Hepatitis E virus in lettuce and water samples: a method-comparison study

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Abstract

The hepatitis E virus (HEV), which is an increasing cause of acute viral hepatitis in Europe, is a zoonotic virus that is mainly transmitted through contaminated water, consumption of raw or undercooked meat from pigs or wild boar, blood transfusion, and organ transplantation. Although the role of HEV transmission through contaminated produce has not been confirmed, the presence of HEV has been reported in irrigation waters and in vegetables. The present study used a World Health Organization (WHO) international standard and clinical samples to evaluate the performance characteristics of three RT-qPCR assays for detection and quantification of HEV. Two of the evaluated assays provided good analytical sensitivity, as 250 international units (IU)/ml could be detected. Then, experiments focused on evaluating the elution conditions suitable for HEV release from vegetables, with the method proposed by the ISO 15216:2017 selected for evaluation in three types of fresh vegetables. The concentration method proposed by the ISO 15216:2017 combined with the RT-qPCR described by Schlosser et al. (2014) resulted in average HEV recoveries of 1.29%, 0.46%, and 3.95% in lettuce, spinach, and pepper, respectively, with an average detection limit of 1.47×10^5 IU/25 g. In naturally contaminated samples, HEV was detected in sewage only (10/14), while no detection was reported in lettuce (0/36) or in irrigation water samples (0/24).

Keywords

Foodborne virus, HEV, RT-qPCR, sewage, irrigation water, vegetables

1. Introduction

Hepatitis E virus (HEV) is a zoonotic pathogen that causes acute hepatitis which in some cases may progress to chronic hepatitis. HEV is a non-enveloped, single-stranded, positive-sense RNA virus with at least four genotypes known to infect humans (EFSA, 2017; Kupferschmidt, 2016). HEV genotypes 1 and 2, which infect humans only, have been associated with waterborne outbreaks in low-income countries, while genotypes 3 and 4 are zoonotic and have been isolated in different animal species, especially in pigs, as well as in humans residing high-income countries (Van der Poel, 2014). The main transmission routes of HEV are contaminated water, consumption of raw or undercooked meat from pigs or wild boar, blood transfusion, and organ transplantation (Van der Poel, 2014). However, patients suffering from hepatitis E may excrete up to 10^{11} genome copies per gram of feces (Li et al., 2006) prior to being symptomatic; therefore, infection can also occur through the fecal–oral route either by direct contact with an HEV-infected person or by ingestion of contaminated food or water. In endemic regions, contaminated waters are primarily responsible for HEV transmission that result in both sporadic and epidemic outbreaks (van der Poel and Rzezutka, 2017).

HEV is not notifiable in all member states of the European Union, even though it represents an emerging infectious disease. During the past decade, HEV has demonstrated a 10-fold increase in confirmed cases (EFSA, 2017) and transmission via the consumption of raw or undercooked meat from swine, boar, deer, and shellfish has been demonstrated (EFSA, 2017; Van der Poel, 2014). To date, there has been no confirmation of direct HEV transmission through contaminated produce, although its presence in irrigation waters, berries, salads, radicchio chicory, pepper, and bay leaf powder has been reported (Kokkinos et al., 2012; Kokkinos et al., 2017; Loisy-Hamon and Leturnier, 2015; Maunula et al., 2013; Santarelli et al., 2017; Terio et al., 2017). In

fact, irrigation water used for primary production, organic fertilizer, and sewage represent potential contamination sources for vegetables and fruits in the field. One of the major limitations to better understanding the role of HEV transmission through contaminated foods is the lack of standardized and validated methods (EFSA, 2017). RT-qPCR methodologies have been used to detect an quantify enteric viruses in food samples, especially in the detection of human norovirus (HuNoV) and hepatitis A virus (HAV), for which a standard quantification method has been recently issued and validated (ISO 15216-1, 2017; Lowther et al., 2017). In contrast, the detection of HEV in food and in environmental samples has been less investigated, although efforts have recently been made to study the role of seafood and meat in HEV transmission (Guillois et al., 2016; La Rosa et al., 2011; Mansuy et al., 2016; Said et al., 2013; Sarno et al., 2017). For instance, an RT-qPCR assay showed similar performance in sensitivity and quantitative accuracy compared with novel techniques, such as microfluidic digital RT-PCR, in being able to detect and quantify HEV in meat products (Martin-Latil et al., 2016).

The European Food Safety Authority (EFSA) recently categorized the evaluation and standardization methods for HEV detection from pig meat and meat products as a high priority. Additionally, the EFSA stated that extraction methods described in the validated ISO norm to quantify HAV and HuNoV (ISO 15216-1, 2017) should be evaluated in order to demonstrate their suitability for the detection of HEV in other food matrices (including vegetables) (EFSA, 2017).

The initial purpose of this work was to compare the performances of three RT-qPCR assays in the detection of HEV. The suitability of the concentration method described in the ISO15216:2017, specifically to detect HEV in vegetables, was then investigated.

Additionally, lettuce, irrigation and sewage water samples were analyzed for the presence of HEV.

2. Materials and methods

2.1. Virus strains

The first WHO international standard for HEV nucleic acid amplification technique (NAT)-based assays (code 6329/10) was purchased from Paul-Ehrlich-Institut (Germany). This standard corresponds to HEV genotype 3a positive plasma measured in international units and containing 250,000 IU/ml (Baylis et al., 2013). Additionally, four HEV-positive serum samples and one fecal sample from four patients, kindly provided by Dr. M. Jesus Alcaraz (Hospital Clínico Universitario) were included in the study. Mengovirus vMC_0 (courtesy of Prof. Albert Bosch, University of Barcelona) was used as a virus process control.

2.2. RNA extraction, RT-qPCR assays and genotyping

Fecal sample was vigorously vortexed in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), and centrifuged at 1,000 × g for 5 min to obtain a final 10% (wt/vol) suspension. The supernatant was stored at - 80 ºC in aliquots. Viral RNA extraction was carried out on 150 µl of fecal suspension, serum and the International WHO HEV Standard using a NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. Primers, probes and RT-qPCR conditions used in this study are listed in Table 1. RT-qPCRs were carried out in 96-well plates using the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen SA) or alternatively the HepatitisE@ceeramTools™ kit (Biomerieux, France) with a half-scale modification of the manufacturer’s protocol and the

LightCycler 480 instrument (Roche Diagnostics). Quality control of the RT-qPCR process included negative (nuclease-free water) and positive (HEV RNA) controls added to each PCR plate. Additionally, each run includes a positive and negative control for RNA extraction. Each viral RNA was analyzed at least in duplicate. Mengovirus quantification was performed according to the ISO 15216-1:2017; a standard curve was generated by amplifying 10-fold dilutions of viral suspensions by RT-qPCR in triplicate, and the numbers of genome copies were calculated.

Standard curves ranging from 2×10^5 to 2×10^2 IU/ml of the WHO HEV international standard (code 6329/10) were used to determine the limit of detection, efficiency, regression coefficient, slope and intercept of the RT-qPCRs in quadruplicate (Baylis et al, 2013).

Samples that rendered a HEV-RNA positive result after RT-qPCR were selected for genotyping. Nucleotide sequences corresponding to ORF2 gene were obtained by RT-PCR followed by a secondary PCR and direct sequencing as described for the ORF2-2 fragment with minor modifications (Fogeda et al., 2009). Sequences were subtyped by sequence comparison and phylogenetic reconstruction with reference sequences available for HEV subtypes (Smith, Simmonds, et al., 2016) as previously described (Bracho et al., 2011).

2.3. Comparison of protocols to release HEV from lettuce

Experiments to evaluate different protocols to release HEV from vegetables were performed with romaine lettuce (*Lactuca sativa*) obtained from a local supplier. Diluted HEV-positive fecal sample (HEVSt/1) containing approximately 2.0×10^6 IU were seeded by distributing 50 µl over 10 spots on the surfaces of fresh lettuce. Inoculated lettuce samples were air dried in a laminar flow hood for 60 min, and 10 µl of

mengovirus were added to the sample as process control virus to control extraction efficiency. Each experimental condition was analyzed in triplicate and processed the same day. Undiluted and 1/10 diluted RNA was tested to check for inhibitors. Different controls were used, including negative process, extraction and RT-qPCR controls, and controls for extraction efficiency.

2.3.1. Method A (ISO 15216-1:2017)

ISO 15216-1:2017 was applied for artificially inoculated lettuce. Briefly, 25 g of inoculated lettuce samples were transferred into a 400 mL polypropylene sterile blender bags with lateral filter (VWR). Viruses were released from the lettuce surface by gentle shaking (60 oscillations min^{-1}) with 40 ml of TGBE elution buffer (Tris base 100 mM, Glycine 50 mM, 1% beef extract, pH 9.5) for 20 min at room temperature. The rinse fluid was then removed from the filter side, centrifuged at 10,000 × g for 30 min at 4°C and supernatant was adjusted to pH 7.0. The neutralized supernatant was supplemented with 10% (wt/vol) polyethylene glycol (PEG) 8000 and 0.3 M NaCl, and incubated for 1 h at 4°C. Finally, the pellet was recovered by centrifugation at 10,000 × g for 30 min, with a further centrifugation step at 10,000 × g for 5 min to compact the pellet. The resulting pellet was resuspended with 500 µl of PBS and retained for RNA extraction.

2.3.2. Method B (Modified ISO 15216-1:2017)

ISO 15216-1:2017 standard was applied as previously described except that 90 ml of TGBE elution buffer (pH 9.5) was used in order to cover the 25 g of lettuce. Once incubated for 20 min at approximately 60 oscillations min^{-1}, lettuce samples were rinsed again with 5 ml of TGBE as for Method C (Sanchez et al., 2012). Additionally, the volume of the PEG solution was adjusted accordingly.

2.3.3. Method C

174 Lettuce samples were processed as previously described by Sánchez et al. (2012).
175 Briefly, 10 g of inoculated lettuces were transferred into a 400 ml polypropylene bag
176 containing a filter compartment (VWR) and viruses were released from the lettuce
177 surface with 90 ml of buffered peptone water (BPW) using the Pulsifier equipment
178 (Microgen Bioproducts) for 15 s. The rinse fluid was removed from the filter side, and
179 lettuce samples were then rinsed with 5 ml of BPW. The resulting filtrate was
180 supplemented with 10% PEG 8000 and 0.3 M NaCl, and incubated for 1 h at 4°C.
181 Finally, the pellet was recovered by centrifugation at 10,000 × g for 30 min and
182 resuspended with 500 µl of PBS.
183
184 **2.3.4. RNA extraction and virus detection from lettuce**
185 For each concentrated sample, 150 µl of sample was added with 25 µl of Plant RNA
186 Isolation Aid (Ambion) and 600 µl of lysis buffer from the NucleoSpin® RNA virus kit
187 (Macherey-Nagel GmbH & Co.) and subjected to pulse-vortexing for 1 min.
188 Afterwards, the homogenate was centrifuged for 5 min at 10,000 × g to remove the
189 debris. The supernatant was subsequently processed using the NucleoSpin®RNA virus
190 kit according to the manufacturer's instructions. RNA was analyzed using the RNA
191 UltraSense One-Step (Invitrogen SA) and RT-qPCR performed as described by
192 Schlosser et al. (2014) and ISO 15216-1 (2017), for HEV and mengovirus, respectively.
193 Undiluted and 10-fold diluted RNA extracts obtained from each sample were tested in
194 duplicate. HEV quantification was calculated by plotting the quantification cycles (Cqs)
195 to an external standard curve built with the International Standard WHO HEV RNA
196 (250,000 International Units/ml). Moreover, extraction efficiencies were calculated and
197 used as quality assurance parameters according to ISO 15216-1 (2017).

198 **2.4. Detection limit and efficiency of the procedure to release HEV from vegetables**

199 Experiments were performed using romaine lettuce, spinach (*Spinacia oleracea*) and
200 peppers (*Capsicum annuum*) obtained from a local market. Briefly, 25 g of vegetables
201 were artificially inoculated with different concentrations (approximately 6, 5 and 4 log
202 IU) of HEV-positive fecal sample (HEVSt/1). Samples were then processed as
203 described above (Method A=ISO 15216-1:2017). Undiluted and 1/10 diluted RNA was
204 tested to check for inhibitors. Different controls were used, including negative process,
205 extraction and RT-qPCR controls, and controls for extraction efficiency.

206 **2.5. Analysis of naturally contaminated samples**

207 Two sewage samples were monthly collected from the municipal wastewater treatment
208 plant of Quart Benager (Valencia, Spain) from March to September 2017. Thirty-five
209 ml of sewage (n=14) were concentrated to a final volume of 500 µl by
210 ultracentrifugation as previously described (Randazzo et al., 2017; Rodriguez-Diaz et
211 al., 2009). RNA extraction was performed as described above. HEV RNA was detected
212 and quantified by the three different RT-qPCR assays as described above. Moreover,
213 mengovirus was included as process control virus to monitor extraction efficiency for
214 each sample.

215 In addition, irrigation water samples from a secondary effluent of a wastewater
216 treatment plant were collected from the irrigation head of a hydroponic system of a
217 commercial greenhouse located in Balsicas (Murcia, Spain). Water samples (n=24)
218 were collected weekly during the growth cycle of the lettuce plants (26-38 days). Two
219 hundred ml of water were concentrated by filtration as previously described (López-
220 Gálvez et al., 2016). Additionally, weekly collected lettuce samples (n=36) cultivated
221 under sprinkler irrigation were analyzed. Lettuce samples were analyzed following the
222 ISO 15216-1 (2017).

223 **2.6. Statistical analysis**

HEV and mengovirus recoveries were estimated by calculating the $\log_{10}$ (Nt/N_0), where N0 is the titer of the processing control (virus directly diluted into final volume buffer solution) and Nt is the titer of the tested sample. All the quantifications of viral loads were obtained by plotting each Cq to a standard curve for each virus. Data were statistically analyzed by STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) applying one-way analysis of variance (ANOVA) to test the impact of different factors. When significant differences were determined on the means, a multiple comparison procedure (Tukey's honest significant difference (HSD)) was applied to determine which factor was significantly different from the others. In all cases, values of $p < 0.05$ were deemed significant.

3. Results and discussion

3.1. Evaluation of RT-qPCR assays

There is a critical and immediate need to develop a method for detecting HEV in vegetables (EFSA, 2017). To address this challenge, we initially evaluated three different RT-qPCR assays for the detection and quantification of the first WHO international standard for HEV (code 6329/10). Standard curves were established for each RT-qPCR assay using the WHO international standard serially diluted from 2.5×10^5 to 2.5×10^2 IU/ml and amplified in quadruplicate. The efficiency, regression coefficient, slope, and intercept for each assay are shown in Table 2. Curves with the slope between -3.10 and -3.60 (corresponding to amplification efficiencies of ~90 % to 110 %) were used for calculations. The performance of assays B (HepatitisE@ceeramTools™ kit) and C (Schlosser et al., 2014) were similar, while assay A (Jothikumar et al., 2006) showed a decrease in sensitivity using the WHO HEV standard (Table 2).

248 Applying assay A, positive amplification in all replicates of each RNA dilution was
249 achieved when 2.5×10^4 IU/ml of HEV or more were present, and as few as 2.5×10^2
250 IU/ml could be detected with 100% and 25% probability when using assays B and C,
251 respectively (Table 2). The detection limit reported using assay B is in line with
252 previous studies (Abravanel et al., 2013; Vollmer et al., 2014).
253 Additionally, four Spanish clinical samples, all subtyped as HEV-3f (nucleotide
254 sequences deposited in the GenBank database under accession numbers MG674574,
255 MG674575, MG674576 and MG674577), were analyzed with the three assays, showing
256 that assay A is potentially less sensitive for genotype 3f (clinical samples) and 3a
257 (WHO standard) due to significant differences in Cq values (Table 2 and Fig. 1).
258 Although assay A, originally developed by Jothikumar et al. (2006), is widely used in
259 the detection of HEV because it targets a highly conserved region (Baylis et al., 2013),
260 this assay was later modified in different laboratories (Garson et al., 2012; Giron-
261 Callejas et al., 2015; Martin-Latil et al., 2014). For instance, the use of a degenerate
262 version of the reverse primer and a MGB-modified probe improved the performance of
263 the assay by detecting 250 IU/ml of the WHO HEV standard (Giron-Callejas et al.,
264 2015).
265 Based on these results it is concluded that assays B and C are suitable in the detection
266 and quantification of HEV. Even though assay B (HepatitisE@ceeramTools™ kit) had
267 a slightly better detection limit, assay C, because of its sensitivity and cost efficiency,
268 was further used to evaluate vegetables and water samples.
269

270 **3.2. Comparison of the performances of different eluting conditions for HEV**

271 Virus elution protocols are particularly pertinent with respect to vegetables that are
272 prone to contamination through sewage-contaminated surface water or infected food

handlers during harvesting, packaging, or food preparation, where the viruses are likely to be on the surface of the food. The elution protocol using TGBE buffer and concentration by PEG precipitation has been proposed as a standard method for HAV and HuNoV detection and quantification in berries and vegetables (ISO 15216-1, 2017). In order to establish a method for detecting and quantifying HEV, we compared the ISO 15216-1 (2017)procedure of eluting viruses from vegetable surfaces with a modification of the ISO procedure (method B, increasing the elution buffer to cover the entire vegetable surface) and with method C, the use of the Pulsifier and elution with BPW, which has been shown to be a suitable procedure for the simultaneous detection of enteric viruses and foodborne bacteria (Sánchez et al., 2012). Overall, similar HEV recovery rates were obtained for methods B and C (Table 3), while method A performed slightly better, with HEV recovery rates ranging from 2.5% to 5.2%. Additionally, similar recovery rates were observed in the 10-fold diluted RNA samples, suggesting the absence of PCR inhibitors by using the three methods as a ΔCq of approximately 3.3 was recorded using the ten-fold diluted RNAs (Table 3).

The HEV recovery rates obtained with method C were lower than those reported for other enteric viruses (Sánchez et al., 2012). For instance, the mean recovery rate of HuNoV genotype II (GII) approximately 9% in parsley, spinach, and mix salad, while HAV recovery rates were around 20%. The use of stool samples for the inoculation experiments could explain the low HEV recovery rates obtained with method C, as the samples could contain free RNA molecules and defective particles that are detected in the positive control if submitted to RNA extraction alone; in vegetable samples, however, the free RNAs and defective particles are most likely lost during the concentration step.

298 **3.3. HEV detection limit and recovery**

299 Given the slightly better performance of the standardized ISO 15216-1 (2017) elution
300 procedure (Table 3) and its current use in HEV monitoring in vegetables (Loisy-Hamon
301 and Leturnier, 2015; Terio et al., 2017), further evaluation of other vegetable matrices
302 was performed. The results of HEV recovery after inoculation in lettuce, spinach and
303 pepper using the elution conditions proposed by the ISO 15216-1 (2017) are shown in
304 Table 4. The average recovery rates for the different types of vegetables calculated with
305 undiluted RNA were 1.29 % (0.39%-2.01%), 0.46% (0.04%-0.88%), and 3.95 (2.97%-
306 5.53%) for lettuce, spinach and pepper, respectively. A minimum recovery rate of 1%
307 mengovirus was obtained for all samples, thus validating the results. Although recovery
308 rates of HEV in vegetables are not published, those obtained in this study are consistent
309 with the results obtained for other viruses applying the ISO 15216-1 procedure. For
310 instance, Coudray and collaborators reported average HAV recovery rates of 0.58% and
311 5.92% in lettuce, both in undiluted and 10-fold diluted RNA (Coudray et al., 2013).
312 Conversely, in the present study, HEV recovery rates were found similar in the 10-fold
313 diluted RNA and in the undiluted RNA (Table 3 and 4), perhaps because in our
314 procedure, the use of the Plant RNA Isolation Aid was included in order to remove PCR
315 inhibitors.

316 According to Wilrich and Wilrich (2009), the processing of vegetables with the ISO
317 15216-1 (2017) elution method rendered an average detection limit of 1.47×10^5 IU of
318 HEV per 25 g of produce (Table 4 and Table S1), even though RT-qPCR methods
319 detect the viral RNA of both infectious and inactivated HEV, potentially overestimating
320 the amount of infectious viruses.

321 Systematic comparisons for HEV in fresh vegetables have not been published, although
322 limited comparative studies on the efficiency of selected methods used in meat products

323 are available. For instance, a method based on TRI Reagent® Solution combined with
324 chloroform and a silica-based RNA extraction method resulted in a detection limit of
325 2.9×10^3 and 5.3×10^4 genome copies per 5 g raw sausage and 2 g liver sausage,
326 respectively (Szabo et al., 2015). A method that includes a virus concentration step by
327 PEG reported a limit of detection of 8.7×10^3 and 8.7×10^4 genome copies of HEV in 3
328 g of figatelli and pig liver sausages, respectively (Martin-Latil et al., 2014).

329 **3.4. Analysis of HEV in lettuce, irrigation water and sewage samples**

330 The prevalence of HEV in produce has scarcely been evaluated. In the present study, the
331 presence of HEV was not detected in any of the 36 lettuce samples analyzed using the
332 ISO 15216-1 (2017) elution procedure and the RT-qPCR described by Schlosser et al.
333 (2014). Likewise, HEV was not detected in samples from water used to irrigate the
334 lettuce plants. A minimum recovery of 1% mengovirus was obtained for all samples,
335 thus validating the results. In larger surveys, while using the ISO 15216-1 (2017)
336 elution procedure and the RT-qPCR described by Jothikumar et al. (2006), HEV was
337 detected in 3.42 % (5/146) of lettuce samples collected from Greece, Serbia and Poland
338 (Kokkinos et al., 2012). Similarly, HEV was detected in 2 (pepper and bay leaf powder)
339 out of 230 herbs and spices (0.87%) collected in France using the ISO 15216-1 (2017)
340 elution procedure and the Ceeram RT-qPCR assay (Loisy-Hamon and Leturnier, 2015).
341 In Italy, the occurrence of HEV in ready-to-eat vegetables was 0.6% (6/911) (Terio et
342 al., 2017) using the ISO 15216-1 (2017) elution procedure and the RT-qPCR described
343 by Jothikumar et al. (2006). In another study by Maunula et al. (2013) frozen
344 raspberries were found to be positive for HEV contamination (1/28, 2.6%).

345 Additionally, the presence of HEV in sewage waters from an urban sewage plant was
346 evaluated from March 2017 to September 2017. Of 14 samples, 10 were positive for
347 HEV, indicating an HEV prevalence of 71.43% (Table 5 and Table S2). For the 14

348 samples analyzed, a minimum yield of 1% mengovirus was obtained, thus validating the
349 results. In addition, comparing the three RT-qPCR assays tested in this study (Table 1),
350 different numbers of positive samples, and thus different prevalence rates, were
351 recorded (Table 5 and Table S2). In particular, prevalence rates of 28.97%, 64.29% and
352 71.43% were recorded for assays A, B, and C, respectively. Again, this outcome
353 confirms the higher sensitivity of assays B and C when compared with assay A, as
354 stated by the WHO international standard in the preliminary RT-qPCR evaluation step.
355 Since the first report of HEV detection in sewage (Jothikumar et al., 1993), its frequent
356 presence in sewage has been confirmed by many countries. For instance, 8.7%, 25%,
357 32%, 56%, 32%, and 93% of sewage from Israel, France, Switzerland, India, Spain, and
358 UK, respectively (reviewed by EFSA, 2017; Smith, Paddy, et al., 2016; van der Poel
359 and Rzezutka, 2017). Despite the fact that reports of HEV prevalence in sewage are
360 significant, the quantification data are somewhat limited. Our quantitative results
361 showed HEV contamination in sewage around 4 log IU/l, a finding that is consistent
362 with previously reported levels (Masclaux et al., 2013), even though infectivity of the
363 samples can not be assessed. Amplification and sequencing of three sewage strains was
364 feasible, rendering the same ORF2 sequence (432 nucleotides) of HEV-3f subtype. The
365 nucleotide sequences were deposited in the GenBank database under accession numbers
366 MG674578, MG674579 and MG674580.
367 In conclusion, our results suggest that assays B and C demonstrated the best results for
368 HEV RNA detection and quantification, as 250 IU/ml of the first HEV WHO
369 international standard could be detected. Moreover, the elution procedure proposed in
370 the framework of the ISO 15216-1 is suitable for recovering HEV in vegetable samples.
371 Nevertheless, considering the low HEV recovery rates in vegetables, improvements to
372 the procedure must be undertaken. For instance, some studies have shown that

ultrafiltration is more efficient than PEG precipitation for the recovery of HAV in vegetables (Butot et al., 2007; Hyeon et al., 2011). Additionally, combining RT-qPCR detection with intercalating dyes or RNase pretreatments will be help to better interpret the quantification of infectious HEV (Cook et al., 2017).

Finally, our results indicate that HEV circulates in sewage and has the potential to contaminate shellfish harvesting areas and water used for agricultural irrigation by discharge from wastewater treatment plants or by failure of depuration processes.

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396 **Table 1.** References of the RT-qPCR assays used in this study.

Assay	Amplification region	Primers and probe	Sequence 5'-3'	RT-qPCR conditions	Location*	Reference
A	ORF2/3	JVHEVF	GGTGGTTTCTGGGGTGAC	RT 50ºC for 30 min	5304-5373 (69 nt)	Jothikumar et al. (2006)
		JVHEVR	AGGGGTTGGTTGGATGAA	95ºC for 15 min		
		JVHEVP	FAM-TGATTCTCAGCCCTTCGC-BHQ	PCR (45x)		
				95ºC for 10"		
				55ºC for 20"		
				72ºC for 15"		
B	ORF2/3	NA	NA	RT 45ºC for 10 min	NA	Anonymous, 2016
				95ºC for 10 min		
				PCR (40x)		
				95ºC for 15"		
				60ºC for 45"		
C	ORF3	HEV.Fa	GTGCCGGCGGTGGTTTC	RT 50ºC for 30 min	5296-5377 (81 nt)	Schlosser et al. (2014) with modified probe
		HEV.Fb	GTGCCGGCGGTGGTTTCTG	95ºC for 15 min		
		HEV.R	GCGAAGGGGTTGGTTGGATG	PCR (45x)		
		HEV.P	FAM-TGACMGGGT/ZEN/TGATTCTCAGCC/3IABkFQ	95ºC for 10"		
				55ºC for 25"		
				72ºC for 25"		

397 *Location in reference to WHO International Standard for HEV RNA, HRC-HE104 strain, accession no. AB630970 (Baylis et al., 2013).

398 F: forward primer; R: reverse primer; P: probe

399

400

401

Table 2. Limits of detection, efficiency, regression coefficient, slope, and intercept three RT-qPCR assays

	Concentration	**Assay A** Jothikumar et al. 2006		**Assay B** HepatitisE@CeeramTools		**Assay C** Schlosser et al. (2014)	
	(IU/ml)	(+/total)	Mean Cq ± SD	(+/total)	Mean Cq ± SD	(+/total)	Mean Cq± SD
WHO HEV standard (6329/10)	2.5×10^5	4/4	31.14±0.30[a]	4/4	28.62±0.17[b]	4/4	28.82±0.10[b]
	2.5×10^4	4/4	34.33±0.81[a]	4/4	29.56±0.46[b]	4/4	32.43±0.15[c]
	2.5×10^3	3/4	37.45±0.70[a]	4/4	32.65±0.25[b]	4/4	35.55±0.30[c]
	2.5×10^2	0/4		4/4	35.70±0.55[a]	1/4	36.98[b]
Efficiency (%)			105.82		96.69		98.23
Regression coefficient			0.9502		0.9963		0.9914
Slope			-3.190		-3.404		-3.365
Intercept			40.012		35.577		38.329

Crossing point (CP) represents the PCR cycle at which the probe-specific fluorescent signal can be detected against the background.

[a,b.c]For each raw, different letters denote significant differences among assays ($p<0.05$).

414 **Tabla 3**. Comparison of elution methods for HEV detection on lettuce

Concentration method	Undiluted RNA			10-fold diluted RNA			
	HEV samples (+/total)	Cq± SD	Mean HEV recovery (min-max) (%)	HEV samples (+/total)	Cq± SD	Mean HEV recovery (min-max) (%)	Reference
A	3/3	29.62±0.47[a]	3.98 (2.56-5.20)	3/3	33.51±0.88	2.89 (1.72-4.85)	ISO 15216-1 (2017)
B	3/3	31.27±0.22[b]	1.21 (1.10-1.37)	3/3	34.65±0.73	1.21 (0.50-1.50)	modified ISO 15216-1 (2017)
C	3/3	31.43±0.69[b]	1.15 (0.46-1.51)	1/3	34.47*	1.25	Sánchez et al. (2012)

415 Each procedure was analyzed in triplicate and each sample analyzed in duplicated by RT-qPCR. Quantification cycle (Cq): the PCR cycle at
416 which the target is quantified in a given RT-qPCR reaction. *One replicate out of two. [a,b]Different letters denote significant differences among
417 concentration methods ($p<0.05$).

418

419

Table 4. Detection of HEV by RT-qPCR in artificially inoculated fresh vegetables following ISO 15216-1:2017 elution procedure and the RT-qPCR assay described by Schlosser et al. (2014).

	Levels of inoculated HEV (IU/25 g)						Mean mengovirus recovery (min-max) (%)	$LOD_{95\%}$[a] (IU/25 g)
	Undiluted RNA			10-fold diluted RNA				
	1.9×10^6	1.9×10^5	1.9×10^4	1.9×10^6	1.9×10^5	1.9×10^4		
Lettuce	3/3 (1.29±0.81)*	2/3	2/3	3/3 (1.38±0.68)*	1/3	0/3	11.08 (5.20-18.12)	2.7×10^5
Spinach	3/3 (0.46±0.34)	3/3	2/3	2/3 (0.71±0.62)	1/3	0/3	9.64 (3.04-17.36)	5.2×10^4
Pepper	3/3 (3.95±1.12)	3/3	1/3	3/3 (1.21±0.65)	1/3	0/3	10.11 (3.61-21.07)	1.2×10^5

*Numbers in parentheses indicate the percent of HEV recovery.

[a]Calculated according to Wilrich and Wilrich (2009).

427 **Table 5.** Comparative HEV detection in sewage by three different RT-qPCR.

428

#	**Sample**	**Assay A** Jothikumar et al. 2006	**Assay B** HepatitisE@CeeramTools	**Assay C** Schlosser et al. (2014)	**Concentration Averages* (Log IU/l)**
1	March-1	**2/2**	**1/2**	**2/2**	4.07 ± 0.32
2	March-2	**2/2**	**2/2**	**1/2**	<LOQ
3	Abril-1	0/2	0/2	**2/2**	<LOQ
4	Abril-2	**2/2**	**1/2**	**2/2**	<LOQ
5	May-1	0/2	0/2	0/2	nd
6	May-1	0/2	**2/2**	**1/2**	<LOQ
7	June-1	0/2	0/2	0/2	nd
8	June-2	0/2	0/2	0/2	nd
9	July-1	0/2	**2/2**	**2/2**	4.82 ± 0.02
10	July-2	0/2	**2/2**	**1/2**	4.49
11	August-1	0/2	**1/2**	**1/2**	4.99
12	August-1	0/2	0/2	0/2	nd
13	September-1	0/2	**1/2**	**1/2**	4.86
14	September-2	**2/2**	**2/2**	**2/2**	<LOQ
Total		4/14	9/14	10/14	
Prevalence (%)		28.57	64.29	71.43	

429 *, data quantification based on Assay C.

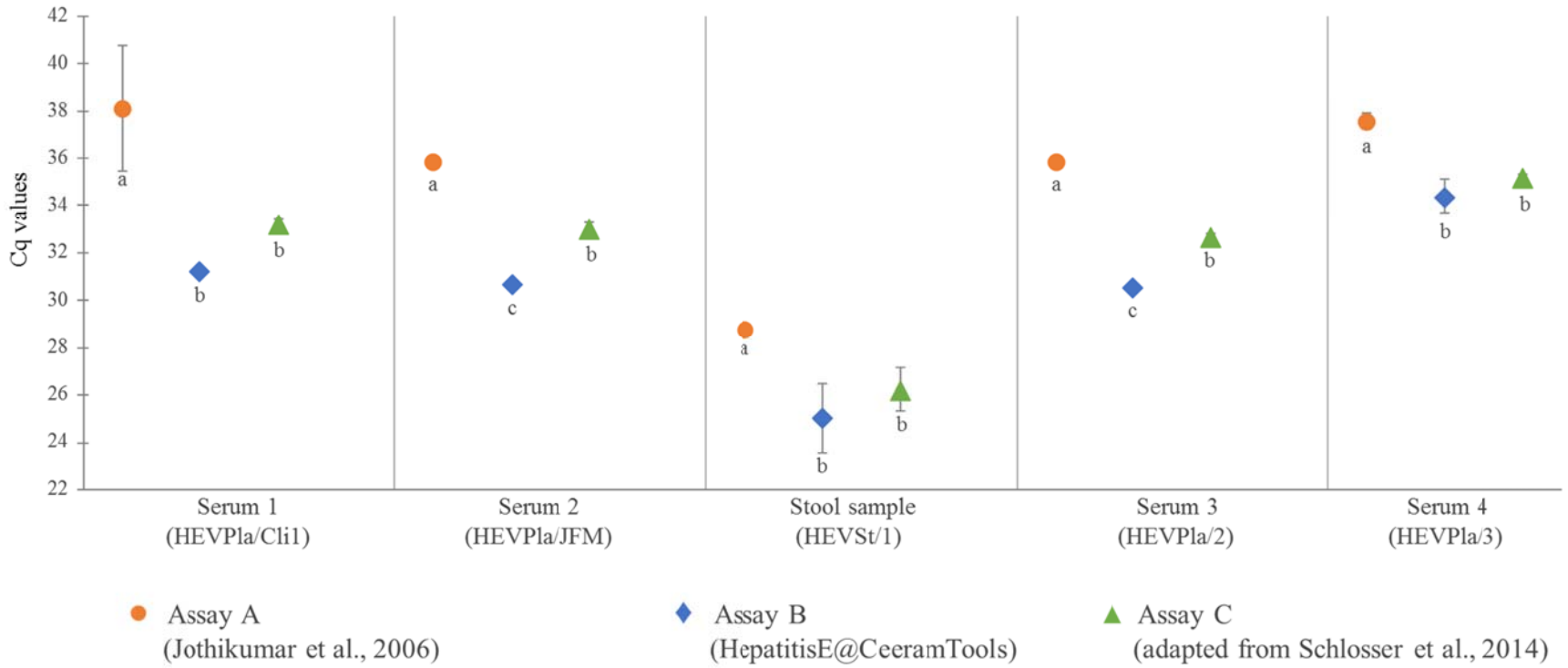
430 <LOQ, below the limit of quantification; nd, not detected.

431

432

433 **Figure 1.** Comparison of three RT-qPCR assays (A, B, and C) in detecting and quantifying HEV RNA on human fecal and serum samples

434 [a,b,c]Different letters denote significant differences among assays for each sample ($p<0.05$).



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