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Monitoring of pork liver and meat products on the Dutch market for the presence of HEV RNA

Ingeborg L.A. Boxman[a,*], Claudia C.C. Jansen[a], Geke Hägele[a], Ans Zwartkruis-Nahuis[a], Aloys S.L. Tijsma[b,1], Harry Vennema[c]

[a] Food and Consumer Product Safety Authority (NVWA), Akkermaalsbos 4, 6708 WB Wageningen, the Netherlands
[b] Food and Consumer Product Safety Authority (NVWA), Catharijnesingel 59, 3511 GG Utrecht, Utrecht, the Netherlands
[c] National Institute of Public Health and the Environment (RIVM), Anthonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, the Netherlands

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ABSTRACT

The aim of the present study was to assess pork liver and meat products present on the Dutch market for the presence of hepatitis E virus (HEV) RNA. HEV RNA was detected in 27.3% of 521 products sampled from Dutch retail stores in 2016. 12.7% of livers were positive for HEV RNA (n = 79), 70.7% of liverwurst (n = 99), 68.9% of liver pate (n = 90), but in none of the pork chops (n = 98), fresh sausages (n = 103) or wild boar meat (n = 52). The highest level of HEV RNA contamination was observed in a liver (reaching up to 1×10^6 copies/g), followed by ready to eat liverwurst and liver pate (up to 3×10^4 copies/g and 7×10^4 copies/g respectively). Sequence analyses revealed mainly genotype 3c, but also some 3a, 3e and 3f strains. One strain derived from a liver sample was 100% (493 nt) identical with one isolated from a HEV case with onset of disease close in time and geography, although no direct epidemiological link could be established. Despite liverwurst and liver pate undergo heat treatment (information dd. Mid 2017) that may be sufficient to inactivate HEV, persons at risk, including Dutch transplant recipients, have been advised to avoid the consumption of raw liver as well as liverwurst and liver pate.

1. Introduction

Hepatitis E is a liver disease caused by infection with a small, non-enveloped, single stranded RNA virus known as hepatitis E virus (HEV). The incidence of hepatitis E in European countries has increased in recent years similarly to as in the Netherlands (Adlhoch et al., 2016; Aspinall et al., 2017). Most of the infections are asymptomatic and self-limited, locally acquired and caused by HEV strains belonging to genotype 3 (gt 3). Immunocompromised patients, including transplant recipients and patients using immunosuppressive medication, are more susceptible to complications and persistent HEV gt 3 infection may occur (Kamar et al., 2013). The recognition of increasing incidence in the general population as well as consequences for infections in vulnerable persons urged monitoring of food products, that might be sources for HEV infection, by viral analyses.

Domestic pigs are a well-recognized reservoir of HEV gt 3. Foodborne transmission of HEV via consumption of raw and undercooked liver, meat, or sausages from domestic pigs has been documented (Colson et al., 2010; Yazaki et al., 2003) and the presence of infectious HEV was demonstrated in pork liver sausage and livers (Berto et al., 2013; Feagins et al., 2007).

For the detection of HEV RNA in food of animal origin, however, there is not a standardized method such as has been published for the detection of norovirus and hepatitis A virus in food (ISO 15216-1, 2017). The prevalence studies that have been performed to assess the presence of HEV RNA in porcine liver, liver products, pork and pork products differ in many aspects, such as the sample volume taken into analysis as well as extraction methods applied. Strategies that have been used to release the virus from the food for extraction have either been manual homogenization using a micropestle (Wenzel et al., 2011), mechanical disruption using beads (Bouwknegt et al., 2007; Di Bartolo et al., 2012), a probe (Mykytczuk et al., 2017), or a stomacher (Martin-Latil et al., 2014; Szabo et al., 2015; Moor et al., 2018). The media in which the foods were homogenized were either chaotropic lysis buffers (Bouwknegt et al., 2007; Di Bartolo et al., 2012; Szabo et al., 2015; Moor et al., 2018) or neutral PBS or high alkaline glycine buffers

* Corresponding author.
E-mail addresses: i.l.a.boxman@nvwa.nl (I.L.A. Boxman), c.c.c.jansen@nvwa.nl (C.C.C. Jansen), g.hagele@nvwa.nl (G. Hägele), j.t.m.zwartkruis@nvwa.nl (A. Zwartkruis-Nahuis), tijsma@viroclinics.com (A.S.L. Tijsma), harry.vennema@rivm.nl (H. Vennema).
[1] Current address of Aloys Tijsma is Viroclinics Biosciences B.V., Marconistraat 16, 3029 AK Rotterdam, The Netherlands.

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combined with polyethylene glycol (PEG) precipitation (Martin-Latil et al., 2014; Mykytczuk et al., 2017). Following virus extraction, nucleic acids were frequently isolated using silica-based methods (Di Bartolo et al., 2012; Martin-Latil et al., 2014; Moor et al., 2018; Szabo et al., 2015; Wilhelm et al., 2014) and HEV RNA was detected using the RT-real-time-PCR, often using the HEV specific oligoset as described by Jothikumar and co-workers (Jothikumar et al., 2006).

The aim of the present study was to assess the presence of HEV RNA in liver and pork products available on the Dutch market, as recent prevalence data are absent and meat preparations differ per country. For this, food samples of animal origin were homogenized in high alkaline Tris-Glycine-buffer with beef extract (TGBE) using a mechanical disruptor (FastPrep24-5G, MP Biomedicals). Subsequently nucleic acid extraction was performed on clarified homogenates, followed by detection of HEV RNA using RT-qPCR. Prior to application to field samples, the method was characterized by the determination of the limit of detection as well as the linearity of the detection of HEV RNA in liver, liver pate, bratwurst sausage and pork chops. Subsequently, the presence of HEV RNA in a variety of liver and pork products on the Dutch market was investigated. In addition, the HEV copy numbers in samples were quantified and HEV strains detected typed by sequence analyses. Finally, these sequences were compared to those retrieved from Dutch patients without a recent travel history in the same time period.

2. Materials and methods

2.1. Virus preparations

A viral stock was prepared from a HEVgt 3c positive porcine fecal sample (Acc No. MF185108) as described earlier (Boxman et al., 2017) and quantified at 1.2×10^7 IU/ml by RT qPCR using serial-dilutions of the WHO HEV genotype 3a standard (PEI code 6329/10) at the VirooScience group of the ErasmusMC Rotterdam (Dr. S. Pas) (Pas et al., 2012). Murine norovirus (MuNoV) was kindly provided by Dr. H. Virgin IV, Washington University, St. Louis, Missouri and quantified by cell culture method at 4×10^7/ml $TCID_{50}$ by Dr. E. Duizer, RIVM, the Netherlands (Tuladhar et al., 2012).

2.2. Sampling

Samples were collected in 2016. Pork livers (n = 79) were collected in April and May, liverwurst (n = 99) in May until July, liver pate (n = 106) in June and July, pork chops (n = 98) in August, fresh sausages (n = 103) in August and September, and wild boar meat (n = 52) in November and December. Sampling was performed in retail stores by inspectors of the Netherlands Food and Consumer Product Safety Authority (NVWA). Samples were aseptically packaged to avoid cross-contamination and kept at 5 °C during transport and were processed before the expiry date. The majority (> 80%) of the liver, liverwurst, fresh sausages, and wild boar samples were produced or packed in the Netherlands. Liver pates were either produced or packed in the Netherlands (47%) or Belgium (41%). For the remainder of the samples this information was missing on the label.

2.3. Viral and nucleic acid extraction

Three pieces of 1 cm^3 taken from different locations of the sample were manually chopped using a surgical blade. For virus extraction, a subsample of 0.3 gram product was aseptically weighed, and transferred to a 2 ml Lysing matrix S tube with stainless-bead (MP Biomedical-Bio-Connect) and either stored frozen at −20 °C or processed immediately. 1 ml of TGBE buffer (100 mM Tris, 50 mM Glycine, 1% (w/v) beef extract, pH 9.5) and 10 µl of MuNoV (4×10^4 $TCID_{50}$), as process control virus, were added to each sample. Mechanical disruption was performed (FastPrep 24-5G, MP Biomedical-Bio-Connect) in four cycles of 30 s each one at the speed of 6 ms^{-1} interrupted by periods without beating for 5 min at room temperature. For liver samples, a subsample of 0.1 g was disrupted in one cycle of 40 s at the speed of 6 ms^{-1}. All lysates were clarified by centrifugation (10,000*g* for 20 min at 4 °C). The aqueous intermediate layer, without the upper fat layer or food pellet, was transferred to a clean 50 ml tube and TGBE buffer was added to reach a total volume of 2 ml. After mixing and clarification (10,000*g* for 20 min at 4 °C), the supernatant was transferred to another new tube and nucleic acid extraction was performed using Nuclisens Magnetic Extraction Reagents kit (BioMérieux) according to the manufacturer's instruction. Negative extraction control samples (buffer only) were run through all stages of the analytical process (except mechanical disruption) at a frequency of one in between each set of three liver(-containing) samples, or one in between each set of five meat product samples.

Table 1
Linearity of the method for the detection of HEV RNA on inoculated liver and meat products.

	Liver (0.1 g)	Liver pate (0.3 g)	Fresh sausage (0.3 g)	Pork chop (0.3 g)
R^2	0.95	0.98	0.94	0.99
Slope	−3.81	−3.22	−3.50	−3.45

2.4. Detection and quantification of HEV RNA

RNA of MuNoV and HEV was detected by RT-qPCRs using oligonucleotides (Baert et al., 2008; Jothikumar et al., 2006) after in-house optimization for the CFX96 platform (Boxman et al., 2017). Viral extraction efficiency was calculated using a MuNoV RNA standard curve (ISO 15216-1, 2017), setting the minimal recovery for each sample to be ≥1%. According to ISO 17025 all reagents were tested before use against the previous batches. Despite the detection of MuNoV RNA in the presence of matrix with < 0.3 Cq difference with the previous batch in a direct comparison experiment, one particular batch of magnetic silica (BioMerieux) used in present study was notified to have a reduced extraction efficiency for RNA viruses in the absence of matrix (BioMerieux, Field Safety Notice 3203). The (likely) overestimated extraction efficiency values for samples extracted with this particular batch were therefore dismissed from Table 3. Each sample was also tested for inhibition using the ssRNA HEV standard in a separate reaction well (Diez-Valcarce et al., 2011), and evaluated as described in ISO 15216-1 (2017). For quantification, each sample was rerun against serial dilution (10^1 to 10^5 HEV genome copies/µl) of linearized plasmid (Boxman et al., 2017). Samples were considered positive when confirmed in this RT-qPCR with Cq values below 40 and amplification plots of the real time signals showed a S curve.

2.5. Determination of the limit of detection of HEV in inoculated food samples

Liver (0.1 g), liver pate (0.3 g), fresh sausage (bratwurst) (0.3 g) and pork chop (0.3 g) samples were inoculated with various amounts of HEV and MuNoV. For this, 10-fold serial dilutions were made in duplicate and two samples were inoculated at each inoculation level (10 µl). In total four extractions were performed for each inoculation level resulting in four cycle threshold (Cq) values. This experimental set-up was performed twice. The recovery rate was set equal to $2^{(-\Delta Cq)} \times 100\%$, where ΔCq is the difference between the Cq of the matrix sample and Cq of the inoculate. HEV genome copies (gc) in nucleic acid extracts of the inoculate (10 µl) were quantified using the dsDNA HEV standard.

Table 2
Detection limit of the method for the detection of HEV RNA on inoculated liver and meat products.

Inoculate HEV gc per sample	Liver (0.1 g)			Liver pate (0.3 g)			Fresh sausage (0.3 g)			Pork chop (0.3 g)		
	Pos n/N[c]	Cq avg ± sd	%[d]	Pos n/N	Cq avg ± sd	%	Pos n/N	Cq avg ± sd	%	Pos n/N	Cq avg ± sd	%
3.8×10^{3}[a]	4/4	39.0 ± 0.3	1.5	4/4	35.4 ± 0.5	18.4	4/4	35.8 ± 0.4	13.5	4/4	35.4 ± 0.1	17.5
3.8×10^{2}[b]	2/4	41.3 ± 0.4	6.6	4/4	38.5 ± 0.8	49.8	4/4	38.6 ± 0.1	40.5	4/4	38.8 ± 1.0	41.2
3.8×10^{1}[b]	0/4	–	–	3/4	40.5 ± 1.1	47.9	1/4	40.1	52.9	3/4	39.9 ± 0.3	52.5
Blank	0/2	–	–	0/2	–	–	0/2	–	–	0/2	–	–

[a] Genome copy (gc) in inoculate as determined using the dsDNA standard.
[b] Genome copy in inoculate as calculated after 10-fold dilution.
[c] Number of samples positive/number of samples tested.
[d] HEV recovery rate.

Table 3
Detection of HEV RNA in liver and meat products on the Dutch market in 2016.

Type of product	HEV pos n/N (%)	Cq values avg ± sd	gc HEV/g avg ± sd	gc HEV/g range	% amplification efficiency avg ± sd	% MuNoV recovery avg ± sd
Liver	10/79 (12.7)	34.9 ± 5.9	$3.4 \times 10^{5} \pm 5.3 \times 10^{5}$	2.1×10^{2}–1.2×10^{6}	88.6 ± 20.7	14.6 ± 16.2
Liverwurst	70/99 (70.7)	36.4 ± 2.1	$3.7 \times 10^{3} \pm 5.5 \times 10^{3}$	9.4×10^{1}–3.0×10^{4}	102.7 ± 27.3	139.2 ± 59.4
Liver pate	62/90 (68.9)	34.8 ± 2.9	$1.0 \times 10^{4} \pm 1.4 \times 10^{4}$	1.1×10^{2}–7.0×10^{4}	82.7 ± 18.6	62.4 ± 54.7[a]
Fresh sausage	0/103 (0)	nd	nc	nc	136.0 ± 46.4	41.1 ± 23.6[b]
Pork chop	0/98 (0)	nd	nc	nc	106.9 ± 32.6	22.4 ± 10.1[c]
Wild boar	0/52 (0)	nd	nc	nc	104.3 ± 25.6	51.0 ± 15.3
Total	142/521 (27.3)					

[a] Including six liver pate samples that tested HEV positive but a recovery rate below 1%.
[b] Recovery calculated on 35 samples only.
[c] Recovery calculated on 10 samples only.

2.6. Typing of HEV RNA positive samples and comparison to human HEV strains

HEV presumptive positive samples were re-amplified using a nested RT-PCR using codehop primers targeting ORF2 with a final 493 nucleotide fragment for sequence analyses (Boxman et al., 2017) and were typed according to Smith et al. (2014) using the HEV typing tool (https://www.rivm.nl/mpf/typingtool/hev/). Sequences have been submitted to GenBank with accession numbers MF996383-MF996450.

Subsequently phylogenetic analyses was used to compare HEV sequences obtained in this study with HEV gt 3c sequences identified in Dutch acute hepatitis cases without a travel history with sampling dates between March and November 2016 to investigate the genetic relationship between the HEV sequences retrieved from food and diagnostic samples. A Maximum Parsimony tree was calculated with Bionumerics software.

3. Results

3.1. Evaluation of the sensitivity of the method for detection of HEV RNA in inoculated liver and meat products

Prior to the assessment of HEV RNA in liver and meat products, performance characteristics of the method were determined. Preliminary experiments using in 0.3 gram samples indicated that the recovery of HEV and MuNoV was very low for liver samples. Reducing the volume of liver samples to 0.1 g resulted in a 20 times higher virus recovery. Further studies were therefore performed using 0.1 gram liver samples. To investigate the linearity of the detection assay for HEV RNA in the products, samples were inoculated with various amounts of virus. A linear relation was observed between Cq values obtained and HEV inoculate (log copies) over at least 3 logs in liver, liver pate, fresh sausage and pork chop (Table 1). Subsequently the lowest amount of HEV that could be detected in at least half of the samples was determined for the four food matrices within one experiment. From two independent experiments the LOD was estimated at 3.8×10^{3} and 1.3×10^{3} copies per gram respectively for raw liver (0.1 g) and fresh sausage (0.3 g) samples (Table 2, representing data of one of the experiments). For liver pate and pork chops (0.3 gram samples) this amount was estimated at 1.3×10^{2} copies per gram. The recovery of the inoculated HEV from the samples varied per product but was lowest for liver. Additional experiments indicated that the amplification efficiency of the external amplification control was above 74% in all inoculated samples.

3.2. Monitoring liver and meat products for the presence of HEV RNA

In total 537 samples were first analyzed for the presence of MuNoV RNA to determine the recovery rate in each sample. This recovery rate varied for various products and within groups (Table 3). In total 515 samples met the recovery minimum of 1%. To these samples, six liver pate samples were added as they subsequently tested HEV RNA positive, despite a recovery rate below 1%. Sixteen other liver pates were excluded from the study as the extraction efficiency was < 1% and HEV RNA was not detected. None of the samples were excluded because of reduced amplification efficiency.

Twenty-seven percent (142/521) of the remaining samples tested positive for the presence of HEV RNA. HEV RNA was detected in 12.7% of livers (n = 79), 70.7% of liverwurst (n = 99), 68.9% of liver pate (n = 90), but in none of the pork chops (n = 98), fresh sausages (n = 103) or wild boar meat (n = 52). The quantitative results showed differences in the level of contamination between products and batches, revealing the highest average copy number in liver followed by liver pate and liverwurst (Table 3).

3.3. Typing of HEV RNA detected in liver and liver products

HEV nested typing PCR fragments (ORF2, 493 nt) were obtained for 6 of the 10 HEV positive liver samples. For 5 samples, sequences could be typed as derived from HEV gt 3c (n = 4) and 3f (n = 1). Nested PCR

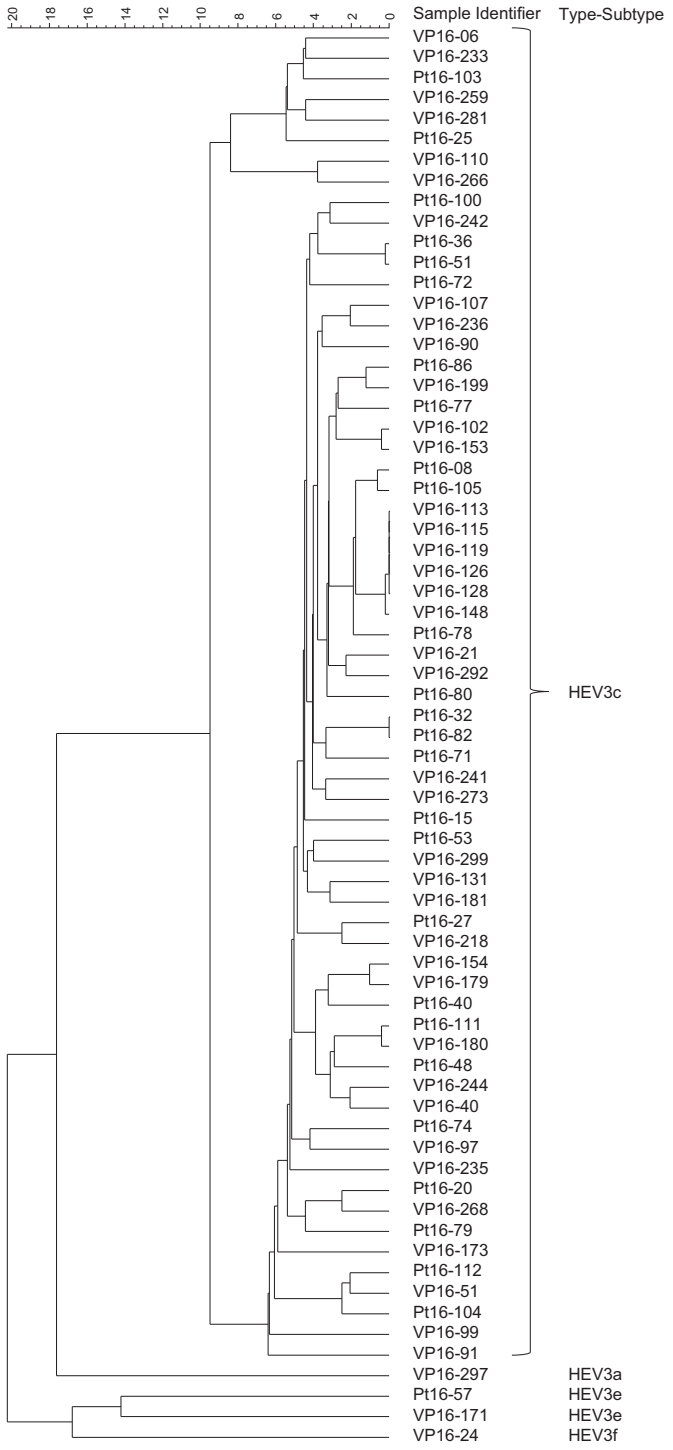

Fig. 1. Phylogenic analyses of HEV strains retrieved from porcine (liver) products.
Nucleotide HEV sequences of a 493-nt open reading frame 2 fragment (positions 5941–6472 of reference sequence NC_001434) from liver (products) (n = 69) were used to produce a UPGMA tree with Bionumerics software.

fragments were obtained for 45 of 70 (64%) liverwurst samples, resulting in 37 single-read HEV sequences. Thirty-five single read sequences in liverwurst were typed as HEV gt 3c, one as gt 3a and one as gt 3e. The remaining eight sequences obtained showed double nucleotide peaks at the third nucleotide of several codons in both forward and reverse reads, indicating more than one strain to be present in the RNA sample, or were otherwise difficult to reliably interpret. Sequence analyses appeared most successful for liverwurst samples that had tested positive with Cq values below 37. For this reason, a selection was made of 39 liver pate samples with Cq values below 37 to further analyze. For all selected 39 samples nested RT-PCR fragments were obtained, resulting in 26 single-read HEV sequences. Twenty-five of these were typed as gt 3c, and one as gt 3e. The remaining seven liver pate samples presumably contained multiple HEV strains. All liverwurst and liver pate samples with presumably multiple strains (n = 15) were excluded for further phylogenetic analysis. In this analysis (Fig. 1) two clusters of identical sequences were identified, one consisting of five liverwurst samples and another of two liver pate samples. Trace back revealed that three out of these five liverwurst samples had been produced in the same factory in the same period, possibly pointing to liver ingredients from the same source. No such relation was found for the other two liverwurst samples or the two liver pate samples.

3.4. Comparison to sequences from Dutch patients in the same period

HEV gt 3c sequences of liver (n = 4), liverwurst (n = 35) and pate (n = 25) samples were compared to gt 3c sequences obtained from 71 Dutch patients with acute hepatitis E without a recent travel history in 2016 for similarities to investigate the genetic relationship between the HEV sequences retrieved from food and diagnostic samples (Fig. 2). Sequences detected in pork liver products and diagnostic samples were interspersed showing a variety within the gt 3c group. Between gt 3c sequences in liverwurst or liver pate and the clinical samples a minimum difference of 2 nucleotides in an overlapping region of 493 nucleotides was observed, which is 0.4% difference. This difference was at least 5.3%, 12.7%, and 9.3%, respectively for gt 3a, 3e or 3f sequences detected in liver products and clinical cases.

For sequences retrieved from liver samples, one gt 3c sequence was identical over 493 nt to a strain obtained in a patient sample. The collection of this sample (VP16–40) (April 2016) in an outlet shop of a national supermarket chain preceded the onset of disease (June 2016), whereas the geographical distance between point of collection and patient residence was only 35 km. Retrospectively, it remained unclear whether this patient had consumed raw or cooked liver despite use of standardized questionnaires.

4. Discussion

Following other reports on the detection of HEV RNA in pork liver and meat products (EFSA BIOHAZ Panel et al., 2017), the analyses of products from the Dutch market revealed a surprisingly high prevalence of HEV RNA in liverwurst (70.7%) and liver pate (68.9%) samples, a high prevalence in liver samples (12.7%), and non-detectable levels in pork chops, fresh sausages and wild boar meat. Contamination levels in raw pork liver and pork liver derived products reached 1×10^6 gc/g and 3 to 7×10^4 gc/g, respectively, but the associated risk with consumption of these food items remains unclear as RT-qPCR is non-discriminatory for detection of RNA derived from infectious and non-infectious virus particles. Currently, an assay to determine the HEV infectivity in food extracts is not yet available.

The prevalence of HEV RNA in Dutch raw pork livers in the present study (12.7%) has meanwhile been affirmed by analyses of 68 Dutch pork liver samples collected retail in 2017 (11.8%) (data not shown). Both outcomes are higher than the prevalence reported in the study on Dutch pork livers in 2005 (6.5%) (Bouwknegt et al., 2007). This may indicate an increase in the number of Dutch pigs with an HEV infection at moment of slaughter in time, although an increase due to differences in methods cannot be excluded. The prevalence of HEV in Dutch pork livers was also higher as compared to recent reported prevalence in European (Di Bartolo et al., 2012; Wenzel et al., 2011) and Canadian studies (Mykytczuk et al., 2017; Wilhelm et al., 2014) ranging from 4% to 11% in liver samples collected between 2010 and 2012. The observed viral load of positive liver samples in the present study

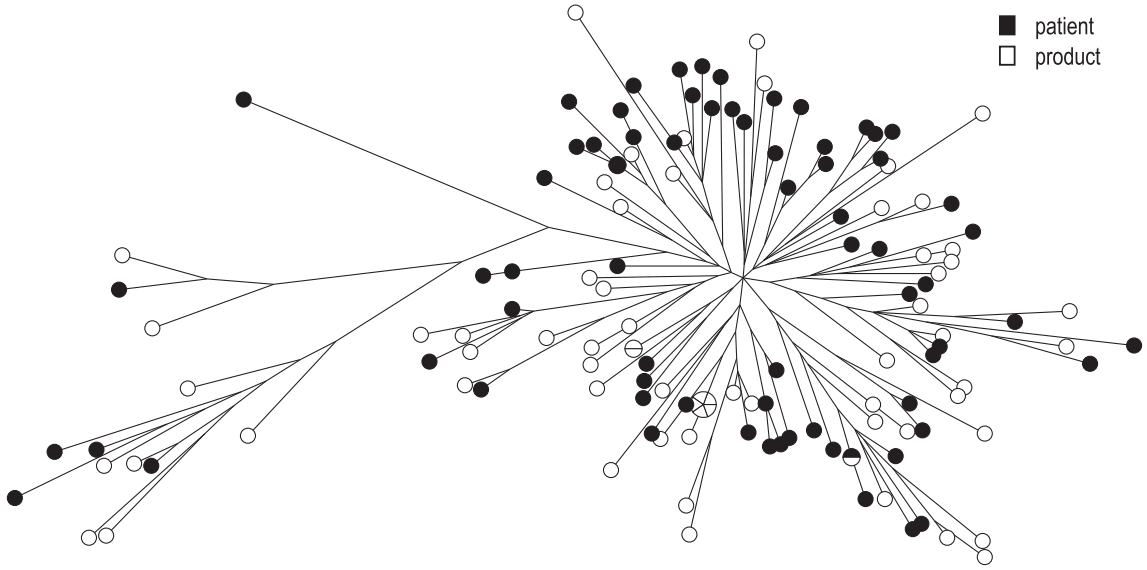

Fig. 2. Phylogeny of genotype 3c strains from porcine liver (products) and patients with acute hepatitis in the Netherlands, 2016.
Nucleotide HEV gt 3c sequences of a 493-nt open reading frame 2 fragment (positions 5941–6472 of reference sequence NC_001434) from liver (products) (n = 64) or from patients (n = 71) with acute hepatitis E in the Netherlands in 2016 were used to produce a Maximum Parsimony tree with Bionumerics software.

(2.1×10^2–1.2×10^6 gc/g) was however comparable to the few datasets that are reported earlier, 1.0×10^3–4.6×10^6 gc/g (Wilhelm et al., 2014).

Dutch liverwurst or liver pates differ in composition and production process, but both are composed of a mixture of livers derived from many animals. A small number of HEV positive livers with a high viral load are therefore likely to contaminate the whole batch of these products, which would be an explanation for the high percentage of liverwurst and liver pate samples testing positive for HEV RNA in the present study. These percentages were higher than those reported earlier in German 'Leberwurst' samples (22%) (Szabo et al., 2015), in French liver sausages (27%–29%) (Martin-Latil et al., 2014; Pavio et al., 2014), or in Brazilian (36%) (Heldt et al., 2016) and Canadian (47%) (Mykytczuk et al., 2017) liver pates. The viral loads in liver pate samples in the present study were higher than in the study of Mykytczuk and coworkers, (2017) (3–500 gc/g), but the viral loads in liverwurst samples were lower than those (1×10^2–2×10^6 gc/g) reported by Pavio and co-workers (Pavio et al., 2014). Results are however difficult to compare as methods and production processes may vary between studies, and some of the liver sausages are sold to consumers to be eaten after cooking (Pavio et al., 2014), whereas Dutch and German liverwursts are a ready to eat product.

Assuming a serving size of about 15 g of Dutch liverwurst or pate, the total intake would reach about 5×10^5–1×10^6 genome copies. It is however currently unknown whether these viral copies are derived from infectious particles. Proper heat treatment will inactivate HEV and therefore reduce the risk for an HEV infection after consumption (Barnaud et al., 2012). Requested information on the industrial production process, received half 2017, indicated heat treatments of liverwurst and liver pate at > 70 °C for > 2 h. HEV particles in these products may therefore be inactivated (Barnaud et al., 2012), however, complete inactivation may also be dependent on the initial viral load and the exact composition of the food matrix (Cook and Van der Poel, 2015). There is thus an urgent need for the development of an effective, easy to perform and reproducible cell culture system, to be used as a HEV infectivity assay that is compatible for food extracts.

Interestingly, ready to eat spreadable liver products have been identified as one of the independent risk factors for HEV infection in multivariate analyses in a case-control study (2012–2014) in Germany (Faber et al., 2018) and a case-control study (2015–2017) in the Netherlands (Tulen et al., 2019), suggestive that, at the time of these case-control studies, there was a risk for HEV infection after consumption of ready to eat spreadable liver products. These studies state that 24% (Faber et al., 2018) and 18% (Tulen et al., 2019) of the acute hepatitis E cases were attributable to these products.

The observation that mainly HEV genotype 3c strains were detected in the liver(products) was expected as they are also most prominent in Dutch patients (Adlhoch et al., 2016) and in porcine blood product used as ingredient in meat products (Boxman et al., 2017). The variations within the HEV genotype 3c strains were not reflected in changed amino acids, and were thus synonymous changes, as earlier reported for HEV 3a in Canadian food samples (Mykytczuk et al., 2017). Comparison of HEV strains from liver(products) with those isolated from Dutch clinical samples with onset of illness in the food sampling period led only to one match of identical sequences (493 nt) between a liver sample and a case. The epidemiological information was unfortunately not strong enough to conclude whether there was a causal relationship. The absence of identical matches between liverwurst/pate samples and cases, despite similarity (up to 99.5%), may be related to the diversity within HEV genotype 3 strains, to the fact that most infections are subclinical and/or to the limited number of sequences in the comparison of sequences derived from food and clinical samples. Moreover, HEV strains that are best detected in food are not necessarily the strains that are best replicated in humans as has been described for detection of norovirus strains in oysters implicated in oyster-related outbreaks (Rajko-Nenow et al., 2014). As none of the tested products in this study was epidemiologically linked to individual patients a technique such a cloning of the PCR fragments was not performed to reveal multiple strains in the products. Such approach or NGS would however be interesting to find strains in a suspected product consumed by a patient. Linkage may also require more data (sub) clinical samples and HEV strains from the environment (manure, as well as water, fruits vegetables, shellfish) (Yugo and Meng, 2013). A recently (October 2017) launched HEVnet database by ECDC, hosted at the National Institute for Public Health And Environment (RIVM), www.hevnet.nl can be used for future epidemiological investigations on a European level.

In none of the other investigated products HEV RNA was detected. For pork chops this result was expected as only 1% prevalence was reported in the Canadian study with only weak signals (Wilhelm et al., 2014) and 0 to 3% prevalence was reported in European meat (Berto et al., 2012; Di Bartolo et al., 2012). The negative results were less expected for Dutch fresh sausages, as fresh sausages have been reported

to be associated with HEV cases in UK (Said et al., 2014) and 10% of such fresh sausages have been tested positive (Berto et al., 2012), but the finding may be due to differences in compositions of the product. Another unexpected result was that none of the wild boar meat samples tested positive whereas the majority (83%) of meat/musculature samples of German wild boars did (Anheyer-Behmenburg et al., 2017). The latter samples were collected near the Polish border > 700 km apart from the Dutch wild boar population. Figatelli sausages, another product which has frequently been tested HEV RNA positive (30%–36%) (Martin-Latil et al., 2014; Pavio et al., 2014) as it contains raw liver, were not included in the present study as figatelli are hardly available on the Dutch market.

A limitation of extraction method used in the present study is the small volume of test material. This may easily underestimate the prevalence in products with low viral load, such as the pork chop and fresh sausages. The chosen test volume was however comparable to the volume used most cited studies, with few exceptions of more recent studies (Martin-Latil et al., 2014; Szabo et al., 2015; Mykytczuk et al., 2017). For analyses of food with low estimated viral loads, extraction of larger volumes e.g. using Tri-reagent may be considered (Szabo et al., 2015) in future experiments, despite the reagent is less environmental friendly and safety precautions are needed.

In the present study a variety of liver and meat products have been tested for the presence of HEV. Thirteen percent of the raw livers tested positive for HEV RNA, of which some at a high level of copy per gram. Proper heat treatment during food preparation is already a promoted intervention to reduce the risk of HEV infection. The safety of the ready to eat liverwurst and liver pate products however is dependent on proper and well controlled industrial heating process. Awaiting risk analyses or infectivity assays to prove that products are completely safe, the results have been used to inform vulnerable individuals such as transplant recipients and persons with pre-existing chronic liver disease via dieticians in hospitals (Voedingscentrum, 2019).

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