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HEV detection in raw pig liver products using a quantitative RT-PCR method

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Hepatitis E virus (HEV) is responsible for acute hepatitis outbreaks in humans in countries with poor sanitation. More recently, many sporadic cases of acute hepatitis E, not linked with travel to endemic regions, have been reported in developed countries. In France, foodstuffs containing raw pig liver were suspected on several occasions to be the cause of autochthonous cases of HEV infection occurring between 2007 and 2009. Within the framework of a national official surveillance plan (DGAl/SDSSA/N2010-8330) for HEV contamination of food containing raw pig liver conducted in 2011, a quantitative detection method was developed, validated and used for HEV genome quantification on 70 food samples. This method consists of three steps: 1) elution-concentration of virus, 2) viral RNA extraction, and 3) a one-step duplex RT-qPCR for detecting HEV and the murine norovirus (MNV-1). MNV-1 was used as a process control for monitoring the quality of the whole extraction procedure. The results showed that about one third of figatelli and pig liver sausages tested positive for HEV genome and that the viral load was more than 103 genome copies of HEV per gram in 55% of samples.

Introduction

Hepatitis E virus (HEV) is transmitted mainly through the gastrointestinal tract after ingestion of contaminated food or water. HEV is recognised as the main causative agent of acute hepatitis in countries with poor sanitation, where it follows an endemo-epidemic pattern. Genotypes 1 and 2 are found in humans in endemic regions while genotype 3, and less frequently genotype 4, are related to sporadic cases of acute hepatitis in developed countries. In 2009, the French Food Safety Agency (AFSSA) issued two reports (Requests 2009-SA-0101 and 2009-SA-0146) pointing to the risk of HEV infection following ingestion of figatelli (raw sausage made from pig liver). The recommendations made in these reports and incrimination of products containing pig liver in autochthonous cases of hepatitis E highlighted the importance of having a sensitive and reliable method for the detection of HEV in foods.

Implementation of detection methods in the area of viral diagnosis in food safety is based on detection of viral RNA using sensitive and specific RT-qPCR methods. The two main difficulties related to detection involve the low concentration of viruses in foods and the presence of substances in the sample that inhibit the PCR reaction. In 2013, technical specifications concerning the detection of norovirus and hepatitis A virus in foods were published(ISO/TS_15216-1:2013; ISO/TS_15216-2:2013). Hepatitis E virus, still considered an emerging virus, was not identified as a priority when work began to standardise detection methods in food virology. However, the general recommendations of the European Committee for Standardization (CEN/TC275/WG6/TAG4) concerning viral detection in food safety provide for a series of controls, including a negative extraction control, virus extraction process control, positive and negative RT-PCR control, and inhibition of RT-PCR control. Adding a process control for each test sample is essential because it helps to determine the effectiveness of processing and to assess the presence of inhibitors for PCR amplification reactions. Determining the mean yield obtained for the process control in a given substrate also enables determination of the acceptable level of yield to validate analysis of the sample.

As part of the surveillance plan carried out in 2011, the objective of this study was to develop and validate a method to extract and detect the HEV viral genome by quantitative RT-PCR in food substrates containing raw pig liver (figatelli and liver sausages) and to analyse 70 samples from the four categories of food potentially posing a risk for the consumer (figatelli, liver sausages, salted dried pig livers, quenelles).

Materials and methods

Hepatitis E virus, murine norovirus, and samples from the surveillance plan

To develop and validate the detection method for the HEV viral genome, artificially contaminated samples were prepared using a viral suspension of HEV (genotype 3f; Genbank accession number: JF718793) obtained from a faecal extract from an infected pig, provided by the Laboratory for Animal Health (ANSES, Maisons-Alfort). The faecal sample was suspended in a 10 mM PBS buffer at pH 7.4 (10% final (w/v)) and centrifuged at 4000 g for 20 minutes at 4°C. The supernatant containing HEV viral particles was aliquoted and stored at -80°C. The number of copies of HEV RNA in the viral suspension was determined by RT-qPCR using a standard curve obtained with HEV RNA transcribed *in vitro*.

Murine norovirus MNV-1 (CW1) was amplified then titred in RAW 264.7 cells (murine macrophage line, ATCC TIB-71).

As part of the surveillance plan for HEV contamination of delicatessen products containing raw pig liver (DGAL/SDSSA/N2010-8330) covering 400 samples (Pavio *et al.*, 2013), 70 samples were selected in order to maintain the initial distribution of the 400 samples in terms of geographic origin and type of product (figatelli, liver sausages, salted dried liver, quenelle paste) then analysed by quantitative RT-PCR.

Quantitative HEV detection method in food samples containing raw pig liver

Elution / concentration of viral particles and extraction of viral RNA

The food sample (3 g) was cut into pieces using a scalpel and placed into a Stomacher bag. After adding the MNV-1 used as

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a process control (50,000 TCID50), 30 mL of distilled water was added and the sample was ground in the Stomacher bag (2 min, 260 rpm). Elution was performed at room temperature under agitation for 10 minutes. The homogenate was clarified by centrifugation (8000 g, 15 min, 4°C) and viral particles were then precipitated with 0.25 volumes of polyethylene glycol (PEG) for 2 h at 4°C and concentrated by centrifugation at 8000 g for 30 min. Viral particles were lysed directly to extract viral RNA using an automated extractor (NucliSens® easyMAG™).

Quantitative RT-PCR

The molecular model for detection of HEV is based on the model described by (Jothikumar *et al.*, 2006), targeting the ORF2/ORF3 region. The molecular model for detection of MNV-1 targeting ORF1 was determined using Beacon Designer software (Bio-Rad, Marnes-la-Coquette, France). Taqman probes for HEV and MNV-1 detection were labelled respectively with ROX or 6-FAM in 5', and BHQ2 or BHQ1 in 3'.

For HEV detection, the sequences used for TaqMan probes and primers were:
HEV-5260-F: 5'-CGGTGGTTTCTGGGGTGAC-3',
HEV-5330-R: 5'-AGGGGTTGGTTGGATGAATATAG-3'
HEV-5280-T: 5'-ROX-GGGTTGATTCTCAGCCCTTCGC-BHQ2-3'.

For MNV-1 detection, the sequences used for TaqMan probes and primers were:
MNV-3193-F: 5'-CCGCCATGGTCCTGGAGAATG-3',
MNV-3308-R: 5'-GCACAACGGCACTACCAATCTTG-3'
MNV-3227-T: 5'-FAM–CGTCGTCGCCTCGGTCCTTGTCAA-BHQ1-3'.

All quantitative RT-PCR runs were performed using the CFX96™ system (Bio-Rad, Marnes-la-Coquette, France). Reactions were carried out using the RNA UltraSense™ One-Step Quantitative RT-PCR System (Fisher Bioblock Scientific, Illkirch, France). Positive controls containing RNA extracted from virus suspensions and negative controls containing all the reagents except the RNA extract were included in each set of experiments. The thermo cycler programme for the one-step RT-qPCR was 60 min at 55°C for reverse transcription of viral RNA; a denaturation step of 15 min at 95°C, then 40 cycles of PCR (15 s at 95°C, 1 min at 60°C and 1 min at 65°C). Each RNA extract was tested undiluted and ten-fold diluted in duplicate. All the samples were characterised by a cycle threshold (Ct). Negative samples did not have a Ct value. A standard curve for each viral target was produced using serial dilutions of the viral suspension. The slopes (S) of the regression lines were used to calculate the amplification efficiency (E) of the RT-qPCR reactions, using the formula: E = 10|-1/s| -1. The extraction yields for HEV and MNV-1 were calculated for each sample on the basis of the corresponding standard curve.

Statistical analysis

Statistical analyses were performed using MATLAB software (version 6.5.1).
To test method validation, the impact / effect of adding the MNV-1 process control was first evaluated based on extraction yields for HEV using a univariate analysis of variance (ANOVA). A multivariate ANOVA was then performed to evaluate the effect of four experimental factors on the extraction yields for HEV: sample dilution, type of food (figatelli, liver sausages), quantity of HEV and inter-assay variability. Concerning extraction yields obtained for MNV-1, three variables were tested, i.e. sample dilution, type of food (figatelli, liver sausages) and inter-test variability.

Results

Validation of the HEV detection method in figatelli and liver sausages

The HEV detection method was validated for figatelli and liver sausages that were artificially contaminated since they accounted for more than 75% of the samples collected during the surveillance plan. The limit of detection (LOD) for HEV and mean extraction yields for HEV and the MNV-1 process control obtained from four replicate experiments are shown in **Table 1**.

Table 1 : Mean extraction yields for HEV and MNV-1
Four experiments were performed and each sample was analysed by RT-qPCR in duplicate and the number of positive tests is indicated in brackets. The LOD_{100} corresponding to the detection of HEV in the four replicate experiments is highlighted in grey. nd: not detected.

Inocula / 3g		Mean extraction yields in figatelli (%) (positive Ct values / 8)		Mean extraction yields in sausages (%) (positive Ct values / 8)	
HEV	MNV-1	HEV	MNV-1	HEV	MNV-1
0	0	nd	nd	nd	nd
875	0	43.9 ± 26.9 (2/8)	nd	nd	nd
1,750	0	22.8 ± 11.0 (4/8)	nd	9.8 (1/8)	nd
8,750	0	14.7 ± 11.3 (7/8)	nd	3.2 ± 2.1 (2/8)	nd
17,500	0	9.6 ± 6.5 (7/8)	nd	4.9 ± 2.5 (6/8)	nd
87,500	0	8.7 ± 2.5 (8/8)	nd	2.7 ± 2.1 (8/8)	nd
0	50,000	nd (0/8)	11.6 ± 7.0	nd	1.6 ± 0.9
875	50,000	28.6 ± 9.4 (3/8)	13.0 ± 6.6	nd	2.0 ± 1.3
1,750	50,000	35.7 ± 33.4 (6/8)	13.4 ± 7.1	nd	1.2 ± 1.1
8,750	50,000	7.6 ± 5.6 (5/8)	12.1 ± 4.1	3.0 ± 1.7 (2/8)	3.1 ± 1.2
17,500	50,000	5.6 ± 5.7 (6/8)	15.8 ± 7.7	2.9 ± 2.1 (2/8)	3.2 ± 0.4
87,500	50,000	6.6 ± 2.0 (8/8)	12.9 ± 3.8	1.1 ± 1.0 (7/8)	6.5 ± 6.5
Mean extraction yields (%)		**18.4**	**13.1**	**3.9**	**2.9**

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Table 2: Results of analyses on 70 samples in the PS-VHE plan, broken down by type of food
The number of positive analyses for HEV and the obtained extraction yields for the MNV-1 process control are shown.

Types of products	Total number of analyses	Number of HEV-negative analyses	Number of HEV-positive analyses	Extraction yields for MNV-1 (%)			
				< 0.1%	0.1-1%	1-10%	>10%
Figatelli	33	21	12	0	6	20	5
Liver sausages (dried or fresh)	27	17	10	1	4	22	2
Salted dried liver	5	5	0	2	3	0	0
Quenelle and quenelle paste	5	5	0	3	2	0	0
Total	70	48	22	6	15	42	7

The presence of the MNV-1 process control did not affect extraction yields for HEV (univariate ANOVA; p-value = 0.10). Mean extraction yields obtained from figatelli were 13.1% for MNV-1 and 18.4% for HEV. In liver sausages, mean extraction yields were 2.9% for MNV-1 and 3.9% for HEV. Extraction yields for HEV and MNV-1 were not improved after ten-fold dilution of RNA samples (results not shown).
Results were analysed using a statistical test of multivariate ANOVA which showed that (i) extraction yields were higher in figatelli than in sausages, both for HEV and the MNV-1 process control; and (ii) virus extraction from these two types of food samples did not generate significant inhibition of amplification of the two viral genomes by PCR.

Quantitative detection of HEV in 70 samples in the surveillance plan (DGAI/SDSSA/N2010-8330)

The results of analyses on 70 samples containing raw pig liver are given in **Table 2**. Presence of HEV genomes was found in 12 figatelli out of 33, and in 10 liver sausages out of 27, indicating prevalence of about 36% for HEV contaminated food. Quantitative data for the HEV-positive samples are provided in **Table 3**. The HEV viral load in figatelli and liver sausages was between 4 x 10^1 and 2 x 10^6 genome copies of HEV / g. The quantity of HEV found was >10^2 genome copies / g in 95% of positive samples, > 10^3 genome copies / g in 55% of positive samples, and > 10^4 genome copies / g in 27% of positive samples.
The MNV-1 process control was used to check all the steps of the analysis from viral extraction from the food substrate to quantitative detection by RT-qPCR. The extraction yield ranges for MNV-1 obtained for the 70 samples containing raw pig liver are presented in **Table 2**.
The extraction yield of the MNV-1 process control obtained from most of the figatelli and liver sausages analysed was > 1%. However, extraction yields obtained for MNV-1 from the other two food categories, i.e. salted dried liver and quenelles, were all < 1%. For 5 samples out of 10, the extraction yield obtained for MNV-1 was even lower than 0.1%.

Table 3: Quantity (genome copies) of HEV detected in positive samples in the PS-VHE plan

Ct values	Copies of HEV genome / 3 g	Number of Figatelli	Number of liver sausages
< 30	> 2E+05	3	1
30 - 36	1,6E+03 - 2E+05	7	2
36,1 - 40	1,2E+02 - 1,6E+03	2	7

Discussion

As part of the surveillance plan on hepatitis E virus contamination of delicatessen products containing raw pig liver at the production stage, this study was intended to develop and validate a method for quantitative detection of the hepatitis E (HEV) viral genome in at-risk food substrates in order to quantitatively assess 70 samples.
The preliminary tests carried out during development of the method for HEV detection highlighted the importance of including an elution / concentration step for viral particles by polyethylene glycol (PEG) before extraction of viral RNA (results not shown). The virus concentration step is generally essential since contamination levels of food by enteric viruses are low.
As a result, concentration of enteric viruses (enterovirus, hepatitis A virus, norovirus, adenovirus, and astrovirus) from a wide variety of food products such as pasta salads, meat, whipped cream, and shellfish, is carried out most commonly by viral precipitation in PEG (Stals *et al.,* 2012). This was also the approach adopted by the European working group CEN/TC275/WG6/TAG4 to detect norovirus and hepatitis A virus in vegetables and mixed berries (ISO/TS_15216-1, 2013; ISO/TS_15216-2, 2013).
Validation experiments for the HEV detection method carried out on artificially contaminated figatelli and liver sausages showed that the mean extraction yields for HEV from figatelli (18.4%) are statistically higher than those obtained from liver sausages (3.9%). These extraction yields are consistent with those described for the detection of hepatitis A virus and norovirus from plants and fruits (Blaise-Boisseau *et al.,* 2010; Martin-Latil *et al.,* 2012a).
To validate viral diagnosis in food safety, it is essential to use a process control virus as described in many studies and review articles (Baert *et al.,* 2011; Coudray *et al.,* 2013; Martin-Latil *et al.,* 2012b; Stals *et al.,* 2012). The process control is added in a defined amount to the test sample before viral extraction and technical specifications (ISO/TS_15216-1, 2013; ISO/TS_15216-2, 2013) propose that viral diagnosis should be validated if process control yields ≥ 1%. The virus selected as a process control must be cultivable, non-enveloped, positive polarity ssRNA (single stranded), and of similar size to the target virus in order to provide a suitable morphological and physico-chemical model. It must also show persistence in the environment similar to the target viruses, and should not be found naturally in foods under normal circumstances. For this study, murine norovirus (MNV-1) was selected as the process control virus, like in many previous studies (Coudray *et al.,* 2013; Martin-Latil *et al.,* 2012a, b; Stals *et al.,* 2009). Extraction yields

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of the process control (MNV-1) higher than 1% were obtained in 80% of the figatelli and in 83% of the liver sausages analysed. For quenelles (5 samples) and dried salted liver (5 samples), extraction yields of the MNV-1 process control obtained were lower, at < 1%. These results confirm the need to use a process control virus to validate the detection method for HEV in each type of food and suggest that improved detection of HEV in quenelles and salted dried liver should be pursued in future studies.

The high prevalence, with a third of tested products showing positive for HEV, associated with high levels of contamination (55% of positive samples had a contamination level greater than 103 genome copies per gram), support the possibility of HEV transmission to humans via these foods. Although the validated detection method is based on determination of viral genomes and does therefore not provide information on the presence of infectious HEV particles in the analysed samples, the infectivity of HEV from figatelli has been demonstrated in cell models (Berto *et al.,* 2013).

In conclusion, development of routine methods showing the infectivity of HEV is required to obtain a better understanding of the viral risk. Nonetheless, it appears warranted to monitor animal reservoirs of HEV, to issue recommendations to avoid entry of HEV into foods, and to emphasise good cooking practices to limit the risk of human contamination. HEV diagnostic methods should be validated based on the type of food in order to determine the prevalence of HEV in all at-risk substrates in a reliable manner. Lastly, additional studies to determine the probability of infection depending on the quantity of HEV genome copies present in a sample may be needed to better assess the viral risk.

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