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EDITED BY
María Belén Pisano,
Universidad Nacional de Córdoba, Argentina

REVIEWED BY
Ilaria Di Bartolo,
National Institute of Health (ISS), Italy
Maria Guadalupe Vizoso Pinto,
CONICET Higher Institute of Biological Research (INSIBIO), Argentina
Annamaria Pandiscia,
Dipartimento di Medicina Veterinaria Bari, Italy

*CORRESPONDENCE
Julie Jean
✉ julie.jean@fsaa.ulaval.ca

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High occurrence of hepatitis E virus in raw pork liver and pork liver pâté produced in the Canadian province of Quebec

Eva Chatonnat[1], Mariya Julien[1], Eric Jubinville[1], Valérie Goulet-Beaulieu[1], Nicole Pavio[2] and Julie Jean[1]*

[1]Institute of Nutrition and Functional Food (INAF), Université Laval, Québec City, QC, Canada, [2]French Agency for Food, Environmental and Occupational Health and Safety (ANSES), Maisons-Alfort, France

The Hepatitis E virus is widespread throughout the world and causes sporadic cases of liver disease. In industrialized countries, its transmission is zoonotic, with pigs, deer, and wild boar being major reservoirs. Humans can be infected by ingesting raw or undercooked contaminated meat products. In the Canadian province of Quebec, HEV has been detected previously in pork liver and chops. In the present study, we analyzed 83 pork liver pâtés sold in local grocery stores and 79 raw pork livers obtained from Quebec hog processors. HEV RNA was extracted and detected by RT-qPCR. HEV RNA was amplified in 29% of the pâtés and 4% of the raw pork livers. Out of all amplified PCR-positive samples, only one showed a product during electrophoresis and was sent for sequencing. The resulting sequence matched a strain belonging to genotype 3 (HEV-3). This pilot study shows that HEV is present frequently in pork liver-based products in the province of Quebec.

KEYWORDS

epidemiology, hepatitis E virus, pork liver products, RT-qPCR, zoonotic transmission, foodborne viruses

1. Introduction

The hepatitis E virus (HEV) is a single-stranded positive RNA virus that causes hepatitis (Balayan et al., 1983). It was recognized as a new disease in the 1980s, and the genome was cloned and characterized in 1991 (Tam et al., 1991; Khuroo, 2011). Since then, HEV has become endemic in developing countries due to a fecal–oral transmission facilitating rapid spread wherever sanitation systems are deficient (Khuroo et al., 2016). HEV is now regarded as an emerging foodborne virus (Harrison and DiCaprio, 2018), and sporadic cases of hepatitis E, not related to travel in endemic countries, are increasingly reported in industrialized countries. Many of these autochthonous cases are associated with zoonotic transmission (Lewis et al., 2010) after the consumption of raw or undercooked food products from infected animals. The WHO estimates the number of HEV infections at 20 million per year worldwide and the number of associated deaths at 44,000 (World Health Organization, 2022). Although most HEV infections are asymptomatic, severe forms such as chronic hepatitis, fulminant hepatitis, and neurological illness may develop in susceptible populations (Webb and Dalton, 2019). HEV is particularly dangerous to pregnant women, with up to 25% mortality during the third trimester of pregnancy in some endemic regions (Navaneethan et al., 2008).

The hepatitis E virus (family *Hepeviridae*, subfamily: *Orthohepevirinae*, genus: *Paslahepevirus*) has eight known genotypes. Among these, four can infect humans (HEV-1 to HEV-4), HEV-1 and HEV-2 infect only humans, and HEV-3 and HEV-4 are transmitted back and forth between humans and animals (Smith et al., 2014). Swine are natural hosts and an important reservoir of HEV (Meng et al., 1997; Li et al., 2022), but the virus is also detected in wild animals, for example, deer, wild boar, and hare (Pavio et al., 2010; Smith et al., 2014). In zoonotic transmissions, humans become infected mainly by eating raw or undercooked meat (Raji et al., 2022).

HEV is liver-cell-tropic, and the food products most often involved in transmission are charcuteries or prepared meals containing a large proportion of pork liver. Since 2010, numerous studies on the prevalence of HEV in pork liver products have been conducted, especially in Europe. The prevalence of HEV ranges from 5 to 70% depending on the type of product and the geographical region (Pavio et al., 2014; Boxman et al., 2019; Pallerla et al., 2021). In Canada, 47% of pork liver pâtés and 10.5% of raw pork livers sampled in Ottawa, the Canadian federal capital, were found positive for HEV RNA (Mykytczuk et al., 2017). To date, no study has focused on the presence of HEV in food in the province of Quebec. Furthermore, cases of patients who developed hepatitis after liver transplantation have been reported in the province and a foodborne origin has been suspected. This hypothesis was supported by the close genomic similarity of the viral strain found in the patients with a strain isolated from swine raised in Quebec (Halac et al., 2012).

We report here the results of a pilot study on the prevalence of HEV RNA in pork liver pâtés sampled in retail stores in Quebec City (QC, Canada) and raw pork liver provided by a local hog processing operation.

2. Methods and materials

2.1. Sampling of pâtés sold commercially

Pork liver pâtés or country-style pâtés were purchased during the summer of 2022 (June 15 to August 17) at 10 grocery stores in Quebec City (QC, Canada). All 83 pâtés were from different lots and contained pork liver as one of the first three ingredients listed. Samples were aliquoted (2.0 g ± 0.1 g) and stored at −30°C until processing.

2.2. Sampling of raw pork liver

Whole raw pork livers were provided by a local business that collected them from hog processing plants throughout the province of Quebec. All 79 were from pigs aged 6 to 7 months and weighing on average 112 kg. Samples were aliquoted (3.0 g ± 0.1 g) and then stored at −30°C until processing.

2.3. Sample processing positive controls

Stocks of purified Mengo virus strain vMC0 and murine norovirus 1 (MNV-1) strain ATCC VR-1937 already in our collection were used as positive controls for sample processing. Viral titers were estimated by RT-qPCR and were 2.16×10^6 genome copies/µl for the Mengo virus and 1×10^6 genome copies/µl for MNV-1. The viral stock suspensions were stored at −80°C until processing.

2.4. Extraction of virus from pork liver pâté

A method of extracting HEV from sausages (Szabo et al., 2015) was adapted to pâté. Briefly, 2.0 g ± 0.1 g of sample was blended in a 50 ml plastic centrifugation tube with 10 µl of Mengo virus suspension and 7 ml of TRI Reagent Solution® (Thermo Fisher Scientific, Waltham, MA, USA) by vortex mixing at maximum speed for 2 min. The resulting mixture was centrifuged at 10,000 × *g* for 20 min (at 4°C), and the supernatant (below a thin layer of liquid fat) was diluted 1:10 with sterile water and vortexed for 15 s. Nucleic acid was extracted from 1 ml of this diluted supernatant. The negative control was obtained by repeating the protocol without pâté and with 10 µl of sterile water instead of Mengo virus suspension.

2.5. Extraction of virus from raw pork liver

Approximately 3.0 g ± 0.1 g of raw pork liver was homogenized with 10 µl of MNV-1 suspension, 7 ml of TRI Reagent Solution®, 2 µl of RNaseOUT™ (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and six glass beads (3 mm, VWR, Radnor, PA, USA) in a Bead Genie device (Scientific Industries, Bohemia, NY, USA) at maximum speed (4,800 strokes per minute) for 2 × 30 s. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant was diluted 1:10 with sterile water. The diluted supernatants were vortexed for 15 s and 1 ml was used for nucleic acid extraction. The negative control was obtained by repeating the protocol without the liver and with 10 µl of sterile water instead of MNV-1 suspension.

2.6. Nucleic acid extraction

All samples (pâté, liver, controls, 1 ml) were treated with 2 ml of NucliSENS® lysis buffer (bioMérieux, Marcy-l'Étoile, France) at room temperature for 10 min, then centrifuged at 1,800 × *g* for 2 min at 4°C. The semi-automated eGENE-UP® system (bioMérieux, Marcy-l'Étoile, France) was used according to the manufacturer's instructions to extract RNA. The extracts of 100 µl thus obtained were diluted 1:10 with NucliSENS® buffer 3 (bioMérieux, Marcy-l'Étoile, France) and stored at −80°C until analysis.

2.7. Detection of HEV by RT-qPCR

HEV, Mengo virus, and MNV-1 were detected using a method described previously (Trudel-Ferland et al., 2021). Genomes were amplified using an iTaq Universal Probe One-step kit (Bio-Rad, Hercules, CA, USA). The primers and probes used are listed in Table 1. The expected size of the HEV amplicon is 72 pb. To quantify HEV, a standard curve was produced in triplicate from 10^5 genome copies/µl to 10^1 genome copies/µl. The MiniGene plasmid pIDTSMART-AMP+ generated by IDT (Integrated DNA Technologies, Coralville, IA, USA) was used (GenBank number MP690161.1). RT-qPCR reactions were performed in 96-well clear PCR microplates (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) sealed with an optical adhesive film (Applied Biosystems, USA). The ABI7500 real-time PCR thermal cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was used as follows: 50°C for 10 min, 95°C for 3 min and 45 cycles of 95°C for 15 s, and 60°C for 30 s. ROX dye was used as the reference dye. Each sample was analyzed in duplicate. All RT-qPCR runs included a negative control (RNase-free water, VWR, Radnor, PA, USA). All qPCR standard curves had an R^2 over 0.985 and efficiency between 90 and 110%. Viral RNA recovery was calculated for all samples using the following formula:

$$Percent\ recovery\ of\ viral\ RNA = \frac{[RNA]_{sample}}{[RNA]_{positive\ control}} \times 100,$$

where [RNA] is expressed in genome copies per sample.

All results were analyzed using SDS Software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Amplification by conventional RT-PCR and sequencing of HEV

For each positive RT-qPCR sample, a conventional RT-PCR was performed using the same RNA used for RT-qPCR.

Briefly, RNA was retrotranscribed into cDNA as follows: 10 µl of RNA extract, 4 µl of 5X iScript reaction mix containing, 1 µl of reverse transcriptase, and 5 µl of PCR water (iScript cDNA synthesis kit, BioRad, Hercules, CA, USA) were mixed and thermocycled at 25°C for 5 min, 46°C for 20 min, and 95°C for 1 min in an Eppendorf® Mastercycler gradient device (Millipore Sigma, Darmstadt, Germany).

Conventional PCR was performed under the recommended conditions as follows: 5 µl of cDNA, 10 µl of iQ Supermix, 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), and 3 µl of PCR Water (iQ Supermix, Bio-Rad, Hercules, CA, USA) were mixed and amplified under the following conditions: 95°C for 5 min, 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 10 min. Specific primer sequences targeting the HEV ORF2 region were used for the amplification: forward primer GTYATGYTYTGCATACATGGCT and reverse primer AGCCGACGAAATYAATTCTGTC (Mykytczuk et al., 2017). The amplicons with an expected size of 348 bp were stored at −80 °C until processing.

An agarose gel (2%) in tris acetate EDTA buffer 1× with 0.01% SYBR® Safe (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used. Each well was loaded with 3 µl of sample and 2 µl of TriTrack DNA loading dye (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA). The amplicon size was compared to a 100 bp DNA ladder (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA) after 45 min of migration at 120 V. A UV-light photo of the gel was taken with the ChemiDocTM MP imaging system (Bio-Rad, Hercules, CA, USA) and processed using Quantity One® software (Bio-Rad, Hercules, CA, USA).

PCR products were Sanger-sequenced after the gel purification at the IBIS Genomic Analysis Platform, Université Laval (Quebec City, QC, Canada) following their recommendations. The Chromas software v2.6.6 was used, and the obtained sequences were aligned using BioEdit v7.0.5.3. The consensus sequence was analyzed with BLAST.

3. Results

3.1. Prevalence of HEV RNA

Of the 83 samples of pork liver pâté, 24 tested positive for HEV RNA (Table 2), indicating a prevalence of 29% with genome copy quantities per gram ranging from 3.34×10^2 to 1.84×10^7 (Table 3). A total of three samples of country-style pâté (P21, P22, and P23) contained conspicuously larger amounts of HEV RNA, and eight samples were withdrawn from the study because the control virus could not be detected. Most of these were of the same brand and one of two products. Of the 79 raw pork livers tested, three were positive for HEV (Tables 2, 3). The estimated prevalence of HEV in raw pork liver produced in the province of Quebec is therefore 4%.

3.2. HEV sequence analysis

A large proportion of the samples found positive by RT-qPCR then transcribed into cDNA and amplified did not show up as positive on an electrophoresis gel. Only one sample showed a PCR product of the expected size (348 nt) and was sent for Sanger sequencing (P22* in Table 3). The sequence was found to match HEV-3 from that in Tokyo, Japan with a sequence similarity of 95% (accession number: LC699232.1). The sequence of the HEV detected in our study appears to be HEV-3 and has been deposited in GenBank (accession number: OQ032868).

4. Discussion

No public data are currently available regarding the number of hepatitis E cases per year in Canada. However, the seroprevalence of HEV in Canadian blood donors is ∼6% (Fearon et al., 2017). Moreover, the virus may circulate among animals and humans that could be infected by ingesting contaminated food products. Food safety is a major public health concern and foodborne viruses, including HEV, are not sufficiently traced in food products. In the present study, we investigated the occurrence of HEV in

TABLE 1 Primers, probe sequences, quencher, and dye used for the detection of the virus by RT-qPCR.

Target	Sequence	References
Mengo virus forward primer	GCGGGTCCTGCCGAAAGT	Pintó et al., 2009
Mengo virus reverse primer	GAAGTAACATATAGACAGACGCACAC	
Mengo virus probe	6FAM-ATCACATTACTGGCCGAAGC- MGBNFQ	
MNV-1 forward primer	CACAGAGGCCAATTGGTAAA	Hewitt et al., 2009
MNV-1 reverse primer	TGCAAGCTCTACAACGAAGG	
MNV-1 probe	6FAM-CCTTCCCGACCGATGGCATC-BHQ1	
HEV forward primer	CGGTGGTTTCTGGGGTGAC	Martin-Latil et al., 2016; Germer et al., 2017
HEV reverse primer	AAGGGGTTGGTTGGATGAATATAG	
HEV probe	6FAM-TGATTCTCAGCCCTTCG-MGBNFQ	

TABLE 2 Detection of HEV in pork liver pâtés collected from grocery stores and in raw livers produced in the province of Quebec.

Food matrix	Total no. of samples	No. of HEV-positive samples	Average recovery from positive controls (%)	Recovery standard deviation (%)
Pork liver pâté	83	24	6.5	1.5–11.5
Raw pork liver	79	3	10.2	5.2–15.2

raw pork liver and pork liver pâté produced in the Canadian province of Quebec. We focused on pork products because swine are known hosts of HEV and because Quebec is the leading pork-producing province, accounting for 30% of Canadian production. The situation of HEV in pork needs to be monitored, and the most recent data for Quebec are now 9 years old. Pork (fresh and frozen) is also the third most consumed meat in the province of Quebec although consumption has been decreasing slightly since 2016. Quebec is the largest exporter of meat and pork products, with a stable share of nearly 40% of the value of total Canadian exports (Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, 2022). Wildlife may also carry HEV (Weger et al., 2017; Lorusso et al., 2022; Takahashi et al., 2022) and sausages or terrines made from deer or others are sold in grocery stores and therefore should be monitored as well. In Canadian pig herds, HEV seroprevalence has been measured at 59.4% and in Quebec at 88.8% (Yoo et al., 2001). Previous prevalence studies conducted in other provinces have concluded similar or higher levels. Between 2011 and 2012, 25 of 283 livers tested (~9%) were reportedly positive or suspected positive for HEV contamination, mainly in the provinces of Ontario and Quebec (Wilhelm et al., 2014). In 2017, Health Canada declared an HEV prevalence of 47% in pork liver pâtés and 10.5% in raw pork livers in the province of Ontario (Mykytczuk et al., 2017). In the USA, the most significant study was conducted in California, which found HEV in 45% of pork livers and 12.6% of ground pork (Harrison et al., 2021). This is also one of the few studies to have found the virus in ground pork.

In the present study, the sampling of pork liver pâtés sold in grocery stores in Quebec City (QC, Canada) included different brands, recipes, and production lots to obtain as much sample diversity as possible. HEV RNA was detected in 29% of the pâtés and in 4% of the raw pork livers. As found in other studies, our results show that liver-based products are more likely to be tested positive for HEV than raw livers (Mykytczuk et al., 2017; Boxman et al., 2019; Pallerla et al., 2021). This is not surprising since many livers (30 to 35) are used to make a large mix that will be then divided into numerous portions of pâté. Our findings are broadly consistent with those in other countries around the world, particularly in Europe where most of the prevalence studies have been conducted. This suggests that sanitation and/or hog processing procedures in Canada and these other countries have a similar impact on the presence of HEV in pork products.

Only the presence of HEV RNA was demonstrated in the present study, which does not provide any indication of the potentially infectious status of virions in the sample. Nevertheless, a link has been previously established between the consumption of pork products and the transmission of HEV to humans (Yapa et al., 2016). The oral infectious dose of HEV is unknown in humans, but it has been estimated experimentally in pigs, where 10^5 HEV RNA copies are sufficient to induce a productive infection (Andraud et al., 2013). In this study, the quantity of HEV RNA found in the products reached up to 1.84×10^7 copies of HEV RNA per gram which is very high and 11 of the positive samples contain more than 10^5 copies of genome per gram. It is therefore possible that these HEV quantities are sufficient to induce an infection.

From the methodological point of view, as far as we have been able to determine, there is still (as of this writing) no standardized method, ISO, or other forms, for processing and analyzing complex samples containing pork liver or pork liver-based products for the presence of HEV. Many protocols are described in the literature and sometimes include similar steps, such as using Trizol®, to dissolve tissues and cells or chloroform to clarify the resulting solutions (Hennechart-Collette et al., 2019; Pallerla et al., 2021; Wang et al., 2021). This methodological diversity creates numerous possibilities for

TABLE 3 HEV RNA detection in samples of pork liver pâté, country-style pâté, or raw liver.

Sample	Product	HEV genome copies/g	Cq	Lot number
P1	Liver pâté	2.62 E+ 04	33.3	2022-06-29
P2	Liver pâté	2.00 E+ 03	36.3	2022-07-08
P3	Country-style pâté	1.30 E+ 03	37.1	2022-07-13
P4	Liver pâté	1.35 E+ 04	35.3	2022-08-03
P5	Liver pâté	1.81 E+ 04	32.5	2022-08-07
P6	Liver pâté	1.53 E+ 05	30.7	2022-08-11
P7	Country-style pâté	1.16 E+ 05	34.4	2022-07-28
P8	Country-style pâté	1.14 E+ 05	32.6	2022-07-29
P9	Country-style pâté	8.05 E+ 04	36.2	2022-07-31
P10	Country-style pâté	8.75 E+ 04	36.1	2022-08-13
P11	Country-style pâté	4.22 E+ 05	34.0	2022-08-12
P12	Liver pâté	3.87 E+ 05	34.8	2022-07-16
P13	Liver pâté	2.17 E+ 05	35.7	2022-08-06
P14	Country-style pâté	1.28 E+ 06	33.1	2022-08-05
P15	Country-style pâté	6.10 E+ 04	30.5	2022-08-22
P16	Country-style pâté	1.33 E+ 04	36.8	2022-09-10
P17	Liver pâté	3.34 E+ 02	38.2	2022-09-10
P18	Liver pâté	5.15 E+ 03	38.0	2022-09-25
P19	Liver pâté	7.70 E+ 02	36.0	2022-09-03
P20	Liver pâté	8.50 E+ 05	31.6	2022-09-16
P21	Country-style pâté	5.10 E+ 06	28.1	2022-08-19
P22*	Country-style pâté	1.77 E+ 06	30.2	2022-09-09
P23	Country-style pâté	1.84 E+ 07	27.2	2022-09-05
P24	Country-style pâté	8.30 E+ 04	35.8	2022-10-03
L1	Raw liver	4.87 E+ 03	38.0	N/A
L2	Raw liver	3.40 E+ 04	32.7	N/A
L3	Raw liver	1.48 E+ 02	41.4	N/A

Cq, quantification cycle; N/A, not applicable; *Sanger sequenced product.

inconsistencies in findings and raises questions about the efficiency of HEV extraction from these food matrices. A straightforward method involving chemical and mechanical homogenization of the samples and a dilution step was performed in this study. The use of the Mengo virus or MNV-1 as control viruses in each method allows us to confirm the efficiency of the protocol. In the absence of a reliable cell culture model, detecting RNA is the only widely available way to confirm that a food sample contains HEV. Since pork-based recipes are the foods most likely to carry a risk of HEV transmission to humans, a standardized method should be developed for this product category. An alternative method for the molecular detection of HEV RNA, based on the integrity of viral capsid, should also be developed to evaluate HEV's possible infectivity (Fraisse et al., 2018).

The HEV RNA sequenced in this study matches genotype 3, frequently associated with HEV zoonotic transmission in industrialized countries (Treagus et al., 2021) and more particularly in North America, Europe, and Japan. In addition, only 1 of 24 positive samples yielded a PCR product that showed up clearly on the electrophoresis gel. HEV genomes could be fragmented during the viral recovery using Trizol®. Our results emphasize the need to develop a standardized method, especially regarding the viral recovery and sequencing methods of HEV in meat products.

Finally, this pilot study provides a glimpse of the potential for contamination with HEV of certain pork liver foods sold commercially in the province of Quebec. It also suggests that monitoring more samples over longer periods is warranted since this virus is now considered an emerging pathogen with potentially severe consequences for growing susceptible populations, such as immunocompromised patients (Damiris et al., 2022) and pregnant women and fetuses (Navaneethan et al., 2008). This research also highlights the continuous presence of HEV in pork liver-based products in Canada over the past 8 years, reinforcing the

need for regular surveillance of its prevalence in food and hence, its epidemiology.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/nuccore/OQ032868.1/.

Author contributions

EC, EJ, VG-B, and JJ: conceptualization. EC and MJ: methodology. EJ, VG-B, and JJ: validation, conceptualization, supervision, and project administration. EC: formal analysis, investigation, resources, and preparation and writing of the original draft. JJ: data curation and funding acquisition. EC, EJ, VG-B, NP, and JJ: writing, review, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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