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Evaluation of methods for elution of HEV particles in naturally contaminated sausage, figatellu and pig liver

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21 **Abstract**

22 Foodborne transmission of HEV is a growing public health concern in industrialised
23 countries, where the disease is mainly autochthonous, caused by zoonotic HEV of
24 either genotype 3 or 4. Foodstuffs containing pig's liver were suspected on several
25 occasions to be the cause of autochthonous cases of HEV infection, while the
26 transmission was associated with animal contact and the ingestion of raw or
27 uncooked meat, especially liver. In assessing the risk related to the presence of HEV
28 in food, detection methods were previously developed but HEV detection rates seem
29 to vary with the type of samples and methods. The ISO procedures (15216-1:2017)
30 only propose standard methods for quantifying NoV and HAV in bottled water,
31 shellfish, vegetables and soft fruit. As foodstuff containing pig liver can be
32 contaminated with HEV internally, an efficient virus extraction procedure is required.
33 The aim of this study was to evaluate six methods for their efficiency in releasing
34 HEV viral particles from figatelli, pig liver sausages and liver samples previously
35 tested positive for the presence of HEV. The ratio weight to volume of elution buffer
36 (1:5) and the FastPrep®-24 homogenizer showed to significantly improve the
37 quantity of HEV genomes released per gram of figatelli and pig liver sausages. To our
38 knowledge, this study is the first to evaluate several methods for elution of HEV
39 particles from naturally contaminated pig liver products, and may be extended for
40 quantifying other viral genomes from food of animal origin.

41

42 **1. Introduction**

43 Hepatitis E virus (HEV) is a positive single-stranded RNA virus classified in the genus
44 Orthohepevirus within the family *Hepeviridae* (Emerson et al., 2004, Khuroo et al.,
45 2016, Smith et al., 2013). HEV has been recognised as a cause of acute hepatitis

occurring during waterborne outbreaks in most parts of Asia and Africa where it is endemic and primarily spread by the faecal-oral route (World Health Organization, 2013). In the industrialized countries, HEV infection leads to sporadic and autochthonous cases of acute viral hepatitis and has increased as a result of zoonotic transmission from the animal reservoir. Among the major HEV genotypes, the genotypes G3 and G4 are known to be zoonotic.

Several cases of hepatitis E have been reported after consumption of contaminated raw or undercooked meat, liver, and liver sausages from infected animals (eg: wild boar, pig) (Colson et al., 2010; Deest et al., 2007; Tamada et al., 2004; Yazaki et al., 2003; Masuda et al., 2005; Tei et al., 2003). A previous study confirmed that HEV detected in pork liver sausage is infectious, highlighting the risk for consumers (Berto et al., 2013). To date, there is no standardised protocol for the detection of HEV in meat products. Various protocols have been published and applied to detect HEV on soft fruits and vegetables, in water, shellfish, and pork products (Brassard et al., 2012; Leblanc et al., 2010; Martin et al., 2012; Thesale et al., 2010; Grodzki et al., 2014; Renou et al., 2014; Wilhelm et al., 2014; Martin-Latil et al., 2014; Di Bartolo et al., 2015; Szabo et al., 2015; Terio et al., 2017). Given that foodstuffs containing pig liver have an animal origin, the virus extraction is a crucial step and requires an efficient grinding for releasing HEV from food with an internal contamination.

The aim of this study was to evaluate six methods for their efficiency to eluate viral particles of HEV from naturally contaminated food samples containing raw pig liver and to determine the most efficient method for extracting HEV from figatelli, pig liver sausages and liver.

2. Materials and methods

2. 1. Food samples containing pig liver

Pig liver samples were collected in the framework of a national official surveillance plan organised by the French Ministry of Agriculture, Food and Forestry in 2011, according to EC Regulation 882/2004 and EC Directive 2003/99/EC (2003; 2004). Of the 400 samples qualitatively analysed to detect HEV (Pavio et al., 2014), 70 samples were quantitatively analysed (Martin-Latil et al., 2014) and conserved at -80°C. One liver, one figatellu and one sausage found positive for the presence of HEV genomes were selected for this study because of their high (>10^6 genome copies per gram) HEV contamination levels.

2.2. Sample processing for recovering HEV

Six elution methods were tested for recovering HEV from sausage, figatellu and liver. Among them, the reference method B was the one previously developed in the framework of a French monitoring program (Martin-Latil et al., 2014). Figure 1 gives an overview of these six methods. Details of the extraction methods are described below.

2.2.1 Method of elution and viral RNA extraction

Three parameters were tested (weight of food sample, ratio of weight-of-sample/elution-volume and homogenisation method) as described in Figure 1.

For each sample of sausage and figatellu, fat was removed manually with a surgical blade. All samples were separated into 3 g (Methods A, B, D, E) or 10 g (Methods C and F) portions, and were homogenised in 15 mL (Methods D and E), 30 mL (Methods A and B), 50 mL (Method F) or 100 mL (Method C) of distilled water with

the FastPrep®-24 tissue (Methods A and D) and cell homogeniser (MP Biochemicals) or with the Stomacher 400 apparatus (Fisher Bioblock Scientific, Illkirch, France) (Methods B, C, E, F). For homogenisation with the stomacher apparatus, food samples were placed in a 400 mL polypropylene bag containing a filter compartment and were homogenised in distilled water using a stomacher apparatus at a normal velocity of 230 rpm for 2 min as previously described (Fumian et al.,2009; Martin-Latil et al., 2014). For the FastPrep®-24 homogeniser, samples were transferred to a 50 mL tube containing lysing matrix D ceramic beads (MP Biochemicals, Solon, OH, USA) and were homogenised using FastPrep®-24 tissue and cell homogeniser for 20 sec at 6 ms^{-1}.

Viral elution was carried out by incubating all samples for 10 min at room temperature with constant shaking regardless the homogenisation method used. The tube sample containing lysing matrix D ceramic beads was transferred to a 400 mL polypropylene bag containing a filter compartment. Then, all filtrates were transferred to a 50 mL centrifuge tube and centrifuged at 8,000 g for 15 min at 4°C to be clarified for removal of particles of debris.

One mL of filtered homogenates was processed on a NucliSens® easyMAG™ platform (bioMérieux) for viral RNA extraction using the “off-board Specific A” protocol according to the manufacturer's instructions.

Nucleic acids were eluted in 70 µL of elution buffer, aliquoted and stored at -80°C.

The experimental step from elution to RNA extraction was performed five times. The same RNA extract (undiluted and 10-fold diluted) was analysed in duplicate with RT-qPCR assay to detect and quantify HEV. Results were expressed as log genome copies of HEV per gram.

120 **2.3.2 Primers and probes**

121 The primers and the TaqMan® probe targeting the ORF2/ORF3 overlapping region
122 of HEV used in this study were adapted from the published model by Jothikumar et
123 al. (2006). The sequence of the primer pairs and the TaqMan probes used was as
124 follows: the sense primer (HEV-5260-F) was: 5′-CGGTGGTTTCTGGGGTGAC-3′, the
125 antisense primer (HEV-5330-R) was: 5′-AGGGGTTGGTTGGATGAATATAG-3′ and
126 the TaqMan probe (HEV-5280-T) was: 5′-ROX-GGGTTGATTCTCAGCCCTTCGC -
127 BHQ2-3′.

128

129 **2.3.3 RT-qPCR conditions**

130 Viral genomes were detected using one-step RT-qPCR, which was performed in
131 duplicate on the CFX96™ real-time PCR detection system from Bio-Rad (Marnes-la-
132 Coquette, France).

133 Reactions were performed in a 25 µL reaction mixture containing 1×RNA
134 UltraSense™ master mix and 1.25 µL of RNA Ultrasense™ enzyme mix, which are
135 components of the RNA UltraSense™ One-Step Quantitative RT-PCR System
136 (Fisher Bioblock Scientific, Illkirch, France), 2 U RNAse inhibitor (Applied
137 Biosystems), 5 µg of bovine serum albumin (Ambion), 600 nM of HEV-5260-F
138 forward primer, 600 nM of HEV-5330-R reverse primer, 250 nM of HEV-5280-T probe
139 and 5 µL of RNA extract. Positive controls containing RNA extracted from virus
140 suspensions and a negative control containing all the reagents except the RNA
141 template were included with each set of reaction mixtures. The one-step RT-qPCR
142 programme was 60 min of reverse transcription of RNA at 55°C, followed by a 5 min
143 denaturation step at 95°C, and finally 45 cycles of 15 sec at 95°C, 1 min at 60°C and
144 1 min at 65°C. The fluorescence was recorded by the apparatus at the end of the

elongation steps (1 min at 65°C) for each amplification cycle. All samples were characterised by a corresponding Cycle threshold (Ct) value. Negative samples gave no Ct value.

The slopes (S) of the regression lines were used to calculate the amplification efficiency (E) of the RT-qPCR reactions, according to the formula: $E = 10|-1/s| - 1$.

To establish the standard curves, the genomic stock of HEV viral RNA was obtained as previously described by Martin-Latil et al. (2014) and had a titre of approximately 1.75×10^6 genome copies/mL. The recovery quantity of HEV in food samples was calculated by reference to the corresponding standard curves obtained from ten-fold dilution of the titrated clarified suspension stock of HEV.

2.3.4 Statistical analysis

The quantitative data of HEV recovered with different elution methods from naturally contaminated figatellu, sausage and liver are expressed in log genome copies per g follow a normal distribution N (μ, σ). The Normal distribution is completely characterized by the sample mean (μ) and sample standard deviation (σ).

The standard normal distribution curve of data is a symmetrical bell-shaped. The peak is located at the sample mean μ and its height indicates weather values in the population fall near the mean value or not. The width of the bell is determined by the population standard deviation (σ) that measures the spread of a data distribution.

A one-way analysis of variance (ANOVA) was further performed with Statgraphics Centurion XVII software to evaluate the influence of the elution method on the recovered quantity of HEV. Quantitative data obtained from undiluted and 10-fold diluted RNA samples were taken into account for this statistical analysis. The result of the ANOVA is a p value associated with the hypothesis that the mean recovery

quantities of all groups (expressed as percentages) were the same. Because the HEV recovered quantities were statistically different depending on the elution method used (ANOVA, $p < 0.05$), a multiple comparison procedure was applied to determine which elution method provided the highest recovery quantity. This post-hoc test allowed to evaluate the influence of three other factors on the recovered quantity of HEV obtained for each sample: (1) the weight of the sample (3 g or 10 g), (2) dilution of the food sample (ratio 1:5 or 1:10 in buffer elution) and (3) the homogenisation of the sample (stomacher or FastPrep).

Graphs plotting the mean and its standard error for each group illustrate the multiple comparison procedure. When confidence intervals of means do not overlap, the difference between two groups of a factor is significant.

3. Results

3.1 Quantification of HEV in naturally contaminated figatellu, sausage and liver samples according to the elution method used

To compare the efficiency of six elution methods in extracting HEV from food samples, HEV genomes were quantified from naturally contaminated figatellu, pig liver sausage and liver. The amounts of HEV genomes recovered from food samples by using each of the elution method were normally distributed as illustrated by the symmetrical bell-shaped of quantitative data obtained with the reference method B (Figure 2). The means and standard deviations of HEV quantities obtained for each food type according to the elution method used are displayed in Figure 3.

HEV genomes were detected in all RNA samples. The quantification of HEV in sausage, figatellu and liver ranged respectively from 8.79 to 9.70, 6.92 to 7.82 and

194 8.64 to 8.96 log_{10} HEV genome per gram whatever the elution method used.
195 Quantitative data calculated from undiluted and 10-fold diluted RNA extracts were
196 similar since their ratio ranged from 0.90 to 0.99 regardless the food type and the
197 elution method used. Thus, there was no relevant inhibition of HEV RNA
198 amplification.
199 Comparisons between quantitative data obtained by using the reference method B
200 with those obtained by using other elution methods indicated that the method D
201 provided the highest quantification for HEV (Figure 3). Indeed, the means of HEV
202 recovered by using the method D were 7.82 and 9.70 log_{10} HEV genome per gram in
203 Figatellu and pig liver sausage respectively, whilst they were 6.92 and 8.94 log_{10}
204 HEV genome per gram with the method B. In addition, a slightly increase in HEV
205 quantification could be also observed by using methods F (9.28 log_{10} HEV genome
206 per gram) and E (9.50 log_{10} HEV genome per gram) for pig liver sausages. On the
207 contrary, HEV levels found in liver were similar regardless the elution method since
208 their ranges were from 8.64 to 8.96 log_{10} HEV genome per gram.
209 The standard deviation values were higher for liver (from 0.37 to 0.68 log_{10} HEV
210 genome per gram) than for figatellu (from 0.15 to 0.34 log_{10} HEV genome per gram)
211 regardless the elution method used. The standard deviation values obtained from pig
212 liver sausage were close to the ones obtained for figatellu (from 0.13 to 0.30 log_{10}
213 HEV genome per gram) except by using the method D (0.68 log_{10} HEV genome per
214 gram) (Figure 3). This result showed that quantitative data of liver samples were the
215 most widely spread, also illustrated by the flattened peak in Figure 2.
216 As a whole, these results indicated that the elution method influenced the amounts of
217 HEV genome recovered from figatellu and sausage; and that the quantitative data

obtained from liver were the most widely distributed regardless the elution method used.

3.2. Assessment of the experimental factors essential to increase HEV genomes recovery from figatellu, pig liver sausage

To determine whether elution method has an impact on HEV extraction from food, statistical analysis was further performed on HEV quantitative data obtained according to the elution method used. Results of the one-way ANOVA showed that the elution method was a significant factor for improving the HEV recovery from figatellu (ANOVA; $p=0.0011$) and from sausage (ANOVA; $p<0.0001$). On the contrary, none of the elution methods could significantly improve HEV recovery from liver (ANOVA; $p=0.5295$).

A multiple comparison test was further performed to identify the most efficient elution method in extracting HEV. As shown in Figure 4, the method D provided the highest HEV recovery from figatellu and pig liver sausage and the methods E and F also showed a better efficiency in HEV recovery from sausages.

In addition, pairwise comparisons of HEV quantities obtained between the reference method B and others could determine the most efficient experimental factors leading to a significant improvement in extracting HEV from figatellu and pig liver sausage.

To evaluate independently the influence of the type of grinding, the sample weight and the ratio weight to volume of elution buffer (w/v), HEV quantities obtained by using the method B were respectively compared to the ones obtained with method A, method C and method E. The ratio weight to volume of elution buffer was the only factor leading to a significant increase in HEV recovery from pig liver sausages.

242 The influence of sample weight as an additional factor to the ratio w/v was further
243 evaluated by comparing HEV quantities obtained using method B with those obtained
244 with method F. Nevertheless, the increase of sample weight from 3 g (method B) to
245 10 g (method F) did not lead to a significant increase in HEV quantities recovered
246 from figatellu. On the contrary, the use of FastPrep homogenizer when elution buffer
247 was added to figatellu samples with a ratio of 1:5 (method D) instead of 1:10 (method
248 B) showed to be a significant experimental factor. This result showed that both
249 experimental parameters are necessary to significantly increase HEV recovery from
250 figatellu.
251 As a whole, the method D proved to be the most effective for HEV extraction from
252 figatellu and sausage whereas none of the elution method had impact on HEV
253 recovery from liver.
254

255 **4. Discussion**

256 The foodborne transmission of HEV is mainly due to consumption of raw or
257 undercooked liver, meat or sausage from infected animal reservoirs such as pigs or
258 wild boar (Yugo and Meng, 2013). The detection of HEV genome was reported in
259 several organs of infected pigs (liver, plasma, bile, hepatic lymph node, etc.) with the
260 highest viral load reported in liver, *i.e.* 10^7 per gram (Leblanc et al., 2010; Son et al.,
261 2014). In assessing the risk related to the presence of HEV in food, detection
262 methods were previously developed and allowed highlighting the high prevalence
263 (*i.e.* up to one third) of HEV in Europe for foodstuffs containing pig liver, (Szabo et al.,
264 2015; Giannini et al., 2018; Di Bartolo et al., 2015; Moor et al, 2018; Martin-Latil et
265 al., 2014). Regarding the quantitative data, virus load of up to 5.7 log10 HEV genome
266 copies per gram were measured (Moor et al., 2018; Giannini et al., 2018; Martin-Latil

et al., 2014). In the framework of a French monitoring programme, the developed method was efficient for the detection of HEV in figatelli and pig liver sausages as most of the samples tested had a recovery rate of MNV process control enough raised to validate analysis. On the contrary, the low recovery rates obtained for MNV from pig dried salted liver and quenelle samples underlined that the performance of the method should be further improved (Martin-Latil et al., 2014).

In this aim, the present study focuses on the extraction of the virus from food, which is a critical step because of the internal contamination of pig liver products with HEV. An improvement in HEV recovery was found for figatellu and sausage samples with the method D in comparison with the previously published method B (Martin-Latil et al., 2014). To validate method D according to the EN ISO 16140, further analysis should be performed by increasing the sampling for every type of food and by taking into account at least, three levels of viral contamination as previously for method B (Martin-Latil et al., 2016). Both crucial parameters were the ratio weight to volume of elution buffer and the type of mechanical cell disruption for figatelli and pig liver sausages. Nevertheless, the ratio weight to volume of elution buffer 1:5 was sufficient to significantly increase the recovered quantities of HEV from pig liver sausages. The use of the FastPrep®-24 homogeniser showed to be an additional significant factor for releasing HEV from naturally contaminated figatellu samples. Since the grinding step is important for extracting virus from food of animal origin, the efficiency of several strategies of mechanical cell disruption was previously described, *e.g.* the use of vortex mixer or stomacher, which is part of the standard equipment in many laboratories, or a FastPrep®-24 instrument, which is a more drastic mechanical homogeniser (Di Bartolo et al., 2015; Szabo et al., 2015; Wilhelm et al., 2014; Berto et al., 2013; Martin-Latil et al., 2014). A more efficient homogenisation of food

292 samples was found with stomacher and FastPrep®-24 by detecting the released pig
293 DNA following the homogenisation process (Szabo et al., 2015). Different cycles of
294 freeze thawing could be also used to disrupt mechanical samples of liver (Serracca et
295 al., 2015). Nevertheless, the strategies of mechanical cell disruption should be
296 chosen to keep intact viral particles for further analysis of HEV infectivity once a
297 suitable cell culture method will be available for routine use.
298 In our study, the weight of sample did not influence the quantity of HEV recovered per
299 gram for any food matrices showing that an increase of sample weight did not
300 hamper the efficiency of the grinding step, neither the detection of HEV genomes by
301 RT-qPCR. Sample sizes of swine liver samples ranging from 250 mg to 10 g were
302 analysed for the presence of HEV in previous studied (Bouwknegt et al., 2007;
303 Leblanc et al., 2010; Son et al., 2014; Martin-Latil et al., 2014). In particular, Son et
304 al. (2014) showed that the detection rate of HEV in swine liver was improved (three
305 times more detection) by increasing the sample size from 1 g to 10 g when a
306 concentration step followed HEV extraction. It should be noted that an increase of the
307 sample size, as an additional step of virus concentration, could lead to an increase in
308 PCR inhibitors. For this reason, Szabo et al. (2015) analysed 2 g of liver sausage to
309 compensate for the expected higher amounts of inhibitory substances in liver-
310 containing matrices. However, no significant evidence of RT-qPCR inhibition was
311 shown in this work for detecting HEV in any food matrices whatever the method used
312 in agreement with our previous studies (Martin-Latil et al., 2014 and 2016).
313 The quantitative data obtained from liver were more widely distributed for liver than
314 for figatellu and pig liver sausage whatever the elution method used as shown by the
315 standard deviation values. The difference in HEV genomes distributions according to
316 food type may reflect the actual physical distribution of HEV in a particular food

317 product. The highest variability in amounts of HEV genomes found in liver could
318 reflect a heterogeneous contamination resulting from a focal infection in swine liver
319 but further analysis should be performed to confirm this statement. Non-homogenous
320 contamination of food has been already discussed since HEV could not be detected
321 in all slices for the same sausage (Di Bartolo et al., 2015). Nevertheless, the level of
322 HEV contamination was at least 2 $\log_{10}$ lower than the figatellu and sausage used for
323 this study and the weight of samples was 250 mg.
324 On the contrary, figatelli and pig liver sausages may be homogeneously
325 contaminated with HEV since both matrices are already made by mixing meat, fat
326 and liver together with salt and spices. The physical distribution of the microbial
327 contamination among batch samples has already been shown to affect the observed
328 microbial frequency distribution (Jongenburger et al., 2012).
329 To our knowledge, this study was the first to evaluate several methods for elution of
330 HEV particles from naturally contaminated food and could be extended for analysing
331 other food types having internal contamination with pathogens. This approach may
332 improve the risk assessment towards HEV in food virology.

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338 pig faeces.
339

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