Detection of Hepatitis E Virus in the Pig Livers and Retail Pork Samples Collected in Selected Cities in China

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Abstract

Hepatitis E virus (HEV) is a biological hazard that must be controlled and is a recognized etiological agent in viral hepatitis. This is a zoonotic virus and can be transmitted through the fecal–oral route. The pig is an important reservoir host of HEV, and is a source of contamination for the consumer after the consumption of raw or undercooked pork products. When detected, the most prevalent genotype of HEV in China is genotype 4 (denoted as HEV-4). To ensure the safety of this food of animal origin, we undertook a survey of HEV contamination in pig livers and pork samples available for sale, in retail outlets in selected cities in China. Viral RNA was purified from samples collected by lysing in Trizol followed by purification using trichloromethane and virus RNA extract kit. An additional step was applied to improve the recovery rate by adding RNase OUT when extracting virus RNA from pig livers, and the RNA productions were washed in 75% (v/v) ethanol to remove inhibitors. In total, 158 pig livers and 80 pork samples were procured and analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After purification of total RNA from all samples taken and analyzed by RT-qPCR, a single pig liver was positive by this method for HEV. The positive rate was calculated as 0.63%. In this study, a single positive sample was detected. Considering the dietary habits of Chinese people, pork is a popular food that on occasion may be contaminated with HEV, thereby posing a threat to consumer health. Ongoing surveillance is required to assess the risk to human health arising from HEV-contaminated pork being offered for sale, at retail outlets, especially in the areas of China where pig production is practiced.

Keywords: hepatitis E virus, RNA extraction, real-time RT-qPCR

Introduction

HEPATITIS E VIRUS (HEV) is one of many important agents that cause acute viral hepatitis in humans. This viral infection is linked to 20 million clinical cases annually, with 56,000 HEV deaths globally (Blasco-Perrin *et al.*, 2016). HEV is a member of the *Hepeviridae* family and contains a single-stranded positive sense RNA genome, which is ~7.2 kbp in size. The viral genome consists of three open reading frames (ORFs), in which ORF1 encodes non-structural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a viroprotein like protein (Himmelsbach *et al.*, 2018). Eight genotypes have been identified and are denoted as HEV-1–HEV-8. Of these, HEV-1, HEV-2, HEV-3, HEV-4, and HEV-7 are reported to infect humans. HEV-3 and HEV-4 are zoonotic genotypes, which could infect both human and other animals, such as pigs, wild boar, and deer (Tei *et al.*, 2003; Lee *et al.*, 2016; Woo *et al.*, 2016). In some African countries, due to poor hygiene conditions, HEV is transmitted

primarily through water, with HEV-1 and HEV-2 being the prevalent genotypes (Smith *et al.*, 2016). In contrast, in European countries, HEV-3 is identified as the main genotype, and in India and China, the prevalent genotype is recorded to be HEV-4 (Kamar *et al.*, 2012).

HEV is an important foodborne virus and it can be transmitted through the fecal–oral route. Pigs are natural hosts for HEV, the virus could be detected in the small intestines, lymph nodes, colons, and livers in the swine model (Williams *et al.*, 2001). Previous studies reported that humans can be infected by HEV after the consumption of food of animal origin (Schielke *et al.*, 2011). Several lines of evidence suggest that HEV can be transmitted through consumption of contaminated pork and wild boar meat, particularly meat derived from the livers of these animals (Colson *et al.*, 2010). In China, serological data indicated that the positive rate of the HEV antibody measured in pig blood was 83.6% (Wang *et al.*, 2002). Similarly Li *et al.* (2019) reported HEV nucleic acid in swine feces, with a positive detection rate of 61.4%.

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Other authors also reported high positive rates of detection of RNA in pig feces (Zhou *et al.*, 2019).

Given the lack of standardized and reliable virus extraction methodologies from food matrices including pork and pig liver, there is limited data describing HEV prevalence in contaminated pig livers and pork samples for sale to consumers at retail outlets in China. In this study, we report on the development of an optimized extraction method designed to aid the isolation and subsequent detection of HEV from pork and pig livers. The latter assay was then deployed to provide new information on the prevalence of HEV contamination in pig livers and pork samples for sale in retail markets in three selected cities in China.

Materials and Methods

Pork and pig livers sampling

Some 158 pig livers and 80 pork samples were randomly purchased and collected from local retail markets in Yangcheng, Hangzhou, Wenling, Taizhou, and Liaoyuan, in September 2018. The samples were from different retailers, but there were no details about the farms from where the samples were collected. The samples collected were stored at -20°C before being transported to the China National Center for Food Safety Risk Assessment (CFSA) laboratory on ice.

HEV nucleic acid purification from the pig livers and pork

A 1 g sample of pig liver or 5 g pork was thawed slowly at room temperature, and then combined together with a volume of 7 mL TRIZOL (15596018; Life Technology), $10\ \mu\text{L}$ 1.0×10^{10} PFU/L bacteriophage MS₂ (LR73141; Longrun Bio-tech), the process control, and $2\ \mu\text{L}$ RNase OUT (10777019; Invitrogen). All were thoroughly stomached in a blender bag by using Stomach Machine BagMixer400CC (024230400S; Interscience). After homogenizing at the six strokes per second velocity for 6 min, the supernatant was removed into a clean sterile tube, and centrifuged again at $1000 \times g$ for 10 min at 4°C . Finally, the supernatant was transferred into a new tube and mixed with 5 mL of 99% (v/v) trichloromethane. The mixture was then centrifuged at $17,000 \times g$ for 15 min at 4°C , and $140\ \mu\text{L}$ of the aqueous portion, recovered carefully by pipette, was transferred into a new tube for viral RNA purification. Viral RNA extraction was performed using a QIAmp Viral RNA Kit (52904; Qia-gen) according to the manufacturer's instructions. The mentioned procedure we used was derived from the method reported before (Szabo *et al.*, 2015). We also did the experiment if the PCR inhibitor was too much to affect the results. Purified RNA was separated into two groups: one group was amplified directly and another group was washed and treated by using 75% (v/v) ethanol.

Amplification controls

Highly purified nuclease-free water was used as the blank control. Phosphate-buffered saline (0.01 mol/L, pH 7.2) was used as the negative process control and was run through all stages of the analytical process. A HEV positive sample from pig feces, prepared previously that was maintained by our laboratory, was included as the test positive control. A volume of $10\ \mu\text{L}$ MS₂ was added into the sample before the RNA

extraction as the process control. The purified HEV RNA, which was from the pig's fecal sample, was used as amplification control for reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Real-time RT-qPCR amplification

Oligonucleotide primers and detection probes employed in this study were designed to detect HEV. These were commercially synthesized by ThermoFisher Scientific Co. according to the method described by Li *et al.* (forward primer: 5'-GGT GGT TTC TGG GGT GAC-3', reverse primer: AGG GGT TGG TTG GAT GAA, probe: FAM-TGA TTC TCA GCC CTT CGC-MGB) (Li *et al.*, 2019). The real-time RT-qPCR assay to detect the HEV was carried out using RNA Ultrasense One-step Quantitative RT-qPCR System (Invitrogen). Purified RNA was screened by RT-qPCR, performed on a CFX96 (BIO-RAD) real-time PCR platform. A final reaction volume of $50\ \mu\text{L}$ in each well was composed of $10\ \mu\text{L}$ purified nucleic acid template or control and $40\ \mu\text{L}$ HEV reaction premix, containing $1.25\ \mu\text{L}$ of forward primer; $2.25\ \mu\text{L}$ of reverse primer, $0.625\ \mu\text{L}$ of detection probe, and $19.875\ \mu\text{L}$ ddH₂O, along with $25\ \mu\text{L}$ $2 \times$ premix buffer and $1\ \mu\text{L}$ enzyme mix according to the manufacturer (Invitrogen, USA).

The thermal profile comprised 50°C for 30 min and 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 65°C for 30 s.

The MS₂ as process control was amplified according to the MS₂ Process Control Real Time RT-PCR Kit Instructions (Longrun Bio-Tech Company, China). Details are as follows: a final reaction volume of $25\ \mu\text{L}$ in each well was composed of $5\ \mu\text{L}$ nucleic acid template together with $17.5\ \mu\text{L}$ premix buffer and $2.5\ \mu\text{L}$ enzyme mix. The thermal profile comprised 42°C for 30 min and 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 65°C for 30 s.

Results

As a background to detection strategies employed, a sample would be determined as being positive when the Ct values obtained after RT-qPCR were <35 U. The Ct values of the negative and blank controls were higher than 40, respectively, whereas the positive control Ct value was <35 . The sample should be reassayed if the Ct value was between 35 and 40. The sample was determined as negative if the Ct value of the sample was >40 . Besides, the recovery rate of MS₂ was $>1\%$ and the amplification inhibition index was <2 . (The amplification inhibition index is the Ct value of the undiluted sample RNA + control RNA minus Ct value of the water + control RNA.)

Process control

The process control MS₂ was recovered from all samples and the recovery rate ranged from 1.27% to 100%. All of the sample RNA amplification inhibition indexes were <2 . The Ct values of the positive control were <35 and the Ct values of the blank control and negative control were >40 . The standard curve of MS₂ is shown in Figure 1, the effective rate of amplification was determined to be 96.4%, with a slope of -3.412 . All of these results indicated that the experimental data obtained were valid.

FIG. 1. Standard curve of MS₂.

The comparison result of whether used or not the RNase OUT

The recovery rate of MS₂ of the group without adding RNase OUT was <1%, whereas it is >1% of the group adding RNase OUT. Results are given in Table 1.

The efficiency of the method used to remove the PCR inhibitor

The amplification inhibition indexes of the group treated by using 75% (v/v) ethanol are <2, whereas those of the untreated group were >2, which means the 75% (v/v) ethanol could remove some of the PCR inhibitors to make sure the results are available. Results of the method used to remove the PCR inhibitor are given in Table 2.

TABLE 1. THE COMPARISON RESULT OF WHETHER USED OR NOT THE RNase OUT

Method name	Recovery rate (%)	Average recovery rate (%)
Not add RNase OUT	0.24	0.21
	0.27	
	0.13	
Add 2 μ L RNase OUT	1.64	1.79
	2.13	
	1.59	

Contamination of HEV in pig livers and the pork from retail market

The standard curve of HEV is shown in Figure 2, the effective rate of amplification was determined to be 103.6%, with a slope of -3.238 . A total of 158 pig livers and 80 pork samples were collected from five cities located in three provinces in China. In total, 158 pig livers were tested and only 1 sample was positive, giving a calculated HEV positive

TABLE 2. INFLUENCE OF THE AMPLIFICATION INHIBITION INDEXES WHEN APPLYING 75% (v/v) ETHANOL TO TREAT THE RNA EXTRACTION

Method name	Ct value	Average Ct value	Amplification inhibition indexes
No treatment	33.61	33.19	9.74
No treatment	33.19		
No treatment	32.76		
75% Ethanol treated	26.33	25.28	1.83
75% Ethanol treated	25.19		
75% Ethanol treated	25.21	23.45	
Water control	23.66		
Water control	23.59		
Water control	23.09		

The amplification inhibition indexes were calculated by RNA extraction Ct value minus the water Ct value, the result is available when the value is <2.

FIG. 2. Standard curve of HEV. HEV, hepatitis E virus.

rate for pig livers of 0.63%. Among the 80 pork samples tested, none of these tested positive for HEV.

Results are given in Table 3 and Figure 3, and the fluorescence amplification curve of the positive sample is shown in Figure 4.

Discussion

HEV is a zoonotic virus, which can infect susceptible individuals after the consumption of raw or semicooked pork.

Based on recent seroprevalence and very recent blood donor data, it is likely that there are at least two million locally acquired HEV infections in Europe every year (Dalton *et al.*, 2018). Seroprevalence studies of humans suggested that even one-third of the world’s population will be infected at some point during their lifetime (Webb *et al.*, 2019). Food products containing raw pork liver are suspected to be vehicles for transmission of HEV (Pavio *et al.*, 2014). Researchers from Korea collected 100 pig livers from a local grocery market, the positive rate of HEV was 3%, all of them belonged to

TABLE 3. RESULT OF HEPATITIS E VIRUS CONTAMINATION IN PORK AND PIG LIVERS

City	Pig livers			Pork		
	No. of pig livers	Positive number	Positive rate	N o. of pig pork samples	Positive number	Positive rate
Yancheng	32	1	3.13%	20	0	0
Hangzhou	34	0	0	20	0	0
Wenling	32	0	0	20	0	0
Taizhou	30	0	0	20	0	0
Liaoyuan	30	0	0	0	0	0
Total	158	1	0.63%	80	0	0

FIG. 3. The sampling place in China.

HEV-4 (Go *et al.*, 2019). Similar research carried out in Italy showed that the positive rates of HEV-3 in the raw and dry liver sausage were 22.2% and 4.3%, respectively (Di Bartolo *et al.*, 2015). In some areas of China, such as in Tibet and Yunnan provinces, consumption of pig livers without cook-

ing is common, thereby exposing the consumer to infection. As the components of these food matrices, such as pig liver, are complex, such inhibiting components contained therein may interfere with test reactions, and the lack of reliable virus extraction method from foods is a direct result.

FIG. 4. The HEV positive result of pig liver. HEV, hepatitis E virus.

Previously, Szabo reported on the development of a method to detect HEV in a pork sample, and when HEV was detected in pig liver sausages, the viral recovery rate was <1%. This suggests that the concentration of PCR inhibitors in the purified viral RNA was too high to ensure the success of this PR-based assay (Szabo *et al.*, 2015).

In this study, we optimized the original method of Szabo. The sample size and the dose of RNase OUT used in RNA extraction were optimized, and 75% (v/v) ethanol was used to remove PCR inhibitor from the purified RNA, a step that improved the method recovery rate and the amplification efficiency. MS₂ was used as process control being added to the sample to monitor the virus extraction efficiency. Also we introduced an amplification control during the PCR amplification to assess the amplification inhibition indexes. All of these minimize the false negative and ensure the experimental data are valid. But since some components in the pig liver, such as protoheme and polysaccharide (Qingqing *et al.*, 2014), would interfere with the PCR results, the detection limitation would be affected, so further research should be carried out to increase the sensitivity of the experiment.

The method was subsequently applied in an investigation of HEV contamination in pork and pig livers in selected cities in China. Previously it has been reported that HEV could infect humans after consumption of uncooked meat or livers taken from wild boars in Europe. In China there was no similar evidence that people could be infected with HEV through eating pork and pig livers. But a serological survey of humans in China indicated that the seroepidemiology of HEV among the rural population was 38%, and the incidence of HEV was 2.8 of 10,000, most of these being identified as HEV-4 (Zhu *et al.*, 2014). The molecular epidemiology of HEV strains isolated and studied from pigs confirmed the most prevalence genotype to be HEV-4, the same genotype was found in HEV patients (Zhou *et al.*, 2019). This observation suggests that susceptible individuals may become infected with HEV from eating foods of porcine origin, such as pig livers. A recent study showed that the HEV was completely disinfected when the food was cooked with the internal temperature reaching 71°C for 20 min (Barnaud *et al.*, 2012). HEV in pig livers may remain infectious after cooking, a feature that is associated with Chinese cooking methods. Therefore, it is risky for consumers in some areas to eat raw or half cooked pig livers, especially pregnant women.

In our study, the positive rate of pig livers is less than that in the earlier research in Hong Kong, which is 1.5% (Chan *et al.*, 2017). The different methods of virus extraction that are used in the two studies and the different areas of the sample collection may induce different results. The sensitivity of the method may still need to be improved to detect lower concentration of HEV in samples. Further research is needed to surveil the HEV contamination of the foods in the retail market especially the areas where the human HEV positive rate was high.

Conclusions

In this study, we optimized the extraction method of HEV in pig livers and pork and applied it to survey HEV in pig livers and pork from the retail market. One positive pig liver sample was detected. It may cause potential harm to consumers' dietary health, considering Chinese eating habits.

The surveillance to HEV contamination in foods should be strengthened, especially in the areas where the human HEV positive rate was high.

Disclosure Statement

No competing financial interests exist.

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