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RESEARCH ARTICLE

Hepatitis E virus cross-contamination on the surface of porcine livers after storage in Euro meat containers in a German pig abattoir

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Abstract

Hepatitis E virus (HEV) is a foodborne zoonotic pathogen and known as the causative agent of hepatitis E in humans. The specific role of porcine liver as a vehicle for human HEV infections has been highlighted in different studies. Nevertheless, gaps of knowledge still exist regarding possible HEV cross-contamination both at consumer and production level. Furthermore, people working in the food production industry, e.g. veterinarians and abattoir employees, are exposed to an increased risk of HEV infection. The aim of the present study was to investigate HEV cross-contamination on the surface of porcine liver in a German abattoir. The sample set included 250 samples of porcine liver parenchyma and the corresponding 250 superficial layer samples of the same livers, which were analyzed for the presence of HEV ribonucleic acid (RNA). Afterwards, the initial status of the tested liver parenchyma was compared with the occurrence of HEV RNA in the corresponding superficial layer. HEV RNA was detectable in 34% (85/250) of superficial layer samples, with 58% (49/85) of the samples originated from initially HEV negative livers. To our knowledge, this is the first study that provides an insight in the potential of HEV cross-contamination at abattoir level in Germany. Furthermore, it could be identified that the joint storage of livers in Euro meat containers has a significant impact on the presence of HEV RNA on the surface of porcine liver.

Keywords Foodborne pathogens · Porcine liverr · Cross contamination · Hepatitis E virus · Pig abattoir · Euro meat container

1 Introduction

Hepatitis E virus (HEV) is known as the causative agent of hepatitis E in humans and is mainly responsible for viral hepatitis worldwide transmitted via the orofecal route (Lapa et al. 2015).

In 2015, there were 21,000 human clinical cases of HEV infections reported in the European Union and the European Economic Area (European Centre for Disease Prevention and Control 2017). In Germany, a dramatic increase from 102 reported clinical cases in 2009 to 3728 in 2019 has been observed in human patients (Robert Koch-Institut 2010, 2021).

As a RNA virus, HEV is classified as *Orthohepevirus A* in the family of *Hepeviridae* (Smith et al. 2014). HEV genotypes 1 to 4 (HEV-1 to HEV-4) can infect humans, with HEV-3 and HEV-4 common in both humans and animals, especially in domestic pigs and wild boars, but also in other mammals (Meng et al. 1997; Johne et al. 2014; Smith et al. 2014; Pavio et al. 2015). Focusing on domestic pigs, the consumption of raw or undercooked pork liver, respectively liver products has been recognized as a main route of zoonotic transmission (Feagins et al. 2007; Colson et al. 2010; Boxman et al. 2019).

In Europe, the HEV seroprevalence in pigs amounts up to 96% at single animal basis and up to 97% at herd level, highlighting that HEV circulates in European pig populations (Wutz et al. 2013; Burri et al. 2014; Caruso et al. 2017; Feurer et al. 2018; Dzierzon et al. 2020). HEV infection in pigs occurs usually without clinical symptoms, so there is no possibility to detect HEV infected pigs macroscopically

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during routine ante- and post-mortem inspection at the abattoir (Meng et al. 1997; van der Poel et al. 2001).

HEV RNA detection in different organs and matrices of pigs during slaughter as well as in abattoir's effluents and on different surfaces along the slaughter line indicates that HEV circulates in pigs and in the abattoir's environment (Fenaux et al. 2018; Feurer et al. 2018; Garcia et al. 2019; Milojević et al. 2019; Dzierzon et al. 2020). It is considered that temperature is the most effective option for inactivation of HEV in food and in the environment (EFSA 2017). However, a time–temperature combination for a safe HEV inactivation could not been determined so far, even though virus inactivity appears to decrease rapidly with temperatures above 65 °C (Schielke et al. 2011; Johne et al. 2016).

Various foods of animal origin have been identified as a source for HEV worldwide (Ferri and Vergara 2021). Focusing on pork, the consumption of products made from raw pork liver is very common in different European countries, where analyses of raw pork products yielded positive results for HEV RNA underlining the specific role of raw pork liver as a vehicle for potential HEV infections in humans (Colson et al. 2010; Pavio et al. 2014; Kubacki et al. 2017; Giannini et al. 2018).

The objective of the present study was to investigate HEV cross-contamination on the surface of porcine liver in a German abattoir. Therefore, the surface of porcine liver was analyzed for HEV RNA after joint storage of livers with different initial HEV status in Euro meat containers.

Additionally, data from our previous publication (Dzierzon et al. 2020) were used to evaluate whether a higher concentration of antibodies in meat juice is associated with the occurrence of HEV RNA in the liver of slaughter pigs.

2 Materials and methods

Liver samples were collected from 250 slaughter pigs at an abattoir in North-West Germany between August and December 2018. Pigs originated from 25 German conventional fattening farms, of which ten slaughter pigs were randomly selected at the abattoir. The HEV seroprevalence and the prevalence of HEV-RNA in the liver of those pigs was previously determined and published (Dzierzon et al. 2020). Therefore, the HEV detection rate of 250 livers was available and considered as the initial HEV status of each liver for the present study.

2.1 Sample collection and processing

The sampling procedure along the slaughter line has been described previously (Dzierzon et al. 2020). In addition, each carcass and liver was numbered before sampling, in order to assign each sample to the pig of origin. For that,

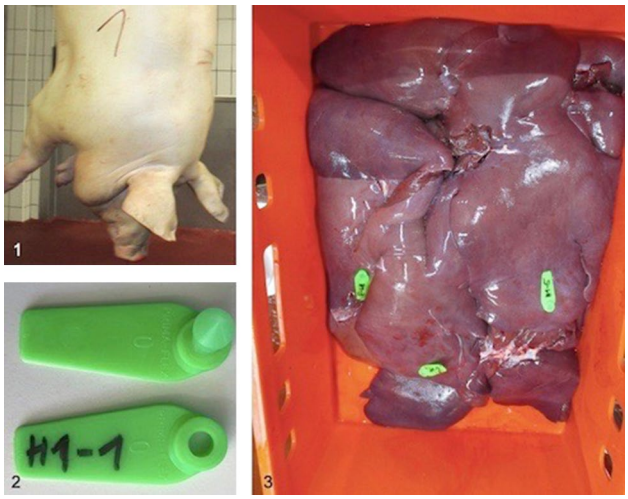

Fig. 1 After tagging every slaughter pig individually with a number (1–10) (1), corresponding livers were labeled using a sterile ear tag (2) and were stored in Euro meat containers (3)

each carcass was tagged with a consecutive number by using a meat marking pencil (Faber-Castell, Stein, Germany). The liver of a numbered pig was collected after the official meat inspection and labeled with a sterile ear tag carrying the corresponding number of the pig carcass (Fig. 1). All 250 collected livers were temporarily stored in a total of 50 standard Euro meat containers, according to the abattoir's procedures for storing livers until further processing or marketing (Fig. 1). In every meat container, ten livers were put together from the same herd but only the five livers numbered were finally selected for sampling.

Samples of liver parenchyma had been excised aseptically and contamination-free as described in Dzierzon et al. (2020). The corresponding superficial layer samples were collected from the *margo acutus hepatis* of each liver by using sterile forceps and Cutfix® single-use scalpels (B. Braun, Melsungen, Germany).

Each sample was stored separately in a sterile Whirl–Pak® plastic bag (Nasco Sampling/Whirl-Pak®, Madison, WI, USA). Afterwards, samples were transported at − 2 °C to the laboratory and were stored at − 80 °C until laboratory examination.

Analysis of the superficial layer samples of porcine livers was focused on the serous membrane and the Glisson's capsule in order to investigate HEV cross-contamination.

2.2 Molecular investigation

Superficial layer samples were analyzed at the same time using the same laboratory methods and real-time RT-PCR protocol as described previously for the liver parenchyma samples (Dzierzon et al. 2020), ensuring comparability of the results. As previously published, the HEV prevalence

in liver parenchyma has been analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR) (Dzierzon et al. 2020).

Laboratory methods used for both types of liver samples are described again in the following.

To prepare both types of liver samples for RNA extraction, the tissues were thawed before taking 30 mg of each sample aseptically for disruption and homogenization. Sterile steel beads of 5 mm in diameter (QIAGEN®, Hilden, Germany) were used for disruption of the tissue, diluted in 600 µl of lysis buffer with beta-mercaptoethanol (β-ME) in a 2 ml SafeLock tube (QIAGEN®, Hilden, Germany). The disruption of sample material was performed by the TissueLyser (QIAGEN®, Hilden, Germany) followed by homogenization via centrifugation. Viral RNA was extracted from 600 µl of the supernatant by use of the RNeasy® Mini Kit (QIAGEN®, Hilden, Germany) on the QIAcube (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions *RNeasy Mini, Animal tissues and cells, Large samples* (QIAGEN® 2010). For quality control of the RNA extraction step, one tube of lysis buffer with β-ME was included in each cycle as a negative extraction control.

Bacteriophage MS2 (5×10^7 PFU/ml) was used as an internal process control virus monitoring the presence of possible inhibitory substances causing false negative results during the RNA extraction and molecular detection step. With exception to the negative extraction control, bacteriophage MS2 was added to each sample after homogenization and before starting the extraction step.

A one-step real-time RT-PCR assay was performed according to Jothikumar et al. (2006) using the QuantiTect™Probe RT-PCR Kit (QIAGEN, Hilden, Germany). A tenfold-dilution of porcine HEV RNA, genotype 3, was used as the positive control and RNase free water was used as the negative control. Both controls were included in each real-time RT-PCR run. A separate real-time RT-PCR, as conducted previously by Althof et al. (2019), was performed to detect bacteriophage MS2 RNA by using different primers and probe according to Dreier et al. (2005).

2.3 Statistical analysis

The obtained data were recorded in Microsoft® Excel® 2016 together with previously acquired ELISA test results and PCR results for liver parenchyma from the same pigs (Dzierzon et al. 2020). All statistical calculations were performed using the software IBM® SPSS® Statistics, version 25 (SPSS Inc., Chicago, IL, USA). Descriptive statistical analysis was carried out to determine the percentages and associated 95% confidence intervals (95% CI).

HEV cross-contamination was analyzed by comparing the initial infectious status of the tested liver parenchyma (Dzierzon et al. 2020) with the occurrence of HEV RNA on the surface of the same liver. After combining PCR results of liver parenchyma (Dzierzon et al. 2020) and PCR results of corresponding superficial layer samples in a 2×2 contingency table, the Cohen's kappa (κ) value was calculated to assess the reliability.

A mixed effects logistic regression model was used to investigate the potential of joint storage of livers in Euro meat containers for HEV cross-contamination. The infectious status of the liver surface was the dependent variable, the infectious status of the liver parenchyma was the fixed factor and the Euro meat container was used as the random factor. Additionally, the odds ratio (OR) and its 95% CI were estimated with a p-value of < 0.05 considered to be statistically significant, quantifying the strength of the association between the occurrence of HEV RNA in porcine liver and the occurrence of HEV RNA on the corresponding surface.

In addition, the previously determined ELISA and PCR results (Dzierzon et al. 2020) were included in a binary logistic regression model to evaluate the association between ELISA OD (optical density) values and the presence of HEV RNA in the liver of slaughter pigs. OR and its 95% CI were calculated with a p-value of < 0.05 considered to be statistically significant.

3 Results

HEV RNA was detected in 34.0% (85/250; 95% CI 28.1–39.9%) of the 250 examined superficial porcine liver samples. The distribution of those HEV positive samples varied regarding the serological status and the presence of HEV RNA in the corresponding liver parenchyma sample (Table 1). Out of the HEV positive superficial liver samples, 57.6% (49/85; 95% CI 47.1–68.1%) originating from livers

Table 1 Distribution of HEV ELISA[a] and real-time RT-PCR results

No. of pigs (%)	HEV ELISA[b] Meat juice[a]	HEV RT-PCR[c]	
		Liver parenchyma[a]	Superficial layer
86 (34.4%)	–	–	–
72 (28.8%)	+	–	–
36 (14.4%)	+	+	+
40 (16.0%)	+	–	+
9 (3.6%)	–	–	+
7 (2.8%)	+	+	–
Total no. of positive samples	**155** (62.0%)[a]	**43** (17.2%)[a]	**85** (34.0%)

[a]Dzierzon et al. (2020)

[b]Detection of HEV antibodies

[c]Detection of HEV RNA

initially evaluated as HEV negative, of which 18.4% (9/49; 95% CI 7.5–29.2%) originated from HEV seronegative pigs.

As previously described, 43 of 250 porcine livers were identified as HEV positive, resulting in a prevalence of 17.2% (95% CI 12.5–21.9%) (Dzierzon et al. 2020). The present study shows the molecular analysis of HEV RNA in the corresponding superficial layer samples in 83.7% (36/43; 95% CI 72.7–94.8%). With a κ-value of 0.43 ($p < 0.05$), the results reveal a moderate concordance for HEV positive porcine liver combined with HEV positive liver surface.

The detection rate of HEV RNA on the surface of initially HEV negative porcine livers was 23.8% (49/207; 95% CI 17.9–29.5%). Initially, HEV negative livers derived from each of the 25 pig herds tested. In eleven herds, none of the involved slaughter pigs had detectable HEV RNA in the liver, representing a prevalence of 0.0% (0/10, 95% CI 0.0–3.8%) (Dzierzon et al. 2020). In 45.5% (5/11; 95% CI 16.0–74.9%) of those HEV negative herds, HEV RNA was detected on the surface of at least one liver. Related to Euro meat containers, 18.0% (9/50; 95% CI 7.4–28.7%) included five initially HEV negative livers but with at least one HEV positive liver surface (Table 2).

Altogether, 79.6% (39/49; 95% CI 68.3–90.9%) of initially HEV negative livers with detectable HEV RNA on the surface originated from ten HEV positive pig herds (40.0%; 10/25; 95% CI 20.8–59.2%) with a HEV prevalence in the livers ranging from 10.0% (1/10, 95% CI 0.0–28.6%) to 70.0% (7/10, 95% CI 41.6–98.4%). All livers had been stored until sampling in 17 Euro meat containers in total (34.0%; 17/50; 95% CI 20.9–47.1%) containing at least one initially HEV positive liver (Table 2).

Mixed logistic regression analysis showed a large association between the occurrence of HEV RNA on liver surfaces and in the liver parenchyma (OR of 11.269; 95% CI 4.024–31.562; $p < 0.001$). Additionally, 64% of the variance was due to variance between the Euro meat containers. In addition, Fig. 2 shows the distribution of HEV negative and positive porcine livers and the corresponding OD values of HEV ELISA of the sampled pigs (Dzierzon et al. 2020).

The percentage of HEV positive livers was higher with high ELISA OD values. The estimated OR of 1.016 (95% CI 1.011–1.021) demonstrated that the probability of detecting HEV RNA in porcine liver increased significantly with an increasing OD value by just one unit ($p < 0.001$).

Table 2 Distribution of porcine livers among Euro meat containers related to HEV status

Euro meat containers (n = 50) No. (%)	HEV status of livers (n = 5) in Euro meat containers	
	No. of initially HEV RNA positive livers	No. of HEV RNA positive liver surfaces
19 (38.0)	0	0
17 (34.0)	≥ 1	≥ 2
9 (18.0)	0	≥ 1
4 (8.0)	1[a]	1[a]
1 (2.0)	1	0

[a]Identical liver

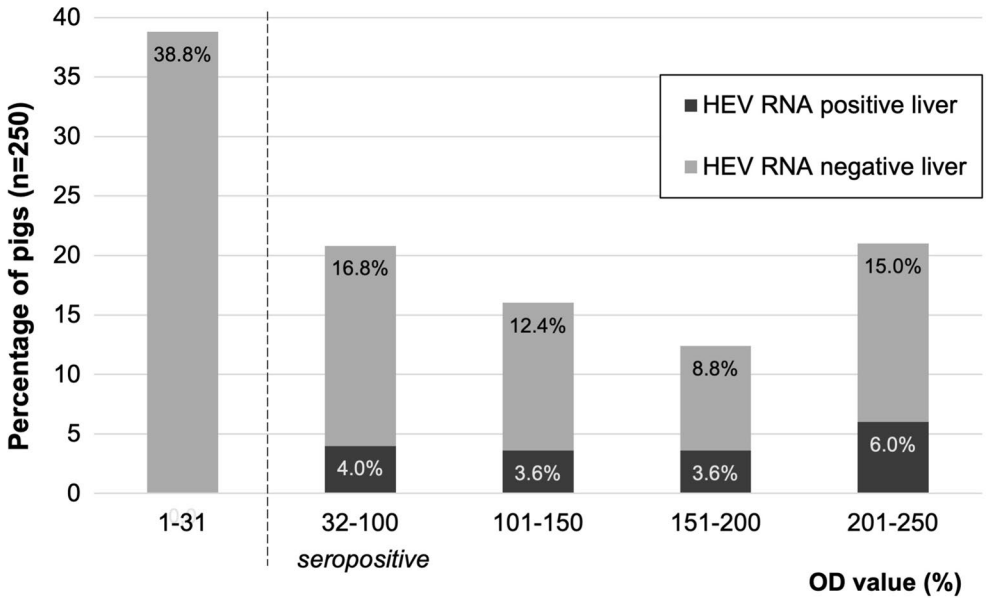

Fig. 2 Distribution of HEV RNA negative and positive porcine livers and the corresponding OD values, measured by HEV ELISA, of the sampled pigs

4 Discussion

To our knowledge, this is the first study analyzing the possible occurrence of HEV cross-contamination at the surface of porcine liver in a German abattoir. Remarkably, this study shows that storing several porcine livers in one Euro meat container at the abattoir can result in a HEV positive liver surface, even when the endogenous HEV status of the liver is negative.

The fact that an initially HEV negative liver had detectable HEV RNA on the surface at the time after storing in a Euro meat container implies that HEV had been transferred to the liver surface during the slaughter and joint storing process. In this context, it must be considered that the entire sampling was performed on days of slaughter with up to 5500 slaughtered pigs per day, when HEV negative and HEV positive herds were slaughtered.

Moreover, 79.6% of initially HEV negative livers with detectable HEV RNA on the surface originated from HEV positive pig herds with a prevalence ranging from 10 to 70%. All livers had been stored until sampling in Euro meat containers with at least one initially HEV positive liver. Mixed logistic regression analysis revealed that the percentage of positive liver surfaces differed largely between the containers, even when the HEV status of liver parenchyma was taken into account. This supports our assumption of cross-contamination during joint storage of HEV positive and negative porcine livers in Euro meat containers.

Bouwknegt et al. (2009) suggested blood of viraemic pigs to be the most likely source of HEV cross-contamination. In our opinion, porcine blood (Boxman et al. 2017), respectively porcine livers comprising blood may possibly contribute to the dissemination of HEV in the food chain.

In this study, the detection of HEV RNA on the surface of initially HEV positive porcine livers (42.4%) and the strong association between HEV positive livers and the occurrence of HEV RNA on liver surface is not surprising, if one considers blood of porcine livers as a source of HEV cross-contamination. Therefore, HEV infected slaughter pigs, harboring HEV RNA in the liver, seem to have a major role in the transmission and cross-contamination of HEV in the abattoir and finally to pig carcasses and organs.

Conversely, seronegative pigs could be a neglected risk regarding foodborne infections in humans (Dzierzon et al. 2020) and cross-contamination in the pork production chain. Therefore, in order to reduce HEV cross-contamination during slaughter and consequently human cases, we recommend logistic slaughter as an intervention measure at slaughter level with an early and separate slaughter of serologically HEV negative herds.

As proven in our previous study (Dzierzon et al. 2020), serological tests for the detection of HEV antibodies in pigs shortly before slaughter have a reliable predictive power on the occurrence of HEV RNA in pork liver, and therefore can be used to create herd profiles regarding HEV risk assessment. Through statistical analysis, the present study demonstrates that an increasing ELISA OD value significantly leads to an increased risk of detecting HEV RNA in the corresponding porcine liver and hence to an increased risk of transferring HEV to humans.

The infectivity of HEV could not be evaluated in the present as well as in other studies due to the fact that simple laboratory methods are lacking and that infection trials with cell cultures are highly sophisticated methods (Cook et al. 2017). Therefore, the survival period of HEV in the slaughter environment, in porcine blood as well as in porcine liver tissue, post-evisceration, is currently unknown. A few studies indicate that HEV shows a high stability to environmental conditions and room temperatures (Schielke et al. 2011; Johne et al. 2016; Baez et al. 2017).

The major potential limitation of this study is the examination of HEV cross-contamination on the liver surface by using superficial layer samples. The reason for choosing surface tissue instead of surface swabs was the goal to achieve optimal comparability of the results with the liver parenchyma (Dzierzon et al. 2020), by using comparable samples and laboratory methods. However, the question arises whether material from the liver parenchyma was accidentally included in the examination of the corresponding surface, thus falsifying the results. However, an initially HEV negative liver with detectable HEV RNA on the surface indicates a HEV cross-contamination. Furthermore, porcine livers, initially evaluated as HEV RNA positive but with no detectable HEV RNA on the corresponding surface (16.3%) also reinforce the method of sample collection and the quality of the present data.

The detected HEV isolates have not been further characterized, as they are intended to provide a scientific basis to further investigations of the potential HEV cross-contamination. Further studies are urgently needed to develop and analyze intervention measures in the field of slaughter hygiene to protect e.g. professional groups such as veterinarians and abattoir employees who are exposed to an increased risk of HEV infection (Hoan et al. 2019; Huang et al. 2019). Additionally, consumer education regarding food safety and good kitchen hygiene practices needs to be pursued and highly prioritized. If HEV RNA is present on the liver surface due to (cross-) contamination, HEV can be transferred to raw edible food during preparation.

5 Conclusion

The present study demonstrates that HEV cross-contamination in an industrial German abattoir can be associated with joint storage of porcine livers in one Euro meat container. This storing practice is common in German abattoirs until further processing or marketing of livers. Therefore, both workers in the food production industry and consumers should be aware of the potential hazard of an HEV infection by HEV positive livers as well as by HEV negative livers superficially contaminated with HEV. More intervention programs and food safety education focused on the risk of HEV cross-contamination in abattoirs, processing plants and consumers´ kitchens are needed to prevent HEV infections in humans. In addition, logistic slaughter as well as raw product steering based on serological HEV herd profiles could represent essential intervention measures to reduce the food-borne infection caused by HEV.

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Author contributions DM conceived the first study conception. All authors contributed to the final study conception and design. JD and VO performed the material preparation and data collection. Laboratory analyses were carried out by JD. Furthermore, JD prepared the first draft of the manuscript. All authors commented on previous versions of the manuscript and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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