathogenic viruses are
Table 4. Screening and viral quantification in clinical samples (stools and viral supernatants from cell culture) by RT-qPCR and novel nanofluidic approaches (RT-qPCR and RT-dPCR). Samples were firstly screened by RT-PCR array and then quantified by RT-dPCR. Absolute viral quantification (by RT-dPCR) was compared to relative quantification (by RT-qPCR).

| Sample number | Nature | Origine | NRC Virus identification | PCR Array 48x48 detection | RT-dPCR quantification | RT-qPCR quantification | Difference between quantification type |
|---------------|--------|---------|--------------------------|---------------------------|------------------------|------------------------|--------------------------------------|
| HM175/18f     | Cell production | ATCC | HAV | 3/3 | 1.51E+09 | 4.50E+10 | 1.47 |
| EchoV         | Cell production | Echovirus 19. Burke strain | EV | 2/2 | 1.61E+09 | 1.75E+10 | 1.04 |
| Adenovirus 40 | Cell production | ATCC VR-931 | Adenovirus | 2/2 | 7.11E+08 | 1.42E+10 | 1.30 |
| Astrovirus GI | Cell production | N/A | Astrovirus | 2/2 | 1.76E+10 | 2.43E+10 | 0.14 |
| 780627147     | stool | HAV/HEV NRC | HAV IA | 3/3 | 1.29E+06 | 2.30E+06 | 0.25 |
| 1181216151    | stool | HAV/HEV NRC | HAV IA | 3/3 | 2.45E+09 | 7.75E+10 | 1.50 |
| 1280210015    | stool | HAV/HEV NRC | HAV IB | 3/3 | 7.52E+07 | 1.50E+09 | 1.30 |
| 1280514230    | stool | HAV/HEV NRC | HAV IB | 3/3 (HAV) | 2.88E+08 | 6.85E+09 | 1.38 |
| 1280511146    | stool | HAV/HEV NRC | HEV 3c | 2/2 | 7.36E+07 | 1.41E+09 | 1.28 |
| 1280418084    | stool | HAV/HEV NRC | HEV 3f | 2/2 | 1.43E+08 | 1.95E+09 | 1.13 |
| 1280530128    | stool | HAV/HEV NRC | HEV 3f | 2/2 | 1.32E+07 | 5.21E+08 | 1.60 |
| 1280615097    | stool | HAV/HEV NRC | HEV 4 | 2/2 | 4.44E+07 | 2.40E+08 | 0.73 |
| 1280522166    | stool | HAV/HEV NRC | HEV 3 | 2/2 | 2.60E+07 | 5.34E+09 | 2.31 |
| E7622         | stool | Enteric viruses NRC | RV G12P8 | 2/2 | 4.84E+09 | 7.28E+11 | 2.18 |
| E8097         | stool | Enteric viruses NRC | RV G1P8 | 2/2 | 7.83E+08 | 4.31E+12 | 3.74 |
|               |       |          |       |     | 1/2 (Aichi virus) | 7.83E+05 | 6.00E+07 | 1.88 |
| E6841         | stool | Enteric viruses NRC | Aichi virus | 2/2 (Adenovirus) | 4.69E+09 | 5.85E+10 | 1.10 |
|               |       |          |       |     | 2/2 (Astrovirus) | 4.42E+08 | 1.65E+11 | 2.57 |
| E5486         | stool | Enteric viruses NRC | NoV GI.4 | 3/3 | 1.98E+07 | 7.79E+07 | 0.59 |
| E5569         | stool | Enteric viruses NRC | NoV GI.1 | 3/3 | 9.58E+06 | 9.91E+07 | 1.01 |
| E8050         | stool | Enteric viruses NRC | NoV GI.3 | 3/3 | 3.96E+07 | 7.40E+08 | 1.27 |
| E6929         | stool | Enteric viruses NRC | NoV GI.4 | 3/3 | 5.53E+07 | 7.00E+08 | 1.10 |
| E6618         | stool | Enteric viruses NRC | NoV GI.7 | 3/3 | 8.29E+05 | 8.03E+06 | 0.99 |
| E7859         | stool | Enteric viruses NRC | NoV GI.6 | 3/3 | 6.94E+07 | 5.24E+08 | 0.88 |
| E7022         | stool | Enteric viruses NRC | NoV GI.3 | 3/3 | 1.95E+07 | 2.91E+08 | 1.17 |

(Continued)
needed for their surveillance in the environment, food samples and for outbreak investigations [28].

Microfluidic digital PCR (RT-dPCR) is an accurate endpoint-sensitive absolute quantification approach that makes it possible to determine the number of target copies without a standard curve. Digital PCR ((RT)-dPCR) was compared to real-time (RT)-PCR for quantifying 19 human enteric viruses and two control process viruses. For detecting viral RNA and cDNA, RT-dPCR assays were often found to be comparable in terms of sensitivity to RT-qPCR. The number of RNA genome copies determined by digital RT-PCR was often lower than the number of copies expected using spectrophotometry. One potential cause of discrepancy between relative and absolute quantification could be errors introduced by spectrophotometric determination of the nucleic acid concentration, leading to an overestimation of the copy genome number [29, 30]. This could explain why samples from viral stocks and stools potentially containing cellular genomes (non-target RNA) and degraded (non-amplified) targets were particularly affected by quantification discrepancies. Both quantification methods were close when DNA targets were tested. One other potential cause of discrepancy might be the RT step, which is not 100% effective, so that all the RNA may not be transcribed into cDNA and therefore is not quantified by the digital PCR. Digital RT-PCR may provide more accurate measurements than RT-qPCR, as it is not dependent on amplification efficiency. Moreover, the advantage of this novel technology is that it is more tolerant to inhibitory substances and may reduce the difficulty of quantifying viruses when inhibitors linked to the matrix-type components analysed in food or environmental virology are present [31, 32, 33].

Recent innovations in PCR miniaturization made it possible to conduct high-throughput qPCR in which the reactional volumes are reduced to a nanolitre, leading to a decrease in the cost per assay per sample. Recently, a microfluidic quantitative PCR (MFQPCR) system was developed to simultaneously quantify 11 major human viral pathogens and two process controls (murine norovirus, mengovirus). This system included a specific target amplification (STA) reaction to increase the amount of target genes prior to MFQPCR [34]. In this study, the RT-qPCR array assays were developed and enabled simultaneous detection of 48 samples with 22 targeted virus assays. The preamplification step was also necessary because low amounts of target molecules had to be detected in very small volumes of reaction (9nl). Thus, RT-qPCR array assays involve three separate steps (RT, preamplification and PCR).

Nineteen enteric viruses and two control process viruses (MNV and mengovirus) were targeted. The sensitivity of the RT-qPCR array assays was lower (by 0.8 to 3.8 log_{10}) than the limits of detection obtained with conventional RT-qPCR and RT-dPCR. However, all the clinical samples tested with the RT-qPCR array assays were identified and matched the NRC results.

### Table 4. (Continued)

| Sample number | Nature | Nature Origine NRC | NRC Virus identification | PCR Array 48x48 detection | RT-dPCR quantification a | RT-qPCR quantification a | Difference between quantification type |
|---------------|--------|--------------------|---------------------------|---------------------------|--------------------------|---------------------------|----------------------------------------|
| E6992 stool    | stool   | Enteric viruses    | NoV GII.1                 | 3/3                       | 1.75E+06                 | 1.52E+07                  | 0.94                                   |
| stool          | stool   | Enteric viruses    | NoV GII.13                | 3/3 (NoV GII)             | 2.95E+06                 | 1.16E+06                  | -0.41                                  |
| + NoV GIV      | stool   | Enteric viruses    | NoV GIV                   | 2/2 (NoV GIV)            | 3.04E+05                 | 1.84E+07                  | 1.78                                   |

a expressed as number of genome copies/g (stools) or genome copies/mL (cell production).

doi:10.1371/journal.pone.0147832.t004
Moreover, two stools contained more than one viral genome, and these results completed the NRC analysis. This assay is therefore useful for rapid sample screening.

In conclusion, a combination of RT-qPCR array and RT-dPCR assays could be applied to screen contaminated samples and quantify pathogenic viruses in case of outbreaks investigation and surveillance. The choice of techniques should take into account the aim of analysis, the number of targets involved and the analytical costs. To date, the RT-qPCR array includes enteric viruses frequently reported as the causes of foodborne outbreaks and some additional viruses of lesser epidemiologic importance. In future, this technology could be updated by extending the range of viral targets to gain information during epidemiological studies. For this purpose, BioMark real-time PCR system (Fluidigm) can be also used for high-throughput microfluidic real-time PCR amplification with 96.96 dynamic arrays (Fluidigm) leading to an increase of detected targets. Concerning RT-dPCR assays, it could be helpful for standardizing the quantification of enteric viruses in samples and might be extended to the quantification of other human microbiological pathogens in foods.

Acknowledgments

We thank AM Roque (NRC for hepatitis A virus, France) and P Pothier (NRC for Enteric Viruses, France) for providing contaminated stools. This work is part of the thesis by Coralie Coudray-Meunier, a PhD student who received financial support from ANSES.

Author Contributions

Conceived and designed the experiments: CCM AF SML SD PF SP. Performed the experiments: CCM AF SD. Analyzed the data: CCM AF SML SD PF SP. Contributed reagents/materials/analysis tools: CCM AF SML SD PF SP. Wrote the paper: CCM AF SML SD PF SP.

References

1. Kotwal G, Cannon JL. Environmental persistence and transfer of enteric viruses. Curr Opin Virol. 2014; 4C: 37–43.
2. Gibson KE. Viral pathogens in water: occurrence, public health impact, and available control strategies. Curr Opin Virol. 2014; 4: 50–57. doi: 10.1016/j.coovi.2013.12.005 PMID: 24440908
3. Koo HL, Ajami N, Atmar RL, DuPont HL. Noroviruses: The leading cause of gastroenteritis worldwide. Discov Med. 2010; 10: 61–70. PMID: 20670600
4. Matthews JE, Dickey BW, Miller RD, Felzer JR, Dawson BP, Lee AS, et al. The epidemiology of published norovirus outbreaks: a review of risk factors associated with attack rate and group/strain. Epidemiol Infect. 2012; 140: 1161–1172. doi: 10.1017/S0950268812000234 PMID: 22444943
5. Nainan OV, Xia G, Vaughan G, Margolis HS. Diagnosis of hepatitis A virus infection: a molecular approach. Clin Microbiol Rev. 2006; 19: 63–79. PMID: 16418523
6. Van der Poel WHM. Food and environmental routes of Hepatitis E virus transmission. Curr Opin Virol. 2014; 4: 91–96. doi: 10.1016/j.coovi.2014.01.006 PMID: 24513966
7. Vaughan G, Goncalves Rossi LM, Forbi JC, de Paula VS, Purdy MA, Xia G, et al. Hepatitis A virus: Host interactions, molecular epidemiology and evolution. Infect Genet Evol. 2014; 21C: 227–243.
8. Bibby K, Peccia J. Identification of viral pathogen diversity in sewage sludge by metagenome analysis. Environ Sci Technol. 2013; 47: 1945–1951. doi: 10.1021/es305181x PMID: 23346855
9. Lindquist L. Tick-borne encephalitis. Handb Clin Neurol. 2014; 123: 531–559. doi: 10.1016/B978-0-444-53488-0.00025-0 PMID: 25015503
10. Malik YS, Kumar N, Sharma K, Dhama K, Shabbir MZ, Ganesh B, et al. Epidemiology, phylogeny, and evolution of emerging enteric Picobirnaviruses of animal origin and their relationship to human strains. BioMed Res Int. 2014; 2014: 780752. doi: 10.1155/2014/780752 PMID: 25136620
11. Oka T, Wang Q, Katayama K, Saif LJ. Comprehensive Review of Human Sapoviruses. Clin Microbiol Rev. 2015; 28: 32–53. doi: 10.1128/CMR.00011-14 PMID: 25567221
12. Atmar RL, Estes MK. Diagnosis of noncultivable gastroenteritis viruses, the human caliciviruses. Clin Microbiol Rev. 2001; 14: 15–37. PMID: 11148001
13. Sánchez G, Bosch A, Pintó RM. Hepatitis A virus detection in food: current and future prospects. Lett Appl Microbiol. 2007; 45: 1–5. PMID: 17594452
14. Lees D, CEN WG6 TAG4. International standardization of a method for detection of human pathogenic viruses in molluscan shellfish. Food Environ Virol. 2010; 2: 146–155.
15. ISO/TS 15216–1. Microbiology of food and animal feed—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 1: Method for quantification. International Organization for Standardization, Geneva, Switzerland. 2013.
16. ISO/TS 15216–2. Microbiology of food and animal feed—Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR—Part 2: Method for qualitative detection. International Organization for Standardization, Geneva, Switzerland. 2013.
17. Lemon SM, Murphy PC, Shields PA, Ping LH, Feinstone SM, Cromeans T, et al. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. J Virol. 1991; 65: 2056–2065. PMID: 1705995
18. Cromeans T, Sobsey MD, Fields HA. Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. J Med Virol. 1987; 22: 45–56. PMID: 3035079
19. Dubois E, Hennecart C, Deboosère N, Merle G, Legeay O, Burger C, et al. Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables. Int J Food Microbiol. 2006; 108: 164–171. PMID: 16387377
20. Cannon JL, Papafragkou E, Park GW, Osborne J, Jaykus LA, Vinjé J. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. J Food Prot. 2006; 69: 2761–2765. PMID: 17133824
21. Wobus CE, Karst SM, Thackray LB, Chang KO, Sosnovtsev SV, Belliot G, et al. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol. 2004; 2: e432. PMID: 15562321
22. Costafreda MI, Bosch A, Pinto RM. Development, evaluation and standardization of a real time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. App Environ Microbiol. 2006; 72: 3846–3855.
23. Coudray-Meunier C, Fraisse A, Martin-Latil S, Guillier L, Perelle S. Discrimination of infectious hepatitis A virus and rotavirus by combining dyes and surfactants with RT-qPCR. BMC Microbiol. 2013; 13: 216. doi:10.1186/1471-2180-13-216 PMID: 24083486
24. Dube S, Qin J, Ramakrishnan R. Mathematical analysis of copy number variation in a DNA sample using digital PCR on a nanofluidic device. PLoS One. 2008; 3(8):e2876. doi: 10.1371/journal.pone.0002876 PMID: 18682853
25. Bhat S, Herrmann J, Armishaw P, Corbisier P, Emslie KR. Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. Anal Bioanal Chem. 2009; 394: 457–467. doi: 10.1007/s00216-009-2729-5 PMID: 19288230
26. Koopmans M, Duizer E. Foodborne viruses: an emerging problem. Int J Food Microbiol. 2004; 90: 23–41. PMID: 14672828
27. Griffin DW, Donaldson KA, Paul JH, Rose JB. Pathogenic human viruses in coastal waters. Clin Microbiol Rev. 2003; 16: 129–143. PMID: 12525429
28. Kocwa-Haluch R. Waterborne enteroviruses as a hazard for human health. PjOEs. 2001; 10: 485–487.
29. Henrich TJ, Gallien S, Li JZ, Pereyra F, Kuritzkes DR. Low-level detection and quantitation of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. J Virol Methods. 2012; 180: 68–72. doi: 10.1016/j.jviromet.2012.09.019 PMID: 22974526
30. Sanders R, Mason DJ, Foy CA, Huggett JF. Evaluation of digital PCR for absolute RNA quantification. PLoS One. 2013; 8: e75296. doi: 10.1371/journal.pone.0075296 PMID: 24073259
31. Coudray-Meunier C, Fraisse A, Martin-Latil S, Guillier L, Delannoy S, Fach P, et al. A comparative study of digital RT-PCR and RT-qPCR for quantification of Hepatitis A virus and Norovirus in lettuce and water samples. Int J Food Microbiol. 2015; 201:17–26. doi: 10.1016/j.ijfoodmicro.2015.02.006 PMID: 25725459
32. Račić N, Morisset D, Gutierrez-Aguirre I, Ravnikar M. One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. Anal Bioanal Chem. 2014; 406: 661–667. doi: 10.1007/s00216-013-7476-y PMID: 24276251
33. Morisset D, Štebih D, Milavec M, Gruden K, Žel J. Quantitative analysis of food and feed samples with droplet digital PCR. PLoS One. 2013; 8: e62583. doi: 10.1371/journal.pone.0062583 PMID: 23658750
34. Ishii S, Kitamura G, Segawa T, Kobayashi A, Miura T, Sano D, et al. Microfluidic quantitative PCR for simultaneous quantification of multiple viruses in environmental water samples. Appl Environ Microbiol. 2014; 80: 7505–7511. doi: 10.1128/AEM.02578-14 PMID: 25261510

35. Martin-Latil S, Hennechart Collette C, Guillier L, Perelle S. Duplex RT-qPCR for the detection of hepatitis E virus in water, using a process control. Int J Food Microbiol. 2012; 157: 167–73. doi: 10.1016/j.ijfoodmicro.2012.05.001 PMID: 22633799

36. Pang XL, Cao M, Zhang M, Lee B. Increased sensitivity for various rotavirus genotypes in stool specimens by amending three mismatched nucleotides in the forward primer of a real-time RT-PCR assay. J Virol Methods. 2010; 172: 85–87. doi: 10.1016/j.jviromet.2010.12.013 PMID: 21185331

37. Pang XL, Lee B, Borousand N, Leblanc B, Preiksaitis JK, Yu lp CC. Increased detection of rotavirus using a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. J Med Virol. 2004; 72: 496–501. PMID: 14748075

38. da Silva AK, Le Saux JC, Parnaudeau S, Pommeuy M, Ellimelech M, Le Guyader FS. Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: different behaviors of genogroups I and II. Appl Environ Microbiol. 2007; 73: 7891–7897. PMID: 17933913

39. Svraškova S, Dužer E, Vennema H, de Bruijn E, van der Vecht B, Dorresteijn B, et al. Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. J Clin Microbiol. 2007; 45: 1389–1394. PMID: 17360839

40. Loisy F, Atmar RL, Guillon P, Le Cann P, Pommeuy M, Le Guyader FS. Real-time RT-PCR for norovirus screening in shellfish. J Virol Methods. 2005; 123: 1–7. PMID: 15582692

41. Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, et al. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol. 2003; 41: 1548–1557. PMID: 12682144

42. Trujillo AA, MaCaustland KA, Zheng DP, Hadley LA, Vaughn G, Adams SM, et al. Use of TaqMan real-time Reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. J Clin Microbiol. 2006; 44: 1405–1412. PMID: 16597869

43. Chan MC, Sung JJ, Lam RK, Chan PK, Lai RW, Leung WK. Sapovirus detection by quantitative real-time PCR in clinical stool specimens. J Virol Methods. 2006; 134: 146–153. PMID: 16427707

44. Drexler J, Baumgarte S, de Souza Luna LK, Eschbach-Bludau M, Lukashev AN, Drosten C. Aichi Virus in water, using a process control. Int J Food Microbiol. 2012; 157: 167–73. doi: 10.1016/j.ijfoodmicro.2012.05.001 PMID: 22633799

45. Gaunt ER, Hardie A, Claas EC, Simmonds P, Templeton KE. Epidemiology and clinical presentations of the enterovirus RNA in sludge samples using single tube real-time RT-PCR. Biotechniques. 2000; 29: 88–93. PMID: 10907082

46. Mohamed N, ElHaffouri A, Fohlman J, Friman G, Blomberg J. A sensitive and quantitative single-tube real-time reverse transcriptase-PCR for detection of enteroviral RNA. J Clin Virol. 2004; 30: 150–156. PMID: 15125871

47. Monpoeho S, Dehee A, Mignotte B, Schwartzbrod L, Marechal V, Nicolas JC, et al. Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. Biotechniques. 2000; 29: 88–93. PMID: 10907082

48. Stöcker A, Souza BF, Ribeiro TC, Netto EM, Araujo LO, Corrêa JI, et al. Cosavirus infection in persons with and without gastroenteritis, Brazil. Emerg Infect Dis. 2012; 18: 656–659. doi: 10.3201/eid1804.111415 PMID: 22469070

49. Xu ZQ, Cheng WX, Li BW, Li J, Lan B, Duan ZJ. Development of a Real-Time PCR Assay for Detecting and Quantifying Human Bocavirus 2. J Clin Microbiol 2011; 49: 1537–1541. doi: 10.1128/JCM.00196-10 PMID: 21325551
54. Martin-Latil S, Hennechart Collette C, Guillier L, Perelle S. Comparison of two extraction methods for the detection of hepatitis A virus in semi-dried tomatoes and murine norovirus as a process control by duplex RT-qPCR. Food Microbiol. 2012; 31: 246–253. PMID: 22608230

55. Pintó RM, Costafreda MI, Bosch A. Risk assessment in shellfish-borne outbreaks of hepatitis A. J Appl Environ Microbiol. 2009; 75: 7350–7355.