

## Hepatitis E virus RNA in commercially available porcine livers in The Netherlands

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### Abstract

Hepatitis E virus (HEV) infections caused by genotype 3 are increasingly observed in industrialized countries, without a distinct source. High similarity between human and swine strains of HEV strongly suggest possible zoonotic transmission. It was reported previously that in 55% of Dutch pig farms HEV-excreting fattening pigs were present. In the current study, presence of HEV RNA in commercially available porcine livers was shown. We examined 62 commercially available porcine livers for HEV contamination. Before examination of livers, the most sensitive combination of tissue disruption and RNA-extraction was chosen from four disruption and seven RNA-extraction methods. Four of 62 livers were shown to be positive for HEV RNA by RT-PCR and Southern blot hybridization, and three sequences were obtained. Phylogenetic analysis showed clustering of the sequences with previously published Dutch HEV genotype 3 sequences from humans and swine. To study infectivity of possible virus, three pigs were intravenously inoculated with suspensions from commercially available HEV positive livers. Two other pigs served as high-dose or low-dose controls. The low-dose control received a comparable viral count as animals receiving inocula from commercially available livers, the high dose control received a viral count that was known to generate infection. Faecal shedding of HEV was observed in the high-dose control, indicating that the control virus was infectious. No faecal shedding of HEV was observed for the low-dose control and the three pigs that were administered the commercially available livers extracts. In conclusion, HEV RNA was found in commercially available porcine livers. Inoculation of susceptible pigs with extracts from HEV-positive livers did not lead to infection, but this may be a dose-dependent effect. The risk for consumers should be investigated further.

### Introduction

Hepatitis E virus (HEV) is an enterically transmitted RNA virus that causes liver inflammation in humans and belongs to the family *hepeviridae* (1). Hepatitis E virus is currently classified into four genotypes (named 1 to 4) (2). Genotype 1 and genotype 2 strains circulate in developing countries and are a major cause of outbreaks and sporadic cases of hepatitis E (3). Genotype 3 and genotype 4 strains circulate predominantly in Western countries and cause sporadic cases of locally acquired hepatitis E, which are increasingly observed in industrialized countries (4, 5). The source of HEV in these locally acquired cases is mostly unknown, but a possible role for swine has been suggested, based on high prevalence of HEV among pigs and high similarity between porcine and human HEV sequences from the same geographical region (6). One route that may lead to zoonotic transmission involves food. Foodborne transmission from wild boar and deer to humans has been observed in Japan (7, 8). Furthermore, Japanese studies suggested the possibility of HEV-transmission through contaminated un(der)cooked porcine liver or intestines (9). In the Netherlands, hepatitis E virus RNA was detected on 55% of 97 finishing pig farms in 2005 (10). This finding raises concern on possibility of foodborne transmission. The objective of this study was to quantify presence of HEV in commercially available porcine livers. Detected HEV RNA was sequenced and infectivity of possible virus present in livers was examined by experimental inoculation of pigs. Prior to detection of HEV in livers, four methods for tissue

disruption and seven for RNA extraction were compared on RNA yield. The method yielding most RNA was used for screening of commercially available porcine livers.

## Materials and Methods

In total, 62 livers were obtained from different butcher shops ( $n=56$ ) and retail stores ( $n=6$ ). The positive control liver was obtained from a pig after experimental infection. The negative control liver was purchased from a local butcher shop.

A standard method for mechanical disruption of tissues is extensive vibration in the presence of small beads. We examined the destructive capability of beads of 1 mm and 2 mm in diameter, vibrating twice at 4 m/s for 40 seconds in the presence or absence of proteinase K (0.35 mg/ml lysis buffer) for additional enzymatic disruption. The method with highest RNA yield in PCR detectable units (PDU) per gram was selected. Estimates of PDU/g were obtained as described (11).

For RNA extraction, seven methods were compared (Table 1). Method 1 was an in-house method based on Boom et al (12), all others were commercially available. The comparison study consisted of three parts. In part one, 150 mg of liver tissue was analyzed with all methods except Method 2, and the method with highest PDU/g was selected. Method 2 was not included, because of a maximum loading capacity of 50 mg. In part two, amount of liver tissue as input (50 mg, 150 mg or 250 mg) was examined for the best method from part one, and for Method 2 (50 mg) and Method 3 (250 mg). Method 3 was included because of a maximum loading capacity of 250 mg. In part three, the effect of a second elution of RNA compared to one elution was examined for Methods 1 and 2. To account for the varying volumes of elution buffer, samples were precipitated using ethanol and RNA was subsequently dissolved in 35  $\mu$ l elution buffer. Subsequently, the method with highest estimated PDU/g was selected for analysis of commercially available livers.

Detection of HEV RNA was done with RT-PCR and Southern blot hybridization of RT-PCR products (11). For comparison of methods for tissue disruption and RNA extraction, RNA was diluted serially in sterile, RNase-free water in 10-fold for part one of the comparison and in 5-fold for parts two and three of the comparison. For HEV detection in commercially available porcine livers, two serial 10-fold dilutions were included. Positive controls were included during extraction and RT-PCR, blanks were included after each dilution series in RT-PCR, and an internal RNA control was added at reverse transcription to examine inhibition (10). The HEV positive RT-PCR products were cloned for sequencing.

To examine infectivity of possible viral particles, three domestic pigs were intravenously inoculated with 3 ml – 4.5 ml of a liver suspension made from commercially available livers. The pigs had been used as untreated controls in another experiment and were about 7-8 weeks old. Prior to inoculation, faeces and sera were collected to examine HEV RNA or anti-HEV antibodies, respectively. One pig was inoculated with 3 ml of a low-dose control inoculum, and one pig with 2 ml of a high-dose control inoculum. The low-dose control contained a viral count equivalent to the inocula made from commercially available livers, as determined by endpoint dilution in RT-PCR. Faecal samples were taken on 0, 3, 7, 10, 14 and 16 dpi. Pigs were sacrificed at 21 dpi and liver, bile and faecal samples were collected. Hepatitis E virus RNA was extracted from a 10% faecal suspension and from undiluted bile using the QIAamp Viral RNA Mini Kit. Liver samples were subjected to the optimized protocol described in this paper.

## Results

For beads of 1 mm and 2 mm in diameter, similar estimates for PDU/g of liver were obtained. Addition of proteinase K increased variation between duplicates and did not increase estimates of PDU/g compared to absence of proteinase K. Subsequently, liver tissue was disrupted with 1 mm beads without addition of proteinase K. Estimates of PDU/g for all RNA extraction methods are displayed in Table 1. In part one of the comparison, Method 1 and Method 6 gave highest estimates of PDU/g for 150 mg of liver as input. For Method 6, however, more aspecific RT-PCR-products were observed in undiluted samples and therefore Method 1 was selected for subsequent parts of the comparison. In part two, methods 1, 2 and 3 were further examined on input of liver. A higher input generated higher estimates of PDU/g for Method 1 and Method 3, without an increase of inhibition in RT-PCR. Method 2 was less sensitive than the other two methods. In part three, a second elution followed by ethanol precipitation was compared to a single elution step for Method

Table 1. Estimated RNA yield in PCR detectable units per g of liver tissue (on a log-scale) for different RNA extraction methods, with various amount of porcine liver as input.

Method Description	Reference	Input of liver tissue		
		150 mg	50 mg	250 mg
In house silica	Method 1	8.0	6.4	8.5
RNeasy <sup>®</sup> Mini kit	Method 2	nd	7.8	nd
RNeasy <sup>®</sup> Midi kit <sup>‡</sup>	Method 3	7.0	nd	8.5
NucliSens <sup>®</sup> Isolation	Method 4	7.0	nd	nd
NucliSens <sup>®</sup> Magnetic Extraction	Method 5	7.0	nd	nd
Trizol <sup>™</sup>	Method 6	8.0	nd	nd
Combination of Method 6 and Method 1	Method 7	6.0	nd	nd

nd: not determined; <sup>‡</sup> A second elution step was done always, followed by ethanol precipitation

1 and Method 2. A second elution step decreased estimated PDU/g for Method 1 with 0.7 log PDU/g for 150 mg and 250 mg of liver, and for Method 4 with 0.6 log PDU/g for 50 mg of liver.

Conclusively, Method 1 was used as optimized protocol in subsequent analyses of commercially available porcine liver, with an input of 250 mg of liver and a single elution of RNA.

Hepatitis E virus RNA was detected in four commercially available porcine livers with RT-PCR and Southern blot hybridization of the RT-PCR products, giving a prevalence estimate of 6.5% (95% exact confidence interval (CI): 1.8% – 15.7%). HEV RNA was detected in the undiluted samples only, yielding an estimated viral load of approximately 65 PDU/g of liver (95% CI: 3 – 580 PDU/g liver).

Sequences from three of four RT-PCR products were obtained and all three sequences clustered within different subgroups of genotype 3. Two of three sequences showed highest similarity to published Dutch swine sequences (94% and 97%), the other to a published UK swine sequence (92%). Comparison of sequences from liver with sequences from locally acquired hepatitis E cases in The Netherlands showed at most 93% similarity.

Pre-inoculation samples of all five pigs were free of anti-HEV antibodies in serum and free of HEV RNA in faeces. Experimental inoculation of pigs resulted in viral excretion by only the high-dose control from 7 dpi up to at least 16 dpi, but not on 21 dpi. No HEV RNA was observed in any of the liver and bile samples collected at 21 dpi.

## Discussion

Hepatitis E virus RNA was present in four of 62 commercially available porcine livers in The Netherlands and three sequences were obtained. In Japan, 7 of 362 (1.9%) pig livers were shown to contain HEV RNA, with six of seven sequences classified as genotype 3 (13). The three viral strains identified in the current study were also classified as genotype 3, which is similar to strains causing locally acquired hepatitis E in humans in The Netherlands (6).

Experimental infection of pigs with an inoculum of commercially available porcine livers did not result in faecal excretion of HEV and a possible explanation is that HEV RNA originated from defective viral particles. Another hypothesis, however, might be that the administered dose was too low to cause infection. Absence of infection was also observed in the low-dose control pig, while this inoculum was a dilution of the infectious inoculum given to the high-dose control pig. Molecular examination of all inocula prior to administration showed presence of viral RNA only in the high-dose control inoculum. HEV RNA was likely to be present in the other inocula, but inside (aggregations of) hepatocytes, because a lysisbuffer was absent during liver disruption. This interferes with a homogenous distribution of virus in the inoculum and hence decreases the detection probability of HEV. In addition, only a small volume of inoculum was examined, further decreasing the detection probability. A dose dependency for HEV in pigs was demonstrated by Meng et al. (14), who observed no infection in pigs after dilution of an infectious HEV-pool. This observation favors the hypothesis that the dose administered to pigs in the current study was too low to establish infection.

Data from a national food consumption survey in 1997 and 1998 indicated that raw porcine liver is handled by consumers on roughly 900,000 occasions annually, but no data on condition of the livers at consumption were available. Effects of preparation methods on viral infectivity likely exist and will influence a possible foodborne risk. For instance, thermal stability of genotype 1 strains of HEV has been examined and the majority of HEV was inactivated at 60°C, although ~1% of the viral particles were still able to infect cells (15). If these results apply to genotype 3 strains,

improper heating of porcine livers may not inactivate all possible viral particles and consumption of undercooked porcine liver may result in ingestion of infectious viral particles.

In conclusion, HEV RNA has been observed in commercially available porcine livers in The Netherlands. Observed sequences belonged to genotype 3, which is the genotype that is associated with locally acquired hepatitis E. A possible dose-dependent relationship for HEV in swine was observed, because the high-dose control inoculum only led to infection in a pig. The risk of foodborne HEV transmission is currently unknown and will be dependent on factors such as infectivity of HEV RNA, method of preparation of liver for consumption, and amount consumed.

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