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Prevalence and Molecular Analysis of Hepatitis E Virus in Pork and Meat Products Distributed in Chiang Rai, Thailand

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Abstract

Hepatitis E virus (HEV) infection is a global disease responsible for causing acute icteric hepatitis. HEV-3 and HEV-4 are considered zoonotic and have been detected in various animal species. The primary reservoirs for HEV include swine, wild boars, and deer. This study aims to determine the prevalence of HEV in food products derived from swine in Chiang Rai, Thailand. A total of 540 pork products were collected from fresh markets between January 2020 and December 2022. The pork products, including meat, liver, and intestine, were screened for HEV RNA using reverse transcription PCR (RT-PCR). The results revealed that 4.63% of the samples were positive for HEV, with pork meat (1.30%), pork liver (2.59%), and pork intestine (0.74%) testing positive. Sequence analysis revealed that all HEV sequences obtained in this study were classified as genotype 3 (HEV-3). Additionally, the data showed that the distribution of HEV in pork products peaked during the winter period in Thailand. Therefore, the prevalence of HEV may be influenced by seasonal patterns. These results suggest that pork products could play a significant role in the transmission of HEV to humans, and swine should be considered a reservoir for HEV in Chiang Rai, Thailand.

Introduction

Hepatitis E virus (HEV) is one of many causes of acute viral hepatitis, with an estimated 20 million cases and 56,000 deaths annually (Blasco-Perrin et al., 2016). Most HEV infections are asymptomatic and tend to resolve on their own. However, they can cause acute hepatitis, leading to severe outcomes in pregnant women and individuals with pre-existing liver conditions. HEV belongs to the *Hepeviridae* family and has a single-stranded, positive-sense RNA genome. There are eight known genotypes of HEV (HEV-1 to HEV-8), and the most common ones that infect humans are HEV-1, HEV-2, HEV-3, and HEV-4. In developed countries, HEV infections are usually seen in middle-aged or elderly men (>55 years), while in developing countries, HEV-1 and HEV-2 predominantly affect young adult males (15-30 years) (Lhomme et al., 2020). Furthermore, HEV-3 and HEV-4 are endemic in pigs, wild boars, deer, and rabbits, as well as in humans (Cossaboom et al., 2011; Pavio et al., 2010). HEV-3 and HEV-4 lead to zoonotic infections, which are primarily transmitted through the consumption

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of contaminated raw or undercooked food and contact with infected animals (Lu et al., 2006).

With great concern for public health, foodborne viruses can remain viable in food products for several months. Consumption of contaminated pork is a primary route of HEV transmission (Pavio et al., 2017). HEV RNA has been detected in animal liver intended for human consumption, as reported in numerous studies. HEV RNA has been detected in pig liver sold as food in Japan and India (Kulkarni & Arankalle, 2008; Okano et al., 2014). A study in Hong Kong also found HEV RNA in pig liver and intestine. Furthermore, the distribution of HEV subtypes in humans and swine showed a high level of similarity (Chan et al., 2017). HEV RNA from pig liver samples was found to have a high identity with HEV isolated from patients with acute hepatitis E in China (Li et al., 2009). In the Czech Republic, Italy, and Spain, a study conducted in 2010 reported a prevalence of HEV ranging from 7% to 53% in the pork production chain, with HEV detected in pig liver and meat (Di Bartolo et al., 2012). In Germany, HEV RNA was detected in pig liver samples from butcher shops and grocery stores, with the sequences showing high homology to HEV from patients with acute hepatitis E in the same location (Wenzel et al., 2011). A study from 2009 to 2012 further supported the role of pigs and wild boars as reservoirs for HEV in France, with HEV RNA detected in both wild boar and pig livers (Jori et al., 2016). Another French study reported a prevalence of 4% to 24% of HEV RNA-positive livers (Rose et al., 2011). The seroprevalence of HEV in Thailand varies across different regions. A nationwide survey among healthy blood donors reported an overall HEV IgG seroprevalence of 29.7%. The prevalence was higher in the northern (28.9%), northeastern (34.8%), and central (35.8%) regions, and notably lower in the southern region (14.2%) (Jupattanasin et al., 2019). Another study focusing on young men entering the Royal Thai Army found an overall HEV IgG seroprevalence of 14%, with provincial rates ranging from 3% to 26% (Gonwong et al., 2014).

As the distribution of HEV varies worldwide, it is important to study the prevalence of HEV to estimate the risk of exposure to HEV. The aim of this study is to determine the prevalence of HEV in swine-derived food products in Chiang Rai, Thailand. In order to show the connection of HEV with foodborne disease and raise the concern about the risk of HEV infection through consumption of raw pork products.

Materials and methods

1. Sample collection

A total of 540 pork products, including 180 meat samples, 180 liver samples, and 180 intestine samples, were collected from five fresh markets in Chiang Rai, Thailand, between January 2020 and December 2022. The samples were obtained from markets in the Mueang Chiang Rai district, specifically from the subdistricts of Wiang, Rim Kok, Bandu, Nang Lae, and Thasut. All the samples were placed on shelves at ambient temperature at the time of collection. Raw pork products were collected monthly to ensure they came from different pig batches. The samples came from different retailers, but there were no details about the farms where they were sourced. The samples were stored at 4°C and processed individually on the same day. The sample size for the prevalence study was calculated based on the assumption that 5% of the samples would test positive for HEV. The analysis of HEV in different markets involved collecting data on the number of positive and negative samples. Statistical measures were calculated to determine the average number and normalized percentage of HEV positive samples, categorized by month and place of collection.

2. Viral RNA isolation and detection of HEV genome

Three hundred milligrams of raw pork products were finely minced and homogenized in a sterile mortar with a Proteinase K solution. The supernatants were then used for total RNA extraction. RNA was isolated from the suspensions using Nucleospin RNA Virus (Macherey-Nagel, Duren, Germany). The supernatant is mixed with lysis buffer, followed by adjusting the binding conditions with ethanol. The mixture is then loaded into the NucleoSpin RNA virus column to bind viral RNA. Next, the column is washed and dried, and finally, pure RNA is eluted using the elution buffer. The extracted viral RNA was converted into cDNA using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). HEV detection was performed using nested-PCR targeting the ORF2 region, with cDNA as the template. The primers and PCR conditions used in this study were optimized in-house for the preliminary detection of HEV in liver samples. The primers were designed to target the HEV ORF2 gene, with the reference sequence sourced from a public database. To maximize efficiency, potential secondary structures such as hairpins, self-dimers, and cross-dimers were minimized. Primer specificity was confirmed using

BLAST to prevent off-target binding. In the first round of PCR, a region of the ORF2 was amplified using the outer primer pair (HEV-ORF2.FO and HEV-ORF2.RO). The sec round of PCR was performed with the inner primer pair (HEV-ORF2.FI and HEV-ORF2.RI) (Table 1). The thermocycling conditions for the first round of PCR was performed at 94°C for 4 min, followed by 35 cycles of 94°C for 40 sec, 55°C for 40 sec, and 70°C for 70 sec, with a final extension at 70°C for 10 min. The sec round of PCR was performed using the same conditions as the first round, except the extension time was reduced to 60 sec. During the PCR optimization process, it was observed that 35 cycles of amplification yielded a sufficient amount of PCR product. The amplification product size from the first round PCR was 333 bp, while the product from the sec round PCR was 212 bp. PCR products were resolved using 1.5% agarose gel electrophoresis, purified with a Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taipei, Taiwan), and sequenced by First BASE Laboratories (Seri Kembangan, Selangor, Malaysia).

Table 1 A list of primers used for detecting and characterizing hepatitis E virus (HEV) in pork products

PCR reaction	Direction	Primer nucleotide sequence (5'-3')
First round	Forward	ACA CCC TAC ACT GGT GCT CT
	Reverse	GAC GGG GCG TGA GTA AAA CA
Second round	Forward	TAC GCG CGT CTC TCG TTA TT
	Reverse	TCA ATT CTG TCG GAA GCC CG

3. Sequence analysis

The ORF2 nucleotide sequences were analyzed using ClustalX and BioEdit software. These sequences were then compared to HEV reference strains using the BLAST server and the NCBI database. A phylogenetic tree was constructed based on the nucleotide sequences using MEGA software, with bootstrapping applied through 1,000 replicates of the data sets.

Results and discussion

1. Prevalence of HEV in pork products

In this study, a total of 540 pork products (180 meat samples, 180 liver samples, and 180 intestine samples) were collected from five markets and screened for HEV RNA. HEV RNA was detected in 25 samples (4.63%), including 7 meat samples (1.30%), 14 liver samples (2.59%), and 4 intestine samples (0.74%) (Fig. 1). Of the 25 HEV positive samples, 28% (7/25) were from pork meat, 56% (14/25) were from pork liver, and 16% (4/25) were from pork intestine (Fig. 2). The amount of sample used for RNA extraction in several previous studies ranged from 200 to 1000 mg (Boxman et al., 2019; Intharasongkroh et al., 2017; Di Bartolo et al., 2012; Wenzel et al., 2011). A preliminary study conducted to determine the optimal sample size for detecting HEV RNA found that 300 mg was sufficient. HEV RNA has been detected in various swine samples, including liver, bile, spleen, meat, and blood. In the Netherlands, HEV RNA was detected in 6.5% of commercial swine livers (Bouwknegt et al., 2007). Similarly, the prevalence of HEV RNA in swine livers was reported at 4% in French slaughter-age pigs (Rose et al., 2011) and 29.9% in bile samples from Italian pigs (Martelli et al., 2010). In Croatia, HEV RNA was identified in blood, spleen, and liver samples, with prevalence rates of 24.5% in domestic pigs and 12.3% in wild boars (Prpić et al., 2015). In India, 0.83% of swine liver samples were found to

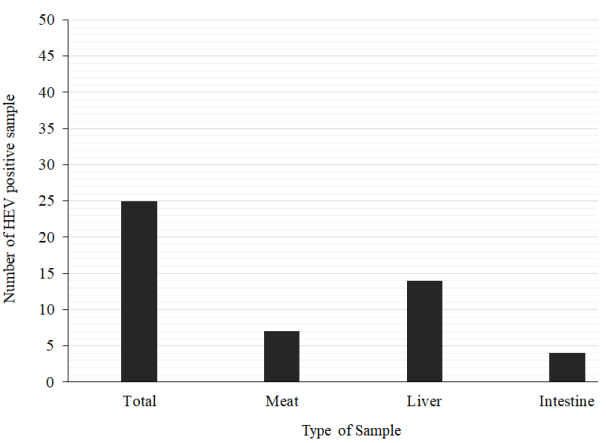

Fig. 1 Number of HEV positive sample in 540 pork products from five local markets in Chiang Rai, Thailand (January 2020 and December 2022)

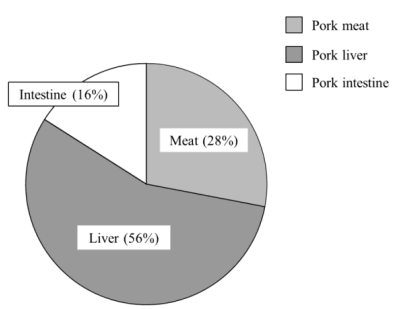

Fig. 2 Percentage of each type of sample in HEV positive pork products. Twenty-five of HEV positive samples including 7 samples of meat, 14 samples of liver and 4 samples of intestine

contain HEV RNA (Kulkarni & Arankalle, 2008), while in Japan, raw pork liver sold as food had a detection rate of 1.9% (Yazaki et al., 2003), while in Japan, raw pork liver sold for consumption had a detection rate of 1.9% (Yazaki et al., 2003). In China, HEV RNA was detected in 4% of swine liver samples (Li et al., 2009) and 22.7% of bile samples (Liang et al., 2014). In Thailand, a study conducted in the Bangkok metropolitan area reported HEV RNA prevalence rates of 0.28% in liver and 0.36% in meat samples (Intharasongkroh et al., 2017).

This study investigated the occurrence of HEV in pork products sold in local markets in Chiang Rai, Thailand. The findings revealed an overall detection rate of 4.63% for HEV RNA. Specifically, the prevalence rates were 1.30% in pork meat, 2.59% in pork liver, and 0.74% in pork intestine. Hepatitis E virus (HEV) is a liver-targeting virus that primarily infects and replicates within hepatocytes, the main cells of the liver (Yin & Feng, 2019). Accordingly, this study observed that HEV was detected more frequently in pork liver than in pork meat or intestines.

2. Association of HEV circulation and season of the year

An analysis of HEV prevalence based on the month of collection revealed that 19 out of 25 samples (76%) had detectable HEV RNA in January, February, November, and December (Fig. 3). The peak prevalence of the HEV positive sample was six samples in December. The average number of HEV positive samples in December, November, January, and February was 2.00, 1.67, 1.33, and 1.33, respectively. Moreover, HEV RNA was detected in pork products in March, July, September and October with an average number of 0.67, 0.33, 0.33 and 0.67, respectively. However, HEV RNA was not detectable in April, May, June and August. Since Thailand experiences three distinct seasons due to its tropical climate, summer lasts from March to May, the rainy season extends from June to October, and winter spans from November to February. The seasonal analysis of HEV positive samples revealed that summer accounts for approximately 0.37% of the total, corresponding to a normalized value of 0.0037. The rainy season contributes around 0.74%, with a normalized value of 0.0074. Finally, winter comprises about 3.52% of the total, with a normalized value of 0.0352 (Table 2). During the winter season in Chiang Rai, the average temperature is lower than in other seasons, ranging from 20-23°C. This cooler temperature may be associated with HEV circulation and could contribute to its distribution. Supporting this, a study on the thermal inactivation of HEV in pork products found that the virus remained infectious in dried sausage stored at 21°C or lower for up to four weeks (Stunnenberg et al., 2023). However, climate change may disrupt the seasonal patterns of HEV circulation, potentially affecting its prevalence and transmission dynamics.

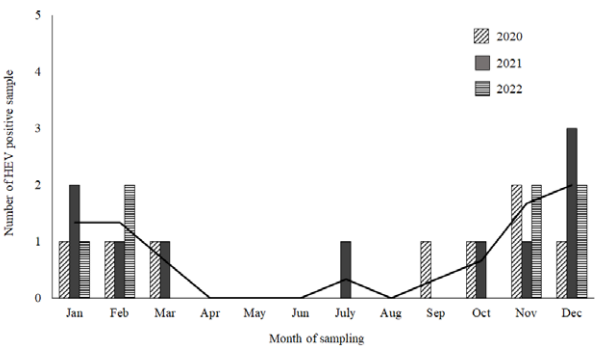

Fig. 3 Number of HEV positive pork products according to the month the pork products were collected. The black line represents the average number of HEV positive samples for each month

Table 2 Normalized percentage of samples with detectable HEV, categorized by the month of collection

Season	Month	Average temperature (°C)	Number of HEV positive sample	Total number of HEV positive sample	Normalized value (percentage)
Summer	March	25	2	2	0.37
	April	27	0		
	May	27	0		
Rainy	June	27	0	4	0.74
	July	27	1		
	August	27	0		
	September	26	1		
	October	25	2		
Winter	November	23	5	19	3.52
	December	20	6		
	January	20	4		
	February	22	4		

3. Distribution of HEV in pork products from different fresh markets

A detailed analysis of the distribution of HEV in different fresh markets showed that 0.93%, 0.93%, 1.11%, 0.73% and 0.93% of market 01, 02, 03, 04 and 05 samples had detectable HEV, respectively. The average number of HEV positive pork products from five markets was five samples. For market 01, five pork products were positive for HEV RNA including 2 meat and 3 liver samples. The amount of HEV positive samples from market 02 was five samples with one of meat, three of liver and one of intestine. Market 03 was the place

with the highest number of HEV positive pork products. Six pork products had detectable HEV RNA including 1, 3 and 2 samples of meat, liver and intestine, respectively. For market 04, HEV was detected in four pork products which include 3 samples of liver and 1 sample of intestine. Five pork products from market 05 had detectable HEV RNA with 3 samples of meat and 2 samples of liver (Fig. 4) Based on the analysis, the proportion of HEV positive samples across markets ranged from 3.70% to 5.56%, with Market 03 having the highest normalized value (0.0556) and Market 04 the lowest (0.0370) (Table3). Considering the location of markets, places more than 10 km apart, such as Market 01 to Market 04 (10 km), Market 01 to Market 05 (17 km), and Market 02 to Market 05 (14.8 km), may source their samples from different origins. However, the number of HEV positive pork products among the five markets showed no significant differences.

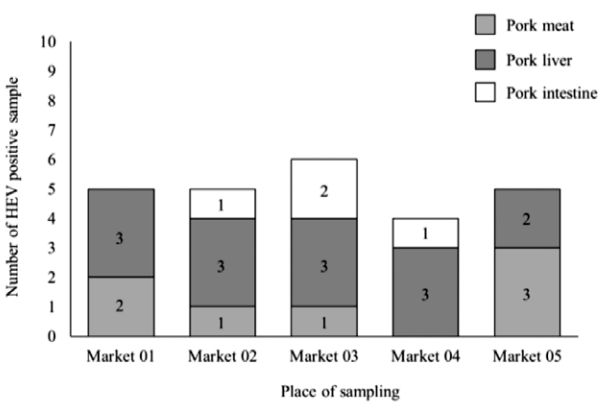

Fig. 4 Number of HEV positive in pork meat, pork liver and pork intestine from five fresh markets

Table 3 Normalized percentage of HEV positive samples categorized by the place of collection

Place	Number of HEV positive sample				Normalized value (percentage)
	Meat	Liver	Intestine	Total	
Market 01	2	3	0	5	4.63
Market 02	1	3	1	5	4.63
Market 03	1	3	2	6	5.56
Market 04	0	3	1	4	3.70
Market 05	3	2	0	5	4.63

4. HEV genetic profiling and phylogenetic assessment

Analysis of twenty-five HEV positive samples was carried out by sequencing the ORF2 region. All sample sequences were compared with HEV reference sequences from HEVnet using the HEV genotyping tool. Sequence analysis showed that all HEV positive samples belonged to genotype 3 (Fig. 5). Overall, the samples exhibited a nucleotide identity of approximately 93-98% with the reference sequences. HEV subtyping was carried out by aligning the samples to reference sequences of HEV genotype 3 subtypes. Phylogenetic analysis of the ORF2 region from HEV RNA detected in this study showed sequence identity with nearby HEV genotype 3 reference strains. Among the samples, 23 (92%) were classified as genotype 3f, while 2 samples (8%) were clustered with genotype 3c. Overall, the analysis demonstrated that the majority of HEV RNA identified in this study was highly similar to hepatitis E virus genotype 3f (Fig. 6). Overall, the analysis demonstrated

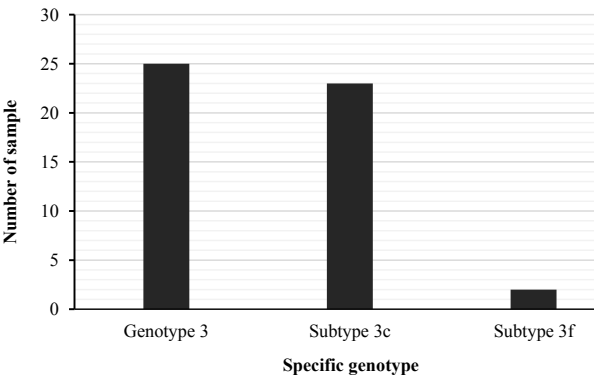

Fig. 5 The specific HEV genotype present in positive pork products

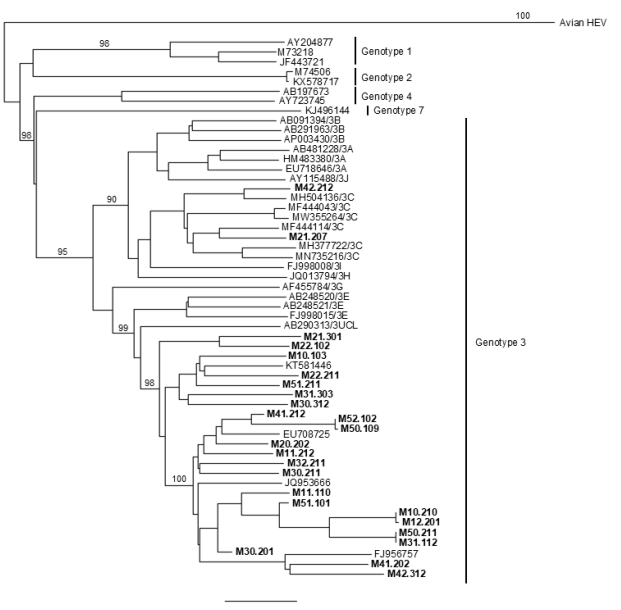

Fig. 6 A phylogenetic tree derived from the nucleotide sequences of the ORF2 gene from the HEV genome was generated using MEGA software, employing 1,000 bootstrap replicates. An avian HEV sequence served as an outgroup, with the 25 HEV isolates from pork products in this study highlighted in bold.

that the majority of HEV RNA identified in this study was highly similar to hepatitis E virus genotype 3f. Taken together, genotyping and phylogenetic analysis of ORF2 sequences revealed that most of the samples testing positive for HEV were identified as hepatitis E virus genotype 3f. As observed in the previous study from 2011 to 2012, HEV samples from pigs in Thailand also belonged to HEV genotype 3f (Keawcharoen et al., 2013).

Conclusion

Altogether, this study provides valuable information on the prevalence of HEV in pork products circulating in fresh markets in Chiang Rai, Thailand. The lower temperatures or winter season may contribute to the circulation of HEV. The occurrence data of HEV in pork products indicate a considerable risk of human transmission through the consumption of raw pork. Foodborne transmission of HEV highlights the need for public health awareness to prevent HEV infection. For future research, the author intends to include more samples from a broader range of locations to evaluate the extent of HEV prevalence in swine. Additionally, real-time PCR is a highly sensitive and quantitative method used to measure the amount of HEV in a sample. Accurate quantification of viral RNA is valuable for establishing a relationship between the infectious dose and the severity of the disease. This information is crucial for understanding the dynamics of infection, the potential for transmission, and the factors influencing the clinical outcome.

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