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High prevalence of acute hepatitis E virus infection in pigs in Dutch slaughterhouses

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ABSTRACT

Hepatitis E is caused by hepatitis E virus (HEV), one of the causes of acute viral hepatitis. Domestic pigs are considered as the main reservoir of HEV-3. The recently reported high prevalence of HEV in liver- and meat products on the Dutch market warranted a cross-sectional prevalence study on HEV infection among 5–6 months old pigs slaughtered in the Netherlands ($n = 250$). For this, liver, caecum content and blood samples were analyzed for the presence of genomic HEV RNA by RT-PCR. In addition, a serological test was performed to detect HEV IgG. Background information was retrieved on the corresponding farms to evaluate potential risk factors for HEV at pig slaughter age.

HEV IgG was detected in sera from 167 pigs (67.6 %). HEV RNA was detected in 64 (25.6 %) caecum content samples, in 40 (16.1 %) serum samples and in 25 (11.0 %) liver samples. The average level of viral contamination in positive samples was $\log_{10}$ 4.6 genome copies (gc)/g (range 3.0–8.2) in caecum content, $\log_{10}$ 3.3 gc/ml (range 2.4–5.9) in serum and $\log_{10}$ 3.2 gc/0.1 g (range 1.7–6.2) in liver samples. Sequence analyses revealed HEV-3c only. Ten times an identical strain was detected in two or three samples obtained from the same pig. Each animal in this study however appeared to be infected with a unique strain. The presence of sows and gilts and welfare rating at the farm of origin had a significant effect ($p < 0.05$) on the distribution over the four groups representing different stages of HEV infection based on IgG or RNA in caecum and/or serum. The observed proportion of tested pigs with viremia (16 %) was higher than in other reported studies and was interestingly often observed in combination with a high number of HEV genome copies in liver and caecum content as detected by RT-qPCR. Data provided will be useful for risk assessment for food safety of pork products, will provide baseline data for future monitoring of HEV infections in pigs and new thoughts for mitigation strategies.

1. Introduction

Hepatitis E is caused by hepatitis E virus (HEV), one of the five main hepatitis viruses (WorldHealthOrganization, 2021). HEV is a small, positive single stranded RNA virus and a member of the *Hepeviridae* family (genus *Orthohepevirus*). Eight HEV genotypes have been described, of which four main HEV genotypes are known to infect humans (Smith et al., 2020). Genotypes 1 and 2 (HEV-1 and HEV-2) are exclusively present in humans and cause endemic outbreaks in developing countries. Genotypes 3 and 4 (HEV-3 and HEV-4) are zoonotic and the main cause of hepatitis E in developed countries. HEV-3 is widely distributed around the world and genetically identified in humans, domestic swine, wild boar, deer, rabbit and mongoose, while HEV-4 is mainly genetically identified in humans, domestic swine, and wild boar in Asia (Kamar et al., 2017; Meng, 2016). HEV can cause a wide range of symptoms, from subclinical or self-limiting icteric hepatitis to hepatic failure (Kamar et al., 2017; Lhomme et al., 2020). People with underlying liver disease and immunocompromised patients are at increased risk for severe or chronic disease after infection (Dalton et al., 2009; Kamar et al., 2008; Péron et al., 2007).

In the period of 2005–2015, the incidence of recognized autochthonous HEV-3 infections has increased in Europe (Aspinall et al., 2017). This increase may reflect a true rise in the incidence of hepatitis E in some parts of Europe suggesting that there has been a change in the risk

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of acquiring HEV. In Dutch blood donors aged 18 to 21 years, e.g. HEV seropositivity increased from 4.3 % to 12.7 % from 2000 to 2011 (Hogema et al., 2014). Laboratory confirmed HEV cases increased from 31 in 2010 to a peak of 307 in 2016 (De Gier et al., 2019).

Domestic pigs are considered as the main reservoir of HEV-3 (EFSA BIOHAZ panel et al., 2017). Food-borne transmission of HEV-3 via consumption of raw pig liver sausage (figatelli) has been supported by epidemiological and virological findings (Colson et al., 2010) and the presence of infectious HEV was demonstrated in pork sausages and pork livers (Berto et al., 2013; Feagins et al., 2007).

Pigs and products thereof may therefore directly pose a risk for food safety. Recently, data for studies on the occurrence and typing of HEV-3 in pigs and pork products up to 2017 have been reviewed (EFSA BIOHAZ panel et al., 2017). Factors that may influence the outcomes of prevalence studies in pigs include methods applied, sampling years, sampling locations and especially age at time of sampling. At finisher farms, the peak of HEV shedding generally occurred at around 15 weeks of age and infection often was cleared at time of slaughter (de Deus et al., 2008; Krog et al., 2019). Farm characteristics that influence infection pressure or delay clearance of the virus may have a direct effect on the proportion of pigs being RNA positive at slaughter (Salines et al., 2020). The recently reported high prevalence of HEV in liver-and meat products on the Dutch market (Boxman et al., 2019; Boxman et al., 2020), warranted data gathering on HEV infection among 5–6 months old pigs slaughtered in the Netherlands. In pigs of that age HEV RNA has been detected in 0–28 % of the tested fecal or caecum content samples, 0.25–15.5 % of the liver samples and 3.5–6.7 % of the serum samples, whereas HEV-antibody response has been reported for 31–93 % of the pigs (Table 1).

As no recent data for HEV infection in pigs at Dutch slaughterhouses was available, a cross-sectional HEV prevalence study was performed in 2019. From 250 pigs, liver, caecum content and blood were collected and analyzed for the presence of HEV RNA. In addition, a serological test was performed to detect HEV IgG to discriminate between pigs that had not yet been infected and those that had already cleared the virus. HEV copy numbers in samples were quantified and HEV strains detected were typed by Sanger sequence analyses. Finally, background information was retrieved on the corresponding farms to evaluate potential risk factors for HEV positivity at slaughter age. The combined results will provide insight in the prevalence of HEV infection in pigs at slaughter with respect to food safety.

2. Methods and materials

2.1. Sample size estimation

The sample size to estimate true prevalence was calculated using a webtool available from https://epitools.ausvet.com.au/prevalencess (Humphry et al., 2004). With a precision of 5 %, an estimated sensitivity of 90 % and specificity of 99 %, a sample size of 201 pigs was needed to determine the prevalence of HEV RNA in liver, for which the assumed true prevalence was set at 12 % based on earlier results (Boxman et al., 2019). A sample size of 116 pigs was needed to determine the prevalence of HEV RNA in serum, for which the assumed true prevalence was set at 6 %. This value was anticipated to be higher than 3.5 % (Grierson et al., 2015) based on the relative high fraction of HEV positive livers on the Dutch market.

2.2. Sampling

Samples were collected from May through December 2019 in Dutch slaughterhouses ($n = 8$) that had been selected for their large slaughter capacity, known for slaughtering pigs raised in the Netherlands only, and altogether covering a large geographical area. In each slaughterhouse, pigs (5–6 month old) were randomly selected, with a sampling plan of two pigs per day, not originating from the same farm, and 32 pigs in total per slaughterhouse in a half year time period. For each pig the unique identification number and date of slaughter was recorded electronically. This unique identification number was used to retrospectively obtain information on the region and regional pig density, presence of sows at the farm of origin, farm size and welfare status (conventional versus one star Better Life Label, (Dierenbescherming, 2021). The Better Life Label is a voluntary scheme. Participants are certified with 1, 2 or 3 stars, the latter being considered as more animal-friendly. Certified organic farms are eligible for a 3-star Better Life label.

Table 1
Studies on the presence of HEV in pigs at slaughterhouses at age of 5–6 months[a].

	HEV RNA			HEV IgG	
	Fecal	Liver	Blood	Blood	
Country	% (N)	% (N)	% (N)	% (N)	Reference
NL	28.0 (50)[b,c]				Rutjes et al., 2009
NL				67.8 (130)[d]	Rutjes et al., 2010
CA	14.0 (43)[b,e]	20.9 (43)[e]	2.3 (43)[e,f]		Leblanc et al., 2010
FR		3.4 (3715)[g]		31 (6565)[d]	Rose et al., 2011
ES	0 (23)[b,c]	6.3 (6/96)[c]		69.2 (120)[d]	Casas et al., 2011
UK	12.5 (40)[b,c]	2.5 (40)[c]			Berto et al., 2012
DE	2.5 (120)[b,c]				Machnowska et al., 2014
UK	14.7 (629)[e,h]		3.5 (629)[e,f]	92.8 (629)[f]	Grierson et al., 2015
SL	0 (400)[b,c]	0.25 (400)[c]			Raspor Lainšček et al., 2017
ES	13.3 (45)[b,c]	15.5 (45)[c]	6.7 (45)[c,d]	73.3 (45)[d]	Garcia et al., 2019
USA			6.3 (5033)[d,e]	39.9 (5033)[d]	Sooryanarain et al., 2020
IT	1.9 (569)[b,e]	2.1 (585)[e]	4.4 (91)[e,f]	76.8 (409)[f,i]	Chelli et al., 2021[j]
NL	25.6 (250)[c,e]	11.0 (228)[e]	16.1 (248)[d,e]	67.7 (247)[d]	this study

[a] Criteria for selection were collection of samples at slaughterhouse of pigs that were at least 5–6 months old.
[b] Fecal.
[c] No quantitative data.
[d] Serum.
[e] Quantitative data.
[f] Plasma.
[g] No individual data.
[h] Caecum content.
[i] Meat juice
[j] In this study pigs were 10 months old.

Carcasses were taken aside at stage of post-mortem inspection and a set of three samples was collected using disposable materials to avoid cross-contamination: approximately 100 g liver, 20 g caecum content, and cardiac blood (ranging from 5 to 20 ml). Samples were kept temperature-controlled during storage and transport (1 to 4 °C) to the laboratory of Wageningen Food Safety Research (WFSR) on the day of sampling, where the samples were stored overnight at 4 °C.

2.3. Sample processing and RNA extraction

Samples were processed after an overnight storage at 4 °C. Coagulated cardiac blood samples were clarified by centrifugation at 1500 *g* and 4 °C for 10 min and supernatants were stored at −80 °C. Prior to extraction, 10 μl (4×10^4 $TCID_{50}$) murine norovirus (MuNoV), was added to 130 μl supernatant as process control virus. MuNoV was kindly provided by Dr. H. Virgin IV, Washington University, St. Louis, Missouri. RNA was extracted using the QIAamp viral RNA mini kit (QIAGEN, the Netherlands) as described by the manufacturer. RNA was eluted in 100 μl and stored at −80 °C until downstream analyses.

Caecum content samples (3 g) were mixed 1:1 with Trypton Soy Broth (30 g per l) with 20 % glycerol and stored in −80 °C. Prior to extraction, the samples were suspended in Hanks' balanced salt solution with gentamycin (5 mg/100 ml) to a final concentration of 10 % (w/v). This mixture was clarified by centrifugation at 3000 *g* at RT for 15 min and supernatants were directly processed. For this, 10 μl MuNoV (4×10^4 $TCID_{50}$) was added to 130 μl supernatant and RNA was extracted using the QIAamp viral RNA mini kit (QIAGEN, the Netherlands) and stored as above.

Liver samples were essentially processed as described previously (Boxman et al., 2019). In brief, three pieces of 1 cm^3, taken from different locations of the liver sample, were manually chopped using a surgical blade. A subsample of 0.1 g was transferred to a 2 ml Lysing matrix S tube with stainless-beads (MP Biomedical-Bio-Connect) and 1 ml of TGBE buffer (100 mM Tris, 50 mM Glycine, 1 % (*w/v*) beef extract, pH 9.5 buffer) and 10 μl of MuNoV (4×10^4 $TCID_{50}$) were added. Mechanical disruption was performed (FastPrep 24-5G, MP Biomedical-Bio-Connect) for 40 s at the speed of 6 ms^{-1}. After clarification (10,000 *g* for 20 min at 4 °C), the aqueous intermediate layer was collected in a new 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 used for nucleic acid extraction (Nuclisens Magnetic Extraction Reagents kit, BioMérieux). Nucleic acids were eluted in 100 μl and stored at −80 °C until downstream analyses.

2.4. Detection of HEV and MuNoV RNA by RT-qPCR

Detection of HEV and MuNoV RNA was essentially done as described previously (Boxman et al., 2017). Each series of virus extractions consisted of a negative extraction control sample in between each set of three samples that was run through all stages (except mechanical disruption) of the analytical process. Water controls and positive target RNA template controls were included in each PCR run.

Viral extraction efficiency was calculated using a MuNoV RNA standard curve (ISO 15216-1:2017) (International Organization for Standardization ISO, 2017), setting the minimal recovery for each sample to be ≥1 %. Each sample was also tested for inhibition using a ssRNA HEV-standard as external amplification control (Diez-Valcarce et al., 2011) and evaluated as described in ISO 15216-2: 2019 (International Organization for Standardization ISO, 2019).

A serial dilution (10^1 to 10^5 HEV genome copies/μl) of linearized plasmid (Boxman et al., 2017) was used for quantification and run in parallel with the samples. Criteria for the standard curves were a $R^2 \geq 0.98$, a slope in between −3.1 and − 3.6, and at least values for three dilutions of MuNoV RNA and four dilutions of HEV dsDNA (ISO 15216-1: 2017). Samples were considered positive when Cq values were below 40 and amplification plots of the real time signals showed an S-curve. Extractions and RT-qPCR detections were performed at WFSR under accreditation of the Dutch Council for Accreditation. The limit of detection (LOD_{50}) for HEV RNA was determined at 6.2×10^2 IU per 130 μl supernatant caecum content, 6.2×10^1 IU per 130 μl serum and 3.8×10^2 IU/0.1 g liver prior to the study (data not shown).

2.5. Typing of HEV RNA

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 and coworkers (Smith et al., 2014) using the HEV typing tool (https://www.rivm.nl/mpf/typingtool/hev/). IQ-TREE 2 was used to calculate a Maximum Likelihood tree (Minh et al., 2020).

2.6. Serological detection of HEV antibodies

Serum samples were tested for the presence of IgG anti-HEV antibodies using the ID Screen® Hepatitis E Indirect Multi-species ELISA, (IDvet, France) validated on swine samples and based on recombinant HEV-3 capsid antigens and a multispecies conjugate. Test procedures were performed according to the manufacturer's instructions. In addition to the positive and negative control from the kit, four extra controls with OD-values ranging between 0.6 and 2.4 were added in each run.

2.7. Interpretation of ELISA results

On each plate, results for kit controls and control sera were used to correct OD-values for plate-to-plate variation by linear regression (Opsteegh et al., 2010). Next, corrected OD (ODc)-values were $\log_{10}$-transformed ($\log_{10}$ODc) and a binary mixture model was fitted to determine the mean and standard deviations of $\log_{10}$ODc for both the positive and negative component. The size of the positive component relative to the total size estimates the prevalence of infection in the study population. Censoring was applied at very low (<−1.7) and very high (> 0.4) $\log_{10}$ODc values (Swart et al., 2021) (Supplementary Fig. 1).

Bayesian statistics were employed for estimating the parameters, including the prevalence, which yields a full uncertainty characterization of the results. In this way, for each individual animal the probability to belong to the positive component (p.pos) was estimated (Swart et al., 2021). Pigs were classified ELISA positive when p.pos ≥ 0.5, corresponding to a $\log_{10}$ODc-value close to −0.35 (see Supplementary Fig. 1).

2.8. Data analyses

RT-qPCR and ELISA results were visualized in an upset-plot using ComplexHeatmap v.2.9.1 in *R* (https://www.R-project.org) and (http://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html) (Gu et al., 2016). Concordance between tests outcomes was analyzed by Pearson's χ2-analyses. According to the combined results for RT-qPCR in serum and caecum content samples and ELISA, pigs were subsequently classified as 1) seronegative pigs without HEV RNA being detected in serum and caecum content, 2) seronegative pigs with RNA being detected in serum and/or caecum content, 3) seropositive pigs with RNA being detected in serum and/or caecum content, or 4) seropositive pigs without HEV being detected in serum and caecum content. Available background data for farm location, farm type and date of slaughter for the tested pigs was evaluated as predictors for the above given classification using contingency tables and Pearson's χ2-tests. In case of an overall significant association, column proportions were compared (z-test) with Bonferroni correction (IBM SPSS Statistics 24.0). Farms sending pigs to slaughterhouse were located based on 3-digit postal code, and were classified to one of four specific provinces with the highest pig production or to 'Other' for the remaining provinces. Other investigated predictors were categorized in either two (sampling date, presence/absence of sows, animal welfare) or four (pig density and

farm size) classes each with equal numbers of seronegative pigs without RNA being detected in serum or caecum content. Cut-off values for categorization are provided with Supplementary Table 2.

3. Results

3.1. Sample description

In the period between May and December 2019, samples were collected from 250 pigs at eight slaughterhouses, with an average of 31 pigs per slaughterhouse (range of 14–52) (Supplementary Table 1). In most slaughterhouses the sampling was performed within the restriction of two pigs per day and sampled throughout the study period. At one slaughterhouse (no. 7), however, sampling started at a later date and the number of samples ranged from four to eight pigs per day in the period between mid-September and October.

Cardiac blood (5–20 ml) could be collected from 248 pigs. One blood sample was used in total for RNA extraction only and was therefore not tested serologically. Farm data was available for 246 pigs. The pigs originated from 215 farms. Twenty-two farms contributed with two, five farms with three and one farm with four pigs. When more than one pig from a farm were enrolled in the study, each pig was slaughtered at a different date.

3.2. Serological results

The presence of HEV IgG in serum was tested by ELISA for 247 pigs. Two components were visible in the histogram of the corrected $\log_{10}$OD ($\log_{10}$ODc) and normal distributions were fitted to calculate the probability of each sample to be positive (Supplementary Fig. 1). Samples were clearly identified as ELISA negative having probabilities close to 0 % or as ELISA positive having probabilities close to 100 %. HEV IgG was detected in sera from 167 pigs (67.6 %, 95 % CI: 61.8–73.5 %).

3.3. HEV RNA detection in liver, serum and caecum content samples

RNA was extracted from 250 liver, 250 caecum content and 248 cardiac blood samples and were analyzed for MuNoV RNA to determine the virus recovery rate in each sample. The average recovery rate for serum (96 %) and caecum content (113 %) samples was much higher than for liver samples (3.3 %). Twenty-two liver samples were excluded from data analysis as extraction efficiency was <1 %, also after re-extraction, and HEV RNA could not be detected. This may have led to an overestimation of the prevalence, as only samples that had not tested positive for HEV RNA were excluded. Despite a too low extraction efficiency, one liver sample was included as it tested positive for HEV RNA. None of the samples were excluded because of reduced amplification efficiency.

In total, HEV RNA was detected in 64/250 (25.6 %, 95 % CI 20.2–31.0 %) caecum content samples, in 40/248 (16.1 %, 95 % CI 11.6–20.7 %) serum samples and in 25/228 (11.0 %, 95 % CI 6.9–15.0 %) liver samples. The average level of viral contamination in positive samples was $\log_{10}$ 4.6 genome copies (gc)/g (range 3.0–8.2) in caecum content, $\log_{10}$ 3.3 gc/ml (range 2.4–5.9) in serum and $\log_{10}$ 3.2 gc/0.1 g (range 1.7–6.2) in liver samples.

3.4. Combined test results

First correlations between results for HEV IgG by ELISA and HEV RNA detection in each of the three samples were studied for the total study group. The presence of HEV IgG was positively associated with the detection of HEV RNA in caecum content only (Table 2A). The presence of HEV RNA in caecum content was positively associated with the presence of HEV RNA in serum and also positively associated with the presence of HEV RNA in liver. In addition, the presence of HEV RNA in serum was positively associated with HEV RNA in liver.

Within the subset of HEV RNA positive pigs (Table 2B), caecum content was more likely to contain HEV RNA in HEV-IgG positive ($p < 0.01$) than in seronegative pigs. Serum was more likely to test positive for HEV RNA in seronegative ($p < 0.05$) than in HEV-IgG positive pigs. For liver, no significant association was observed between results for RT-qPCR and ELISA ($p = 0.310$).

To analyze the correlations between multiple tests, pigs were selected with a complete data set of results for the four assays, 225 pigs in total. Binary results, i.e. detected or not detected, were visualized in an upset plot (Fig. 1), revealing many combinations of results. Of the pigs without HEV RNA being detected in any of the three samples ($n = 137$), 52 were seronegative and 85 were seropositive. Pigs with at least one positive RT-qPCR result ($n = 88$), were either seronegative ($n = 16$) or seropositive ($n = 72$). In total, the majority of the tested pigs 173/225 (76.9 %, 95 % CI 71.4–82.4) had been HEV exposed at slaughter age.

To further examine the viral loads in sample of HEV RNA positive pigs, quantitative results for RT-qPCR and ELISA were plotted per animal (Fig. 2) and grouped for pigs that were seronegative, seropositive or that had not been tested in ELISA. The highest RNA levels in serum, liver and caecum were detected in samples derived from pigs that tested positive in all the three matrices (SV19-ID 075, 179, 185, 208, 245). These five pigs tested also HEV IgG positive. Other pigs that tested RNA positive in all three matrices were either seronegative pigs (ID 017, 095) or pigs with lower RNA levels in serum (ID 184, 243). Remarkably, eight seropositive pigs tested RNA positive in liver samples, while no RNA was detected in serum and caecum content. This suggests that livers may harbor HEV RNA for a long time.

Table 2A
Correlation between test results for total study group.

	p-values χ^2-tests		
Test	Serum RNA	Caecum RNA	Liver RNA
IgG	0.769 (n = 247)[a]	< 0.05 **** [b] (*n* = 247)	0.588 (*n* = 225)
Caecum RNA	< 0.05 ** (*n* = 248)		< 0.05 *** (*n* = 228)
Serum RNA			< 0.05 ** (*n* = 226)

[a] Number of pigs in analysis.
[b] Significance levels are depicted as **($p < 0.005$), ***($p < 0.0005$), ****($p < 0.00005$).

Table 2B
Comparison of HEV detection in serum, liver and caecum content samples in RNA positive pigs with or without HEV IgG.

Test	HEV IgG negative (n/N, %)	HEV IgG positive (n/N, %)	overall agreement	*p*-value χ2-test
Caecum RNA	7/18 (38.9 %)	56/76 (73.7 %)	71.3 %	0.005
Serum RNA	12/18 (66.7 %)	28/76 (36.8 %)	36.2 %	0.021
Liver RNA	6/16 (37.5 %)	18/72 (25.0 %)	31.8 %	0.310

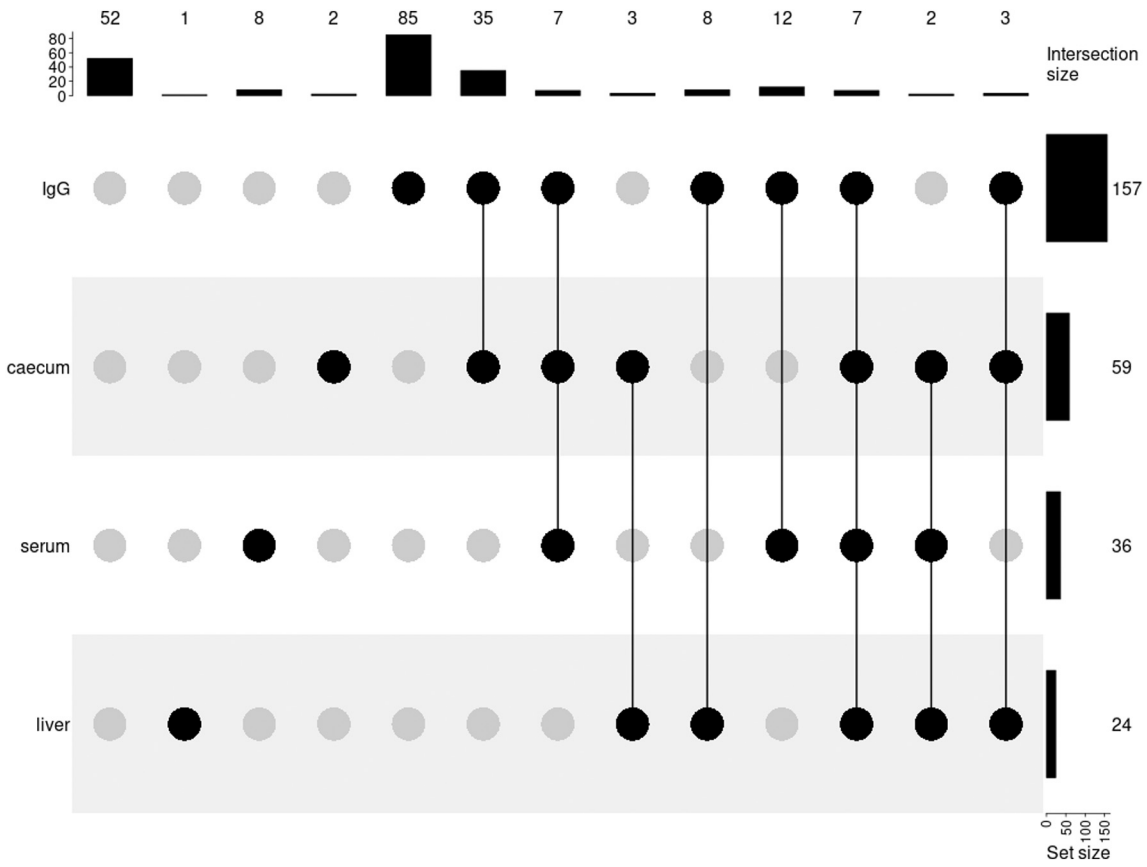

Fig. 1. Number of pigs per combination of test results for RT-qPCR and ELISA
Upset plot of pigs with complete dataset of results for ELISA and RT-qPCR on serum, caecum content, and liver (n = 225) in horizontal rows. Results are indicated in black (detected) or grey (not detected). Connected black dots indicate a set of co-occurrences, the size of each set is indicated at the top. The numbers to the right of the rows indicate the number of samples belonging to that row.

3.5. Sequence analyses

HEV RNA positive samples ($n = 126$) with Cq values ranging from 24 to 40 were subjected to nested typing PCR. Three other positive samples were no longer available for typing. A nested typing PCR product (493 nt) was obtained for 65 samples (51.6 %). Sequence analyses resulted in 61 reads, 29 out of 62 caecum content samples, 17 out of 40 serum samples and 15 out of 24 liver samples. All reads were being subtyped as HEV-3c (Fig. 3, phylogenetic tree). Analyses for the remaining four samples could not be called reliable due to a too weak signal.

First, sequences were compared within the set of samples retrieved from the same animal. In five pigs (SV19-ID 075, 179, 185, 208, 245) an identical strain was detected in the three samples. In five pigs (SV19-ID 017, 032, 095, 138, 235) an identical strain was detected in two samples. The third sample of these latter sets either did not test positive for HEV RNA (three times), was missing (once) or showed a difference of one nucleotide (once, SV19-ID 095). In one animal (SV19-ID 184), two strains with a 28 nt difference (94.3 %) and both unique in this study were retrieved, one strain from serum and the other strain from caecum content. In another animal (SV19-ID 243), a mix of two unique strains with 12 nt difference (97.6 %) was observed in a strong positive caecum content sample ($\log_{10}$ 5.1 gc/g), which could only be individually sequenced using nanopore sequencing (MinION) technology (not further described here).

Subsequently, strains obtained from 41 of the 95 RNA positive pigs were compared. Strains were different for each animal, but some strains were more similar to each other than others. To investigate possible relations between pigs, pig's sequence data was combined with retrieved pig's farm history. Most of the farms contributed to the study with one animal ($n = 187$), but 28 farms contributed with more than one. Six farms contributed each with at least two RNA positive pigs. Sequence analysis was successful for two of these pairs. One pair of pigs (SV19-ID 023 and 059) was slaughtered with a time difference of five months and shared HEV sequences with a similarity of 490/493 nt (99.4 %). Within this period of five months, two other pigs from the same farm tested RNA negative. Another pair of pigs (SV19-ID 006 and 235) from another farm were slaughtered within 28 days and shared HEV sequences with a similarity of 491/493 nt (99.6 %). A high similarity (494/495 nt) was also observed for the pigs SV19 ID 017 and 095 though these pigs originated from different farms.

3.6. Identification of determinants for HEV-exposed pigs at slaughter

Background data of the animal's finisher farm and slaughterhouse ID were combined with outcomes of the test results to identify possible determinants of HEV status at slaughter age. For this, 247 pigs with complete datasets for RT-qPCR in caecum content and serum and ELISA were classified as 1) seronegative without HEV RNA being detected in caecum content or serum, 2) RNA positive only, 3) RNA and IgG positive or 4) IgG positive only. Subsequently a contingency table was made (Supplemental Table 2). For each variable the distribution over the four groups was analyzed using Pearson's χ^2-tests (Table 3). The presence of sows and gilts, welfare rating and slaughterhouse had a significant effect ($p < 0.05$) on the distribution over the four groups representing different states of HEV infection, whereas sampling date, province, pig density and farm size had not. Pair-wise comparison showed that pigs were significantly ($p < 0.05$) more often seronegative (with or without HEV RNA being detected) when raised in the presence of sows and gilts in the

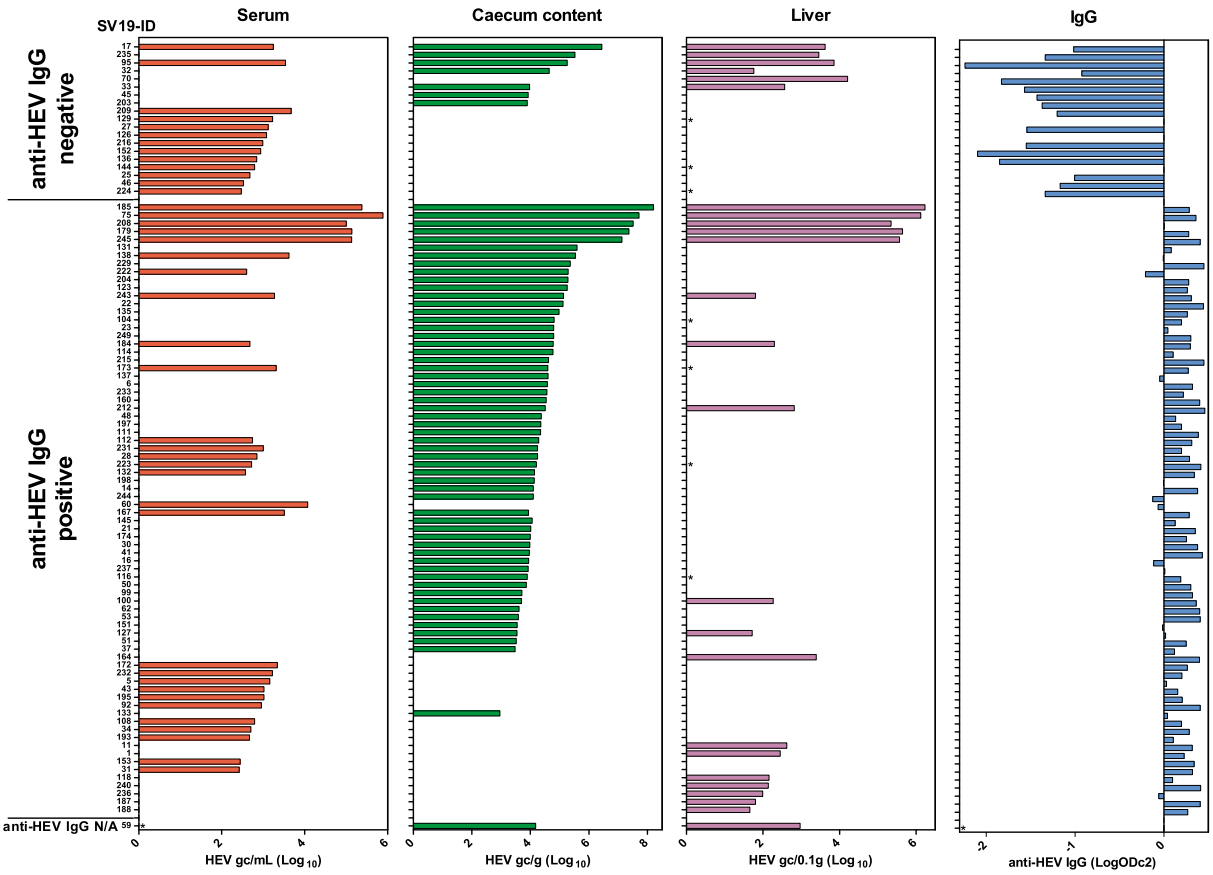

Fig. 2. Quantitative HEV RT-qPCR and HEV IgG ELISA results
Quantitative HEV RT-qPCR results for seronegative pigs that tested RNA positive ($n = 18$); seropositive pigs ($n = 76$) and an animal that was not serologically tested. Results for HEV RNA genome copies (gc) detected in serum ($\log_{10}$ copies/ml)(red), caecum ($\log_{10}$ copies/g) (green), liver ($\log_{10}$ copies per 0.1 g) (purple) and anti HEV IgG (Log_{10}ODc) (blue). IgG positive samples have a $\log_{10}$OD-value above −0.35. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

farm than those raised in the absence of sows and gilts. In addition, pigs tested significantly more frequent HEV RNA and IgG positive when raised under one-star welfare than without. Though an association between slaughterhouse and HEV status was detected, it is less biologically plausible that slaughterhouse directly affects serological response or presence of RNA in serum or caecum content in pigs. In addition, the same analysis was performed for pigs grouped as being HEV RNA positive (irrespective of outcome for ELISA), or HEV IgG positive only or as seronegative without RNA being detected in caecum content or serum (Table 3). This resulted in the same outcomes with comparable *p* values.

4. Discussion

The present study has shown that the majority of the pigs in the Netherlands has been HEV exposed at slaughter age of 5–6 months. Of the pigs entering the food chain 67 % had developed IgG against HEV, while pigs tested RNA positive in 16 % of the serum, 11 % of the liver and 26 % of the caecum content samples. This large set with prevalence data based on quantitative measurements in four assays will be useful for risk assessment for food safety of pork products, and will provide baseline data for future monitoring of HEV infections in pigs.

Sequence analyses on HEV positive samples revealed HEV-3c strains only. Also in previous studies, this subtype dominated in the Netherlands in humans and pigs (Adlhoch et al., 2016; Hogema et al., 2021), as well as in liver and pork (products) on the Dutch market (Boxman et al., 2019; Boxman et al., 2020). All 41 pigs for which a sequence was obtained had a unique sequence. Where two- or more positive samples from one animal were successfully sequenced strains were identical for 10 pigs, but in one pig the strain retrieved from serum was not identical with the one retrieved from caecum content (28 nt). In another pig, two strains were observed within one sample. It is plausible that pigs can concurrently be infected by multiple strains. Identical strains within one pig were described in other slaughterhouse studies, but for only three to four pigs (Chelli et al., 2021; Grierson et al., 2015). Strains from pigs which originated from the same farm seemed to deviate less than strains from pigs that did not share such a recent history. This is in line with a Swedish study that revealed unique farm specific HEV-3 strains that remained unchanged during the sampling period (Wang et al., 2019). Further studies are required to investigate more closely this intra- and inter-farm variability between HEV-3c strains and changes in time and in relation to e.g. the presence of sows and gilts. Despite similarity (up to 99 %) as observed earlier for porcine blood (Boxman et al., 2017), none of the obtained sequences was 100 % identical to Dutch HEV cases up to 19 December 2021 in HEVnet database (Mulder et al., 2019).

The observed prevalence for HEV IgG and RNA in caecum content and liver were as anticipated from earlier studies. The seroprevalence (67 %) in this study was nearly equal to that in pigs at Dutch slaughterhouses in 2004 and 2010 (68–72 %) (Rutjes et al., 2010; Rutjes et al., 2014). The proportion of caecum content (26 %) and liver (11 %) samples being HEV RNA positive was nearly equal to that of caecum content samples in Dutch pigs in 2009 (28 %) (Rutjes et al., 2009) and to that of livers from Dutch retail stores in 2016 (13 %) (Boxman et al., 2019). The result in the present study that really stood out was the percentage of viremic pigs (16 %), a proportion that was much higher than that reported in 2015 (Grierson et al., 2015) or which has been

Fig. 3. Phylogenetic analyses of HEV strains retrieved from pigs
Nucleotide HEV sequences of a 493 nt open reading frame 2 fragment (positions 5961–6454 of reference sequence NC_001434) retrieved from liver, caecum content and/or serum samples from pigs (n = 41) at slaughterhouses were used to produce a Maximum Likelihood tree using IQ-TREE 2, rooted at midpoint for visualization purposes.

published recently (4.4–6.7 %) (Chelli et al., 2021; Garcia et al., 2019; Sooryanarain et al., 2020) (Table 1). This is new information for risk assessment as no earlier data was available for viremia in pigs at Dutch slaughterhouses.

Viremia is believed to be detectable only during a short period after infection, while the onset of the IgG antibody response is about two weeks after infection but remains detectable onto slaughter age (de Deus et al., 2008; Thiry et al., 2017). Using combined test results for presence of HEV IgG and HEV RNA in plasma or serum, pigs at slaughter can be grouped as either non-HEV-exposed, recently exposed with or without detectable serological response or as pigs with a past HEV infection. Such grouping was applied to data of the present study, but also for three other studies reporting HEV RNA in plasma/serum and HEV IgG in 5–6 months old pigs at slaughter (Garcia et al., 2019; Grierson et al., 2015; Sooryanarain et al., 2020) (Supplemental Table 3). About a quarter (27.5 %) of the pigs in the present study was seronegative without HEV

Table 3
Comparison of the distribution over different groups of pigs according to their results in RT-qPCR (serum/caecum content) and ELISA.

		p-values of Pearson's χ2-tests[a]	
Variable	n[b]	Distribution over four groups[c]	Distribution over three groups[d]
Slaughterhouse (n = 8)	247	**0.005**	**0.015**
Sampling (two categories)	247	0.223	0.166
Province (five categories)	243	0.551	0.540
Pig density (four categories)	241	0.866	0.805
Farm size (four categories)	243	0.351	0.369
1-star welfare rating (yes/no)	234	**0.021**	**0.053**
Presence of sows and gilts (yes/no)	243	**0.000**	**0.002**

[a] *p*-values < 0.05 are printed in bold.
[b] For each variable, observations with missing data were excluded from the analysis.
[c] The four groups were 1) RNA and IgG not detected (n = 63); 2) RNA positive only (*n* = 17); 3) RNA and IgG positive (*n* = 68) and 4) IgG positive only (n = 99).
[d] The three groups were 1) RNA and IgG not detected (*n* = 63); 2) RNA positive (*n* = 85) irrespective serological result and 3) IgG positive only (*n* = 99).

RNA being detected in serum. This percentage was comparable to pigs slaughtered in Spain (24.4 %), but much lower than in 5–6 months old pigs in the United Kingdom (45.3 %) and the USA (56.7 %). The proportion of viremic pigs without detectable antibody response in the present study (4.9 %) was higher than in the other studies (1.1–3.4 %), as was the proportion of viremic pigs with antibody response (11.3 %) in comparison to the other studies (2.9–4.6 %). The group of pigs with a past infection in the present study (56.3 %) was lower than the study in Spain (68.9 %), but higher than in the USA (37.0 %) and the United Kingdom (49.0 %). Many factors may have contributed to these apparent geographical differences. Up to now it is not known what induced the high proportion of viremia in pigs in Dutch slaughterhouses. Farming conditions have been reported to affect the exposure of pigs to the virus, and thus possible infection. Using the animal's finisher farm history data an initial predictor analysis was done, despite the study was set up as a prevalence study. Based on test results for IgG and HEV RNA in serum and caecum content (Table 3, Supplemental Table 1), the distribution of pigs over the four HEV infection groups was not significantly affected by sampling date, province, regional pig density and farm size. The presence of sows and gilts at the farm of origin was, however, significantly associated ($p < 0.05$) with seronegative pigs (with or without HEV RNA being detected in caecum content or serum). This may be indicative for a delayed onset of HEV infection as compared to pigs raised in the absence of sows. Sows at the farm can be seen as an indication of on-farm breeding of piglets. These farms breed their own finisher pigs and have no or less introduction of pigs (only breeding gilts) onto the farm, possibly reducing the risk of introduction of HEV virus. Farming under 1-star welfare conditions was also found to affect the distribution of pigs over the four HEV infection groups. Pigs raised under 1-star welfare were significantly ($p < 0.05$) more frequent positive for the combination of HEV RNA and IgG than pigs raised at conventional farms. 1-Star welfare rating requires housing at a lower pig density, more provision of environmental enrichment including some roughage, and omission of castration and is, notably, not comparable to free range or organic production (3-star), which has previously been identified as a risk factor in studies from multiple countries (Jori et al., 2016; Lopez-Lopez et al., 2018; Rutjes et al., 2014). Additional information on the within-herd variation is required for confirmation on the effect of sows and welfare on HEV status, e.g. with an on-farm study.

Pork meat itself has not often been tested positive (Boxman et al., 2019; Feurer et al., 2018; Wilhelm et al., 2014), though (raw) pork sausages have been found positive (Boxman et al., 2020; Martin-Latil et al., 2014; Said et al., 2017; Szabo et al., 2015) as e.g. liver tissue is (unintentionally) present. HEV infection is mainly linked to consumption of raw or under-cooked virus-contaminated liver and processed meat products. Blood from viremic animals could be a potential source of HEV infection if used in food products that are insufficiently cooked (EFSA BioHAZ panel, 2017). Pigs with high virus load in caecum or feces may be a risk for cross-contamination of raw meat when hygienic practices are not in place. In the present study, the viral loads measured varied from not detectable up to $\log_{10}$ 6.2 HEV gc/0.1 g in liver samples, up to $\log_{10}$ 5.9 HEV gc/ml in serum and $\log_{10}$ 8.2 gc/g in caecum contents. Pigs with the highest viremic levels were also the pigs with the highest contamination of liver and caecum content. Moreover, HEV RNA in serum content was positively associated with the presence of HEV RNA in liver, $p < 0.005$. (Severe) viremia, if detected on-site, may therefore serve as a direct indicator for an animal with a (highly) contaminated liver and caecum-intestinal content. Further studies are needed to determine the feasibility and cost-effectiveness of identifying viremic pigs in order to process them separately from non-viremic pigs, undertake even more precautions to prevent cross-contamination of meat and assure that liver, meat, and blood do not enter the food chain unless sufficiently heated. Further studies are also required to investigate variability of viremia between and within herds, which is unclear from the present sampling strategy.

The common use of taking only a small amount of liver into analyses, which is a very small fraction of the total liver, in combination with the relatively low extraction yield, may have led to false-negatives despite meeting quality assurance parameters. This warrants development of a method that takes more liver tissue into analyses and results in a higher virus extraction recovery. To prevent such bias, all results for liver were excluded when comparing HEV status based on farm characteristics.

No international standard for detection of HEV RNA in food is yet available, nor is evidence available whether infectious virus is being detected, as PCR results do not discriminate between RNA from infectious and non-infectious particles. Still the occurrence of HEV in pigs and food is associated with a risk for public health. Vulnerable persons are being advised to avoid identified or suspected high risk products (Voedingscentrum, 2021). Moreover, a costly screening of donor blood products for the presence of HEV RNA has been put in place as 1 out of 762 of Dutch blood donations for production of solvent/detergent (S/D)-treated plasma in 2013–2014 carried HEV (Gezondheidsraad, 2018).

In conclusion, this study has provided insight in the prevalence of (past) HEV exposure and status of HEV infection in pigs at slaughter age in the Netherlands. The observed viremia in 16 % of the tested pigs was higher than in other reported studies and was interestingly often observed in combination with high viral loads in liver and caecum content. Furthermore, sows at the farm of origin were associated with higher fraction of seronegative pigs at slaughter. Data provided will be useful for risk assessment for food safety of pork products, and will provide baseline data for future monitoring of HEV infections in pigs and new thoughts for mitigation strategies.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

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