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RESEARCH

An Efficient, Organic Solvent-Free Method for Extraction and Concentration of Hepatitis E Virus from Pig Liver

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Abstract

The presence of the hepatitis E virus (HEV) in pork products, particularly in pig liver has been frequently described. However, a standardized method is not still available for the detection of HEV in foods, particularly in those difficult food matrixes such as pig meat and pork products. The aim of this study was to design, optimize and evaluate a new method of food-separation and virus concentration for HEV in pig liver samples. This method is based on organic flocculation and avoids the use of organic solvents. The virus recovery rates and analytical sensitivity of the method using murine norovirus MNV-1 as a surrogate were 73.6–82.2% and at least 1×10^3 $TCID_{50}$ per g of liver in 100% of the replicates, respectively. Furthermore, this new methodology was validated by testing the presence of HEV RNA in naturally infected pig liver samples, comparing it with two other commonly used concentration methods. The new extraction method run satisfactory in comparison with the two reference methods; statistically equivalent ($p < 0.05$) to one of the methods used while presented statistically significant better results ($p < 0.05$) compared to the second method. Consequently, our results indicate that the new extraction method can be an adequate cost-effective and ecologically friendly alternative for the food separation and concentration of HEV RNA in pig liver samples.

Keywords Hepatitis E Virus · Food Safety · One Health · Virus Extraction · Pork Liver · Organic Flocculation · Virus detection · RT-qPCR

Introduction

Hepatitis E virus, HEV, is the most common cause of enteric viral hepatitis infection worldwide, and Hepatitis E is now considered an emerging disease in Europe (Ricci et al. 2017). The prevalence of HEV in pig-producing regions in Europe, as well as within domestic pig herds, is usually very high. Consequently, the pig production chain, especially the domestic pig, is considered the main source of zoonotic transmission of HEV in Europe (Ricci et al. 2017).

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Currently, there is no food legislation for HEV. One of the main reasons that prevents the development of legislation in this regard is the lack of a standard method for detecting the pathogen. The detection of HEV in different food matrices poses 2 main challenges, firstly HEV is usually found in very low concentrations in food samples. Secondly, HEV can be found intracellularly and not only as a surface contamination, especially in the pig production chain.

Although there is a cell culture system that allows measuring the infectivity of the pathogen (Johne et al. 2016, Schemmerer et al. 2016), it only works with a specific HEV strain and not with most of the wild-type HEV isolated in different foods from pig and pig products, so the detection of this pathogen in foods is carried out using methods based on RT-qPCR. However, the molecular analysis of HEV, particularly in difficult food matrixes such as pig liver is cumbersome, particularly its extraction from the food matrix and elimination of potentially interfering substances.

HEV has been detected in different pig organs and fluids, but the liver is the organ in which it is most frequently found (García et al. 2020), and different strategies have been

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developed and compared for the recovery of HEV particles in raw liver and derived products (Szabo et al. 2015) (Moor et al. 2018) (Hennechart-Collette et al. 2019). Organic flocculation has been used successfully for the concentration of viruses in water simples (Katzenelson et al. 1976) and solids from wastewater (Safferman et al. 1988), and more recently in strawberries (Melgaço et al. 2016). The stability of HEV, as well as its infectivity in a wide pH range, has been demonstrated (Wolff et al. 2020), so organic flocculation through acidification is a possible solution for the extraction of this pathogen. The European Union's framework program for Research in the period 2021–2027 Horizon Europe has among its main objectives to make the transition towards a prosperous and sustainable future (Horizon Europe 2021) (Horizon Europe 2024); Therefore, the development and subsequently effective implementation of novel analytical methodologies in food safety must follow an ecologically friendly strategy. In this context, furthermore, the development of any extraction and concentration methodology for virus detection in food should also avoid the use of organic solvents. In the present study, a new method for the recovery, concentration and purification of HEV from raw pig liver samples was developed and optimized, and the method was subsequently compared with two commonly used extraction methods (Szabo et al. 2015) (García et al. 2020).

Materials and Methods

Extraction Method

Figure 1 shows a graphical representation of the novel method developed in this study (Fig. 1). Briefly, 2 g of each liver sample was aseptically collected and were finely chopped manually using scalpels with sterile blades. Subsequently, 300 mg of liver was taken into a 15-ml polypropylene tube, and 2 ceramic spheres of ¼″ diameter (MP Biomedicals, Germany) and 250 µl of extraction buffer were added. Four different extraction buffers were tested in this study (Table 1). The liver sample containing tube was subjected to a mechanical disruption (FastPrep 24 5G, MP Biomedicals, USA) for 2 cycles of 40 s at 6 m/s, with a lapse of 300 s between cycle. Another 3.75 ml of buffer was added, and 3 further disruption cycles were performed in identical conditions. Afterwards, tubes were centrifuged at 3740 × g and 4 °C for 15 min, and 3.7 ml of the resulting supernatant was recovered without recovering the superficial fat phase. Subsequently, the pH was adjusted to 3.5 (± 0.1) using 1 M HCl, and the tubes were further centrifuged at 13,250 × g and 4 °C for 20 min. The supernatant was removed and the pellet was immediately used for virus RNA extraction.

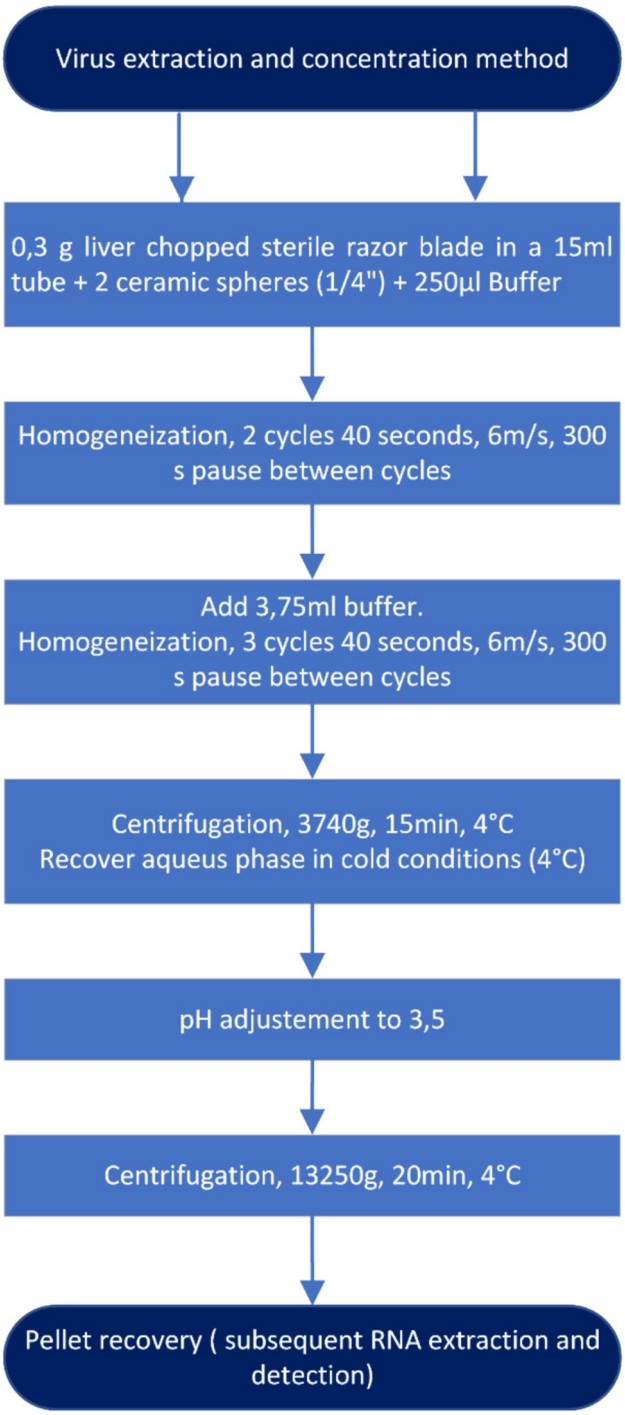

Fig. 1 Diagram of the developed extraction and concentration method

Method Optimization

For the optimization of the new extraction method, after preliminary tests, certain parameters were set (buffers used, amount of homogenized sample), and the method was optimized by studying the behaviour of 3 parameters:

Table 1 The extraction buffers evaluated in this study for the new solvent-free method

Buffer	Reagents (g/L)				
	Na_2HPO_4	KH_2PO_4	NaCl	KCl	$(NH_4)_2SO_4$
J0	11.5	2	80.6	2.2	0
JA3	16.56	0.6	60.44	1.648	0
JB3	26.32	0.6	0	0	70.6
JC	26.32	0.6	60.44	1.648	70.6

centrifugation temperature, pH, and type of sample homogenization. These 3 parameters were analysed one by one, leaving the rest of the parameters fixed. After the tests, it was decided to establish 3 centrifugation temperatures (25, 10 and 4 °C), 5 pH adjustment values (6.5, 5.5, 4.5, 3.5 and 2.5) and 4 homogenization procedures: procedure 1, homogenization in microcentrifuge tubes with 2 g of 1 mm zirconia beads, homogenization of 3 cycles of 40 s, 6 m/s, 300 s lapse between cycles; procedure 2 in 15-ml tubes, with 2¼ ceramic sphere balls, 3 cycles of 20 s, at 4 m/s with a 300 s lapse between cycles; procedure 3 in 15 ml tubes, with 2¼ ceramic sphere balls, 3 cycles of 40 s, at 6 m/s with a 300 s lapse between cycles, adding the buffer before the first homogenization cycle; and procedure 4: in 15-ml tubes, with 2¼ ceramic sphere balls, 5 cycles of 40 s, at 6 m/s with a 300 s lapse between cycles, adding the buffer after the first 2 homogenization cycles. The experiments were performed in triplicate. A pool of homogeneously mixed positive livers was used, from which all aliquots were taken for method optimization tests. Homogeneity Tests were performed with a 95% confidence level for the different groups of results (LSD, Tukey LSD, Scheffe, Bonferroni, Student–Newman–Keuls, Duncan).

Determination of the Analytical Sensitive of the New Method Coupled to a RT-qPCR

To assess the analytical sensitivity of the method, a virus surrogate, murine norovirus-1 (MNV-1), was used (Diez-Valcarce et al. 2011). Briefly, liver samples that had been tested negative for HEV were minced manually using a scalpel and spiked with decimal dilutions of MNV-1 (1×10^4, 1×10^3, 1×10^2, 1×10^1, and 1 $TCID_{50}$ of MNV-1 per g of liver), and the extraction procedure was performed subsequently. For the detection of MNV-1 RNA, a previous RT-qPCR was used (Diez-Valcarce et al. 2011) with a reaction volume of 10 µl (of which 2.5 µl corresponds to RNA), using the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, USA), and ROX as a reference to normalize fluorescence in a Quant Studio 5 instrument (Applied Biosystems). Three independent assays were performed, and 3 replicates of each condition per assay, with a total of 9 RT-qPCRs for each MNV-1 concentration tested.

Comparison of the Performance of the Novel Concentration Method

Samples

Twenty-one samples of pig livers naturally infected with HEV that had previously tested positive for hepatitis E virus RNA were used. The samples come from a study carried out by our research team in which the presence of HEV RNA in liver samples collected from Spanish slaughterhouses with a nationwide representation during 2020–2021 (Santamaria-Palacios et al., submitted for publication). Liver samples were kept at − 80 °C until use.

Sample Preparation

Two grams of each liver sample was homogenized, and aliquots were taken for comparison of the different extraction methods. Liver samples were finely chopped manually using scalpels with sterile blades. A single homogeneous bite was made from each sample, from which different aliquots were separated: 300 mg aliquots were taken for the new method using the 4 different extraction buffers (Table 1) (Methods 1–4); 100 mg was taken for method 5, which is a widely extraction method that uses Quiazol and chloroform (Di Bartolo et al. 2020, Diez-Valcarce et al. 2012, Rodriguez-Lazaro et al. 2015, García et al. 2020); and 300 mg was taken for method 6, which is an adaptation of a reference method that uses Trizol and chloroform (Szabo et al. 2015).

Method 5

Briefly, 100 mg of homogenized liver was taken in a 2-ml Fastprep tube with 200 µl of phospate-buffered saline and 2 g of 1 mm zirconia beads and subjected to 3 mechanical disruption cycles (FastPrep 24 5G, MP Biomedicals, USA) of 40 s at 6 m/s, with a 300 s lapse between cycles. Then, 1 ml of QUIazol (Qiagen, Germany) was added, vortexed for 30 s and incubated for 5 min at room temperature. Subsequently, 200 µl of 24:1 choroform:isoamylalcohol (Sigma Aldrich) was added, vortexed for 30 s and incubated for 5 min at room temperature. The tubes were centrifuged at 12,000 × g and 4 °C for 15 min, and the supernatant was used for virus RNA extraction.

Method 6

The method is described in Szabo et al. (Szabo et al. 2015) with small modifications in the volumes to be comparable. Briefly, 300 mg of liver was taken in a 15-ml polypropylene

tube, 2 ceramic spheres of ¼″ diameter (MP Biomedicals, Germany) and 1.05 ml of Trizol (Invitrogen, USA) were added, and the tube was subjected to mechanical disruption (FastPrep 24 5G, MP Biomedicals, USA) for 5 cycles of 40 s at 6 m/s, with 300 s lapses between cycles. The tube was centrifuged at 10,000 × g and 4 °C for 20 min, the supernatant was taken into a new centrifuge tube, and 200 µl of chloroform was added for each ml of Trizol. The sample was vortexed for 15 s and incubated for 15 min at room temperature. Subsequently, the tube was further centrifuged at 10,000 × g and 4 °C for 15 min, and the supernatant was used for viral RNA extraction.

Nucleic Acid Extraction

For the new extraction method and for the method based on Szabo et al. (2015), the QIAamp Viral RNA mini kit (Qiagen, Germany) was used following the manufacturer's instructions, using an initial volume of 200 µl, and a final elution volume of 60 µl of nuclease-free water. For the extraction method based on García et al. (2020), the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) was used, which includes the Quiazol reagent, following the manufacturer's instructions and eluted in 60 µl of nuclease-free water.

RNA Detection by RT-qPCR

Detection of HEV RNA was performed by RT-qPCR. The system described by Jothikumar et al. (2006) was used with slight modifications (García et al. 2020). RT-qPCRs were performed in triplicate, with a reaction volume of 10 µl (of which 2.5 µl correspond to RNA). TaqMan Fast Virus 1-Step Master Mix (Applied biosystems, USA) was used, using ROX as a reference to normalize fluorescence, in a Quantstudio 5 device (Applied Biosystems). For all experiments, only C_T values < 40 were considered as positive.

Virus Recovery Rates

Murine norovirus-1 (MNV-1) propagated in RAW264.7 cells was used as a surrogate (Diez-Valcarce et al. 2011). To calculate the recovery rates, a dilution of MNV-1 was spiked onto minced liver (3×10^3 $TCID_{50}$ in each sample), immediately before performing the analyses. Liver samples that had tested negative for HEV were used, minced manually using a scalpel.

For the detection of MNV-1 RNA, a previous RT-qPCR was used (Diez-Valcarce et al. 2011) with a reaction volume of 10 µl (of which 2.5 µl corresponds to RNA), and TaqMan Fast Virus 1-Step Master Mix (Applied biosystems, USA), using ROX as a reference to normalize fluorescence were used in a Quantstudio 5 instrument (Applied Biosystems).

Statistical Analysis

Statgraphics Centurion 19 was used for data processing. The RT-qPCR C_T values obtained for method optimization are the average of 3 replicates with the standard deviation of each sample. For the comparison of the extraction methods, a multifactorial ANOVA with interaction was performed for each extraction method and sample factors. Six different extraction methods were considered (the new method with the 4 different extraction buffers, and the two reference extraction methods for HEV in pig products). Subsequently, a normalized multiple regression was performed with a model $y = b0 + b1 \times 1 + b2 \times 2 + b12 \times 1x2$ to study the significant interaction resulting from the multifactorial ANOVA. Finally, a one-way ANOVA was performed to group the samples into homogeneous groups, and a multifactorial ANOVA with interaction was performed in each group. Homogeneity tests were performed with a confidence level of 95% for the different groups of results (LSD, Tukey LSD, Scheffe, Bonferroni, Student–Newman–Keuls, Duncan).

Results

Method Optimization

The RT-qPCR results obtained for the different optimization parameters tested are shown in Table 2. The RT-qPCR results for a centrifugation at of 4 °C, a value of pH of 3.5 and the homogenization type 4 (in 15-ml tubes, with 2¼ ceramic sphere balls, 5 cycles of 40 s, at 6 m/s with a lapse of 300 s between cycles, and adding the buffer after the first 2 homogenization cycles) were significant better ($p > 0.05$) to those using other centrifugation temperatures, pH values and homogenization procedures.

Additionally, the centrifugation temperature parameters were set at 4 °C, and the pH at 3.5, and the 4 procedures of homogenization were tested to calculate the recovery rates using a virus surrogate (MNV-1). The results obtained are shown in Fig. 2. As expected, the homogenization type with the highest recovery rate was type 4 with a value of 82.1% [79.1–89.8 95% CI], although the homogenization procedures 3 and 4 were statistically homogeneous ($p < 0.05$).

Consequently, the centrifugation temperature was set at 4 °C, a pH of 3.5 and the homogenization procedure 4 were selected and used to evaluate the analytical sensitivity of the extraction method and for the validation of the method.

Analytical Sensivity

The analytical sensitivity of the extraction method coupled to a HEV-specific RT-qPCR assay was evaluated using

Table 2 HEV RT-qPCR C_T values obtained using different optimization parameters (temperature, pH and type of homogenization). Mean and standard error and the 95% confidence homogeneous groups are shown

Parameter	Value	C_T
Temperature (°C)	25	29.44 ± 0.26 [a]
	10	27.91 ± 0.15 [b]
	4	25.78 ± 0.07 [c]
pH	6.5	26.71 ± 0.12 [a]
	5.5	25.91 ± 0.6 [a,b]
	4.5	25.68 ± 0.08 [b]
	3.5	24.3 ± 0.12 [c]
	2.5	36.5 ± 0.43 [d]
Homogenization procedure[a]	1	29.71 ± 0.15 [a]
	2	26.57 ± 0.8 [b]
	3	25.60 ± 0.23 [b]
	4	22.91 ± 0.5 [c]

Homogenization procedure 1: homogenization in microcentrifuge tubes with 2 g of 1 mm zirconia beads, homogenization of 3 cycles of 40 s, 6 m/s, 300 s lapse between cycles. Homogenization procedure 2: Homogenization in 15 ml tubes, with 2¼ ceramic sphere balls, 3 cycles of 20 s, at 4 m/s with a 300 s lapse between cycles; Homogenization procedure 3: Homogenization in 15 ml tubes, with 2¼ ceramic sphere balls, 3 cycles of 40 s, at 6 m/s with a 300 s lapse between cycles, adding the buffer before the first homogenization cycle. Homogenization procedure 4: Homogenization in 15 ml tubes, with 2¼ ceramic sphere balls, 5 cycles of 40 s, at 6 m/s with a 300 s lapse between cycles, adding the buffer after the first 2 homogenization cycles

minced liver samples spiked with different concentrations of MNV-1. The results are shown in Table 3. Using the new extraction method, regardless of the extraction buffer used (Table 1), positive RT-qPCR signals were obtained in all the replicates after the inoculation of 10^3 $TCID_{50}$ per g of liver (Table 3). Similar results were obtained for the extraction method based on García et al. 2020, but the extraction method based on Szabo et al. 2015 coupled to a HEV-speficic RT-qPCR only detected 55.56% (5/9) of the RT-qPCR replicates as positive, showing therefore a lower anlytical sensitivity (Table 3). Simialrly, the new extraciton method using the buffers J0 and JA3 did not present statistically significant differences ($p < 0.05$) compared to the extraction method based on García et al. 2020 down to the spiking levels 1×10^3 $TCID_{50}$ per g of liver according to the 95% LSD group homogeneity test. In addition, the extraction method using the buffer JB3 did not show either statistically significant differences ($p > 0.05$) in its sensitivity at the spike level of 1×10^3 $TCID_{50}$ /g liver in comparison to the former mentioned extraction methods.

Performance Comparison of the Extraction Methods

The new extraction method was tested in 21 naturally HEV-infected pig liver samples using four different extraction buffers (Table 1), and the performance values were compared with those obtained by two commonly used methods for the analysis of HEV in liver (Table 4). The statistical analysis, using the extraction method and the samples as factors, indicate that both factors, and their interaction were significant ($p < 0.05$). The homogeneity test of the groups with respect to the method, with a confidence level of 95%, yielded the same results regardless of the statistical methods (LSD, Tuckey HSD, Scheffe, Bonferroni, Student–Newman–Keuls and Duncan methods 4). The LSD statistical analysis, confirmed 3 different homologous groups: (i) the new method using the extraction buffer J0 and JA3, and the extraction method based on García et al. 2020; (ii) the new method using the buffer JC and the method based on Szabo et al. 2015; and (iv) the new method using the buffer JB3.

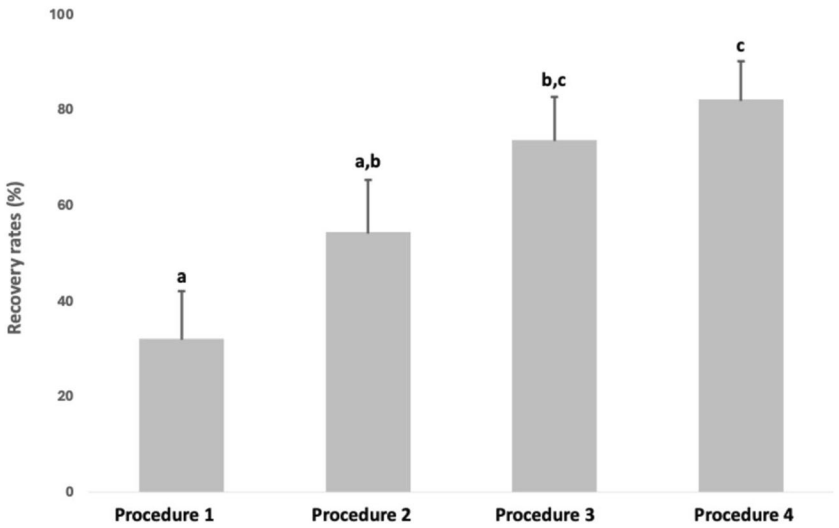

Fig. 2 MNV-1 RT-qPCR recovery rates for the 4 procedures of homogenizations tested. The letters in the types of homogenizations represent the homogeneous groups at 95% confidence

Table 3 Analytical sensitivity of the extraction methods coupled to a HEV-specific RT-qPCR. 95% confidence homogeneous groups were established for each spiking by the 95% LSD method

MNV-1 $TCID_{50}$/g liver	Extraction Method[1]											
	1		2		3		4		5		6	
	C_T values (M ± ES)	+ ve / total reactions	C_T values (M ± ES)	+ ve / total reactions	C_T values (M ± ES)	+ ve / total reactions	C_T values (M ± ES)	+ ve / total reactions	C_T values (M ± ES)	+ ve / total reactions	C_T values (M ± ES)	+ ve / total reactions
1×10^4	$33.81 \pm 0.30^{a,b}$	9/9	$34.14 \pm 0.71^{a,b}$	9/9	34.25 ± 0.57^{b}	9/9	$34.07 \pm 0.53^{a,b}$	9/9	33.65 ± 0.39^{a}	9/9	36.41 ± 0.84^{c}	9/9
1×10^3	36.90 ± 0.41^{a}	9/9	$37.53 \pm 0.43^{a,b}$	9/9	$37.60 \pm 0.73^{a,b}$	9/9	37.77 ± 0.78^{b}	9/9	36.89 ± 0.85^{a}	9/9	39.27 ± 0.58^{c}	5/9
1×10^2	38.55 ± 0.84	3/9	39.18 ± 0.43	3/9	39.76 ± 0.38	4/9	39.61 ± 0.52	2/9	39.75 ± 0.41	4/9	39.80 ± 0.09	2/9
1×10^1	39.31 ± 0.92	2/9	39.65 ± 0.47	2/9	40.06	1/9	39.85	1/9	39.46 ± 0.27	2/9	n.d	0/9
1	n.d	0/9	n.d	0/9	n.d	0/9	n.d	0/9	n.d	0/9	n.d	0/9

[1] Method 1 corresponds to the new extraction method with the J0 buffer (Table 1); Method 2 corresponds to the new extraction method with the JA3 buffer (Table 1); Method 3 corresponds the new extraction method with the JB3 buffer (Table 1); Method 4 corresponds to the new extraction method with the JC buffer (Table 1); Method 5 corresponds to extraction method based on García et al., 2020; and Method 6 corresponds to extraction method based in Szabo et al. 2015

N.d., no detection

Table 4 Multiple range test of group homogeneity for the methods tested according to the C_T values obtained for the 21 naturally HEV-contaminated liver samples tested. Super indexes indicate homogenous groups with a 95.0% LSD

Method[1]	LS Mean	LS Sigma
1^{a}	31.0084	0.0590206
2^{a}	31.1574	0.0590206
3^{c}	32.5653	0.0590206
4^{b}	31.724	0.0590206
5^{a}	31.1657	0.0590206
6^{b}	31.6366	0.0590206

[1] Method 1 corresponds to the new extraction method with the J0 buffer (Table 1); Method 2 corresponds to the new extraction method with the JA3 buffer (Table 1); Method 3 corresponds the new extraction method with the JB3 buffer (Table 1); Method 4 corresponds to the new extraction method with the JC buffer (Table 1); Method 5 corresponds to extraction method based on García et al. 2020; and Method 6 corresponds to extraction method based in Szabo et al. 2015

The performance in group 1 was better than in group ii and iii (Table 4).

When the 6 extraction methods (the new one with 4 extraction buffers, the methods based on García et al. 2020, and the method based on Szabo et al. 2015), were compared in pairs, the new method using the buffer J0 did not show statistically significant ($p < 0.05$) performance differences with the new method using buffer JA3, or the method based in García et al. 2020, but its performance was statistically higher ($p < 0.05$) compared the new method using the buffers JB3 and JC and the method based on Szabo et al. (Table 5).

Subsequently, we clustered the 21 samples tested in 4 groups based in the C_T values range obtained by the extraction method based on García et al. 2020 in a previous experiment in which the samples suitable for this test were selected and homogeneity groups were determined using the 95% LSD group homogeneity test (Table 6). This approach aimed to group the samples initially by the type of C_T and therefore by the expected HEV load.

As expected, the results obtained in samples with expected high HEV load (i.e., expected C_T values ≤ 25) were more similar in the 6 extractions methods, grouping only in 2 categories, and the new method with the extraction buffer J0 and JA3 and the two commonly used methods showing better results (Table 6). A similar situation was observed when samples with expected C_T values ≤ 30, but the results in the new method using the buffers JB3 and JC showed mean CT values below 30; 30.38 and 30.51, respectively (Table 6). Interestingly, similar clustering to the global homogenous grouping shown in Table 3 was observed in samples with expected C_T between 30 and 35 (Table 6). However, a significant difference was observed when the C_T values of samples with very low HEV load were analyzed:

Table 5 Statistical differences between the methods tested[1] using the 95% LSD method

Contrast	Difference	+/− Limits
1 – 2	−0.14902	0.16438
1 – 3*	−1.55670	0.16438
1 – 4	−0.71558	0.16438
1 – 5	0.15734	0.16438
1—6*	0.62826	0.16438
2 – 3*	−1.40793	0.16438
2 – 4*	−0.56656	0.16438
2 – 5	0.00832	0.16438
2 – 6*	0.47925	0.16438
3 – 4*	0.841375	0.16438
3 – 5*	−1.39961	0.16438
3 – 6*	−0.92869	0.16438
4 – 5*	−0.55824	0.16438
4 – 6	−0.08731	0.16438
5 – 6*	−0.47092	0.16438

[1] Method 1 corresponds to the new extraction method with the J0 buffer (Table 1); Method 2 corresponds to the new extraction method with the JA3 buffer (Table 1); Method 3 corresponds the new extraction method with the JB3 buffer (Table 1); Method 4 corresponds to the new extraction method with the JC buffer (Table 1); Method 5 corresponds to extraction method based on García et al., 2020; and Method 6 corresponds to extraction method based in Szabo et al. 2015

* Denotes a statistically significant difference

the new method using buffer J0, JA3 and JC shows significantly better results ($p > 0.05$) that those observed using the 2 commonly used methods. This is very relevant as the load of the HEV in pig products is expected to be very low, and usually with C_T values below 35, particularly in pig liver and derive pork products.

Discussion

In this study, we present a novel methodology for recovery and concentrate HEV virus from pig liver samples. This novel method is based on the mechanical homogenisation using a novel extraction buffer (Table 1). The procedure used for homogenization in the extraction of HEV from porcine food samples is relevant since the intracellular nature of this enteric virus. Some authors have previously determined that the mechanical disruption with beads is the homogenization procedure that generate the best results (Hennechart-Collette et al. 2019) (Zhao and Li 2021). However, the method can be adjusted for other types of samples. In food samples that only present surface contamination, such as vegetables contaminated by irrigation water, the mechanical lysis procedure can be varied, making it gentler by using a stomacher or other agitation systems that recover the viruses from the surface without performing a deep cell lysis, thus avoiding the release of a larger number of interfering substances. Interestingly, the new method developed in this study may be also valid for the use of the recovered viral particles in a subsequent cell culture, since the pH reached does not seem to destroy HEV viability according to the study by Wolff (Wolff et al. 2020).

The sample size was a very important parameter in the first design steps of the method (data not shown). In previous works carried out by our research team, sample amounts from 100 to 350 mg obtained better results in the detection of HEV than larger sample amounts, so it was decided to prepare a 1 g of a minced sample, homogenize it and take a quantity of that homogenate for analysis (García et al. 2020). The most likely explanation is that larger sample sizes present a greater quantity of PCR inhibitory substances, so it was decided to take a homogenate of 300 mg of sample.

Table 6 Comparison of the C_T values (mean ± SE) obtained using the 6 extraction methods tested, clustered in C_T ranges, using the C_T values obtained by the extraction method based on García et al. 2020 in a previous experiment in which the samples suitable for this test were selected. The homogeneity groups were obtained by the 95% LSD group homogeneity test

Method	Ct Groups (Ct mean ± SE Homogeneous Groups)			
	$C_T > 35$ ($n = 5$)	$30 < C_T \leq 35$ ($n = 8$)	$25 < C_T \leq 30$ ($n = 5$)	$C_T \leq 25$ ($n = 3$)
1	34.28 ± 1.93^{a}	$33.88 \pm 2.02^{a,b}$	28.65 ± 1.02^{a}	21.84 ± 2.23^{a}
2	$35.29 \pm 1.01^{a,b}$	33.39 ± 1.22^{a}	28.73 ± 1.60^{a}	$22.35 \pm 1.42^{a,b}$
3	$36.59 \pm 1.40^{b,c}$	34.64 ± 2.03^{b}	30.38 ± 2.04^{b}	23.98 ± 1.01^{b}
4	35.08 ± 1.68^{a}	33.32 ± 2.50^{a}	30.51 ± 2.07^{b}	23.91 ± 3.33^{b}
5	36.99 ± 1.17^{c}	33.01 ± 1.41^{a}	28.43 ± 0.84^{a}	21.10 ± 2.01^{a}
6	$34.98 \pm 3.16^{a,b}$	34.90 ± 1.94^{b}	28.92 ± 2.63^{a}	21.88 ± 1.91^{a}

[1] Method 1 corresponds to the new extraction method with the J0 buffer (Table 1); Method 2 corresponds to the new extraction method with the JA3 buffer (Table 1); Method 3 corresponds the new extraction method with the JB3 buffer (Table 1); Method 4 corresponds to the new extraction method with the JC buffer (Table 1); Method 5 corresponds to extraction method based on García et al. 2020; and Method 6 corresponds to extraction method based in Szabo et al. 2015

These results are consistent with those found by other authors (Son et al. 2014) (Zhao and Li 2021).

The minimum efficiency requirement for the virus recovery rate in food is 1% according to ISO 15216 (Microbiology of the food chain—Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR). All the extraction methods tested have obtained virus recovery rates higher than this reference, ranging from 32.07 to 82.12% recovery efficiency. Recovery rates using the same sample process control (MNV-1) have been reported previously ranging from 14.76 to 100% in a method for detecting HEV in fish (Hennechart-Collette et al. 2023) so the results obtained using this new extraction method, regardless of the extraction buffer used, can be considered equivalent. Similarly, the analytical sensitivity of the extraction method was similar to that reported in another optimized method for extracting and detecting HEV in pig liver, using the *E. coli* phage MS2 (Zhao and Li 2021) as sample process control. However, the data on virus recovery rates and analytical sensitivity can show certain limitations; both generally are calculated using a surrogate, applied on the surface of the food. However, the HEV naturally present in the liver samples is intracellular, so the sensitivity and recovery rates in naturally infected samples may be different, and the extrapolation of the data using surrogate cannot be totally accurate. This was observed in the results obtained during the optimization of the method where samples of several naturally contaminated liver samples were used, and the results obtained in the recovery evaluation, where a surrogate virus, MNV-1, was spiked on the surface of the liver samples (Tables 2 and 3). However, this limitation is present in all studies where not naturally contaminated samples are used (e.g. HEV or surrogates applied to the surface of food), but still nowadays it is cumbersome to generate naturally infected liver samples with adequate HEV titres to be used in optimisation and evaluation steps, so this limitation should be assumed for the calculation of the most relevant performance parameters such as the recovery rates and analytical sensitivity.

Regarding the comparison of the method, the experimental design carried out results in a statistically significant interaction between the sample and method factors. Using naturally contaminated liver samples with a variety of viral loads, which is logical for testing the method, also causes a significant interaction. A possible explanation for this statistical result is that the biological and compositional variability of the liver samples affects the performance of the extraction method used. The validation results indicate that the method can be used for the detection of HEV in pig liver samples. By grouping the samples into 4 C_T ranges (< 25, between 25 and 30, between 30 and 35 and > 35), we observed that the new extraction method is especially effective compared to 2 commonly used methods based on Garcías et al. 2020 and Szabo et al. 2015 in the > 35 C_T range, where there is a statistically significant difference, and it is equally effective in the 30 to 35 C_T range. This supports the validation of the method for naturally infected liver samples, since the range of $40 > Ct > 30$ is where most naturally infected samples are found (García et al. 2020).

Finally, the parameters of the method should be adjusted for use with other types of matrices, for which the sample quantity, centrifugation temperature, type of homogenization and buffer composition parameters should be checked for each type of matrix.

Conclusions

In conclusion, we have optimised a novel method for concentration and separation of HEV from pig liver that does not require the use of organic solvents and is compatible for subsequent molecular and/or cellular analysis. This new method coupled with a subsequent virus nucleic acid isolation and RT-qPCR is able to detect robustly (100% of replicates) down to 1×10^3 $TCDI_{50}$ viral particles per g of liver and down to 10 $TCDI_{50}$ in a percentage of the replicates depending on the extraction buffer used, being the buffer J0 and JA3 (Table 1) the most efficient. The performance of this novel method was compared with two extraction methods routinely used for HEV recovery from liver and pig products using a number of naturally contaminated liver samples and observing that it was equally or even more efficient. As mentioned, the new extraction method avoids the use of organic solvents, which prevents operators from handling these types of products, the use of appropriate facilities for their safe storage and handling, and the generation of waste. This generates economic savings and a lower environmental impact.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

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