

Survey of hepatitis E virus in pork products and pig stools in Nakhon Pathom Province, Thailand

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Abstract

Background: Hepatitis E virus (HEV) is an important public health threat resulting in more than 3 million symptomatic cases and 70,000 deaths annually. HEV is classified into at least eight genotypes, and five are associated with human infection. Genotypes 1 and 2 primarily affect humans, whereas genotypes 3 and 4 circulate in both humans and swine and are considered zoonotic viruses. Previous studies in Central Thailand have reported human HEV isolates with high similarity to swine strains and high seroprevalence in pigs, suggesting the potential for pig-to-human transmission.

Objectives: This study aimed to detect and analyse HEV in pork products and pig stools collected from local markets and pig farms in Nakhon Pathom Province in Central Thailand.

Methods: A total of 177 pig stool and 214 pork product samples were detected for HEV by using RT-PCR amplification. Next, nucleotide sequencing and phylogenetic analysis were performed.

Results: We found one sample of pork products (1/214, 0.5%), which was a pig liver sample (1/51, 2.0%), and 49 HEV-positive samples in pig stools (49/177, 27.7%). Phylogenetic analysis showed that all these HEV sequences belonged to genotype 3, with a high correlation between our samples and HEV from humans and swine was previously reported in Thailand.

Conclusions: This study suggested that the consumption of poorly sanitized or uncooked animal meat or food and frequent exposure to pig stools may be risk factors for HEV infections in humans.

KEYWORDS

hepatitis E virus, pig stools, pork products, Thailand

1 | INTRODUCTION

Hepatitis E virus (HEV) is the main causative agent of acute liver failure or chronic infections in humans worldwide (Meester et al., 2021).

It remains an important global health problem, giving rise to > 3 million symptomatic cases, 70,000 deaths, and 3000 stillbirths annually (Nan et al., 2017). Hepatitis E is not only of concern in developing countries in the Asian and African regions but also in many industrialized

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countries, including the USA and Japan (Aggarwal & Jameel, 2011; Feagins et al., 2007; Ward et al., 2009).

HEV is classified into the family *Hebeiviridae* and genus *Hebeivirus* (Fernández-Barredo et al., 2006). It is a nonenveloped virus with positive-sense RNA of approximately 7.2 kb consisting of three open reading frames (ORF1, ORF2 and ORF3) between short 5' and 3' UTRs (Yazaki et al., 2003). HEVs are classified into at least eight genotypes, and five (1–4 and 7) are associated with human infection (Meester et al., 2021). Genotypes 1 and 2 are found in higher primates, especially humans and are largely distributed in Asia, Africa, Mexico and Nigeria (Keawcharoen et al., 2013; Yazaki et al., 2003). Genotypes 3 and 4 are circulating worldwide, presenting the possibility for zoonotic transmission with domestic pigs as the reservoir host (Kamar et al., 2017; Keawcharoen et al., 2013; Leblanc et al., 2010; Yazaki et al., 2003). Genotype 7 is found in humans who regularly consume camel meat and milk (Lee et al., 2016). This virus is transmitted by the faecal–oral route through the consumption of stool-contaminated food or water or via ingestion of undercooked animal meat, including liver meat (Yazaki et al., 2003). The typical signs and symptoms of hepatitis E include an initial phase of mild fever, jaundice, dark-coloured urine, pale stools, and liver enlargement or hepatomegaly (Boccia et al., 2006; Ward et al., 2009). There is evidence of pig-to-human HEV transmission in Thailand. Viruses recovered from Thai patients were shown to be closely related to genotype 3 and swine HEV. Phylogenetic analysis showed a high genetic sequence similarity of 83.25% between swine and human HEV. These results suggest that swine is a potential zoonotic source for HEV infection (Leblanc et al., 2007; Suwannakarn et al., 2010). Other studies identified a history of pork consumption in hepatitis E patients, supporting the transmission of HEV from contaminated pork or pork products. HEV infection related to undercooked pork consumption has also been reported in Japan, the USA, and France (Berto et al., 2013; Feagins et al., 2007; Yazaki et al., 2003). The prevalence of anti-HEV IgG was 9–22% in adults and lower in children, which may be related to higher pork consumption among the adult population (Y. Poovorawan et al., 1996).

Previously, human HEV infection was associated with close contact with domestic animals in developing countries because high seroprevalence was found in pig farm workers (Fernández-Barredo et al., 2006). From 2006 to 2007, high levels of anti-HEV antibodies were reported in pigs from five commercial pig farms in Central Thailand, including Nakhon Pathom and Ratchaburi provinces (Siripanyaphinyo et al., 2009). Moreover, the sequence analysis of the viral isolates from hepatitis E patients in Bangkok was similar to that of HEV genotype 3, perhaps originating from swine (K. Poovorawan et al., 2014; Suwannakarn et al., 2010).

Although the seroprevalence data of HEV in pigs have been reported previously, there are limited data on the HEV status of pig stools and pork products from pig farms and markets in Thailand. This study aimed to detect HEV in raw pork, pig liver and other pork products from markets as well as pig stool samples from pig farms in Nakhon Pathom Province. Additionally, we phylogenetically analysed the nucleotide sequences of HEV to explore the relationship with previously reported HEV sequences from humans and swine.

Pork is the most consumed meat in Thailand; therefore, the prevention of disease transmission from pigs to humans is crucial. Therefore, the results of this study could be useful in prompting public health authorities to apply a holistic approach, including raising awareness of at-risk populations (e.g., farmworkers, sellers, pork handlers, and consumers), promoting health education (e.g., consuming well-cooked meat, good personal hygiene, and safe manure/animal waste management), and encouraging routine screening for seroprevalence in high-risk populations.

2 | MATERIALS AND METHODS

2.1 | Study site and sample collection

A cross-sectional study was conducted in Nakhon Pathom Province during 2013–2014. This province was chosen because it is one of the largest pig-farming provinces and is located in Central Thailand, 56 km from Bangkok. A total of 177 pig stool samples were randomly collected from four pig farms with permission from the owners. A total of 214 pork product samples were collected using a random sampling method from several local markets that are located close to human communities. These included 70 raw pork meat samples, 51 ground pork meat samples, 51 pig livers, 30 pig intestines, 6 packs of raw pork balls, 3 packs of raw pork sausages, and 3 packs of fermented pork products. The samples were kept in ice boxes and transferred to the laboratory. They were stored at -80°C until processing.

2.2 | Sample processing

The frozen pork samples were homogenized by bead beating using 3–4-mm stainless steel beads in lysis buffer with oscillation frequencies of 20–30 Hz for 2 min using a TissueLyser II (Qiagen, Hilden, Germany). The homogenized samples were centrifuged at 5000 rpm for 3 min at 4°C and the supernatants were collected. Stool samples were prepared by diluting the stool with phosphate-buffered saline and chloroform. The suspension was mixed by vortexing and sonication for 10 min prior to centrifugation at 2000 rpm for 15 min. All procedures were performed at 4°C . The supernatants of pork homogenates and stool suspensions were collected and stored at -80°C for nucleic acid extraction.

2.3 | RNA extraction and conventional RT-PCR

The RNA of pork homogenates and stool suspensions was extracted using a RNeasy[®] Mini kit (Qiagen) and QIAamp[®] Viral RNA Mini Kit (Qiagen), respectively. Nested RT-PCR was performed to amplify ORF2 of HEV as described previously (Huang et al., 2002). Specifically, the forward primer 3156N (5'-AATTATGCYACAGTAYCGRGTG-3') and reverse primer 3157N (5'-CCCTTRTCYTGTCTGMGCATTCTC-3') were used during the first

round. The resulting PCR amplicons were subjected to the second-round PCR using the 3158N (5'-GTWATGCTYTGATWCATGGCT-3') and 3159N (5'-AGCCGACGAAATCAATTCTGTC-3') primer sets. SuperScript® III One-Step RT-PCR with Platinum® Taq (Invitrogen, Carlsbad, CA, USA) and Platinum® Taq DNA Polymerase (Invitrogen) were used in the first and second rounds of nested RT-PCR, respectively. The amplified PCR product of 348 base pairs was visualized in a 2% agarose gel.

2.4 | Nucleotide sequencing and phylogenetic analysis

The nested PCR products of 348 base pairs were excised from the agarose gel and purified separately using a QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were sent for chain-termination DNA sequencing (Macrogen, Seoul, Korea). The nucleotide sequences were aligned with reference sequences of HEV ORF2 using ClustalW in BioEdit version 7.2.6.1. The phylogenetic tree was constructed with the neighbour-joining algorithm with 1000 bootstrap replicates using MEGA 7 software. The designation and accession numbers of reference sequences representing different genotypes circulating in both developing and industrialized countries were retrieved from the GenBank database for analysis of HEV ORF2. The details are shown in Table 1.

3 | RESULTS

3.1 | Detection of HEV RNA in pork products and pig stools

A total of 214 pork product samples were collected from several markets in Nakhon Pathom Province. HEV RNA (corresponding to 348 base pairs) was detected in one sample of pork products (1/214, 0.5%), which was a pig liver sample (1/51, 2.0%). Other pork products were negative for HEV (Table 2). HEV RNA partial ORF2 was also detected in pig stool samples collected from pig farms (Table 3). The highest prevalence of HEV was observed in Farm 4 at 65.4% (17/26 samples) and, to a lesser extent, Farms 1 and 2 at 31.4% (16/51) and 30.0% (15/50), respectively. Farm 3 showed the lowest prevalence at 2.0% (1/50). Overall, 27.7% (49/177) of pig stool samples were positive for HEV.

3.2 | Sequence analysis of partial HEV ORF2

The phylogenetic relationships of the HEV nucleotide sequences were analysed based on the partial ORF2 sequences. Eighteen HEV ORF2 sequences from the pig stool and pork liver samples were submitted to GenBank, and accession numbers were generated, including MH341459 to MH341466 for samples from Farm 1, MH341467 to MH341471 for samples from Farm 2, MH341472 to MH341475 for

TABLE 1 List of reference sequences of HEV open reading frame 2

Genotype	GenBank accession number	Country origin
Genotype 1	D11093	China
Genotype 1	M80581	Pakistan
Genotype 1	M73218	Burma
Genotype 1	X99441	India
Genotype 2	M74506	Mexico
Genotype 3	FJ653660	Thailand
Genotype 3	JX625217	Thailand
Genotype 3	JX625216	Thailand
Genotype 3	GU947815	Thailand
Genotype 3	AY032759	Netherlands
Genotype 3	EU723512	Spain
Genotype 3	AB248522	Japan
Genotype 3	AF503511	UK
Genotype 3	AB290312	Mongolia
Genotype 3	AY115488	Canada
Genotype 3	AB073912	Japan
Genotype 3	AB091394	Japan
Genotype 3	AP003430	Japan
Genotype 3	AF455784	Kyrgyzstan
Genotype 4	AY723745	India
Genotype 4	AJ272108	China
Genotype 4	AY594199	China
Genotype 4	AB099347	Japan
Genotype 4	AB097811	Japan
Genotype 4	EU676172	China
Genotype 4	AB108537	China

samples from Farm 4, and MH341476 for the pig liver sample. The nucleotide sequences of our samples and reference sequences of all HEV genotypes were built into a phylogenetic tree. On the basis of the ORF2 sequence, our samples were clustered into HEV genotype 3 (Figure 1). The nucleotide sequence of HEV in pig liver was closely related to those from the pig stools. Most HEV sequences were related to reference strains FJ653660 and JX625217, which were found in humans and swine in Thailand from 2010 to 2013. Moreover, the nucleotide sequence from a pig stool sample from Farm 2, MH341468, was similar to the human HEV reference strain from Thailand submitted in 2010 (GU947815). We did not detect any other HEV genotypes apart from genotype 3.

4 | DISCUSSION

We reported the prevalence of HEV in pork products collected from local markets and pig stools from four pig farms in Nakhon Pathom Province. Pork products are consumed in many developing

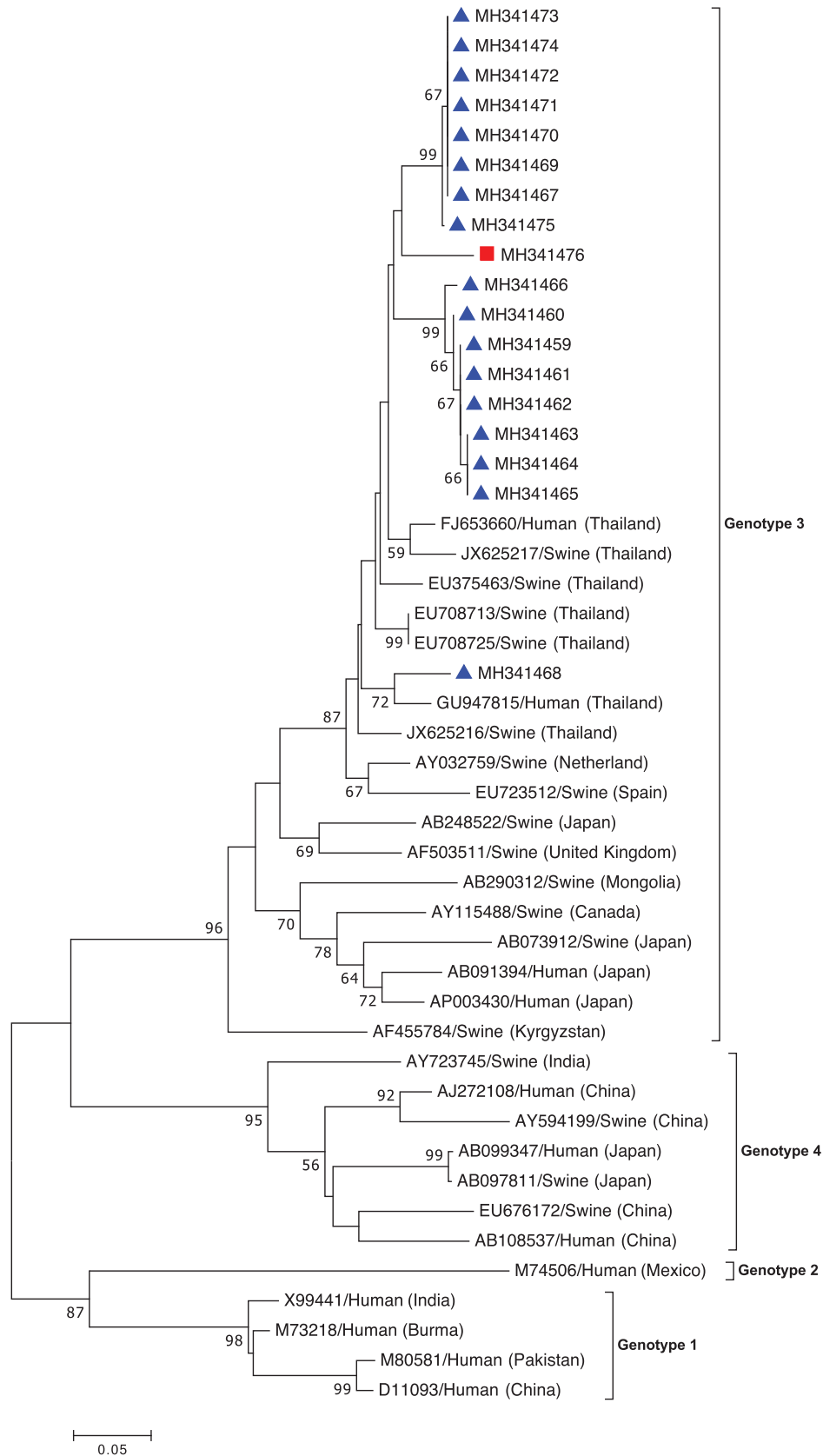


FIGURE 1 Phylogenetic tree of HEV. The neighbour-joining (NJ) algorithm with 1000 replicate bootstraps was performed based on a partial nucleotide sequence of the open reading frame 2 (ORF2) region using Molecular Evolutionary Genetic Analysis (MEGA) software, version 7.0. HEV strains are divided into four genotypes (Genotype 14). The sequences obtained in this study from pig liver and pig stool samples are assigned by the red square and blue triangle symbols, respectively. The strain names are shown as accession numbers, hosts and countries of HEV isolation. Numbers on branches represent bootstrap support values.

TABLE 2 Prevalence of HEV among raw pork, ground pork, pig liver, pig intestine and pork products

Sample type	Number of tested samples	Number of HEV-positive samples	Prevalence of HEV (%)
Raw pork	70	0	–
Ground pork	51	0	–
Pig liver	51	1	2.0
Pig intestine	30	0	–
Pork balls	6	0	–
Pork sausages	3	0	–
Fermented pork	3	0	–
TOTAL	214	1	0.5

TABLE 3 Prevalence of HEV among pig stool in four pig farms

Pig farm number	Number of tested samples	Number Of HEV-positive samples	Prevalence of HEV (%)
1	51	16	31.4
2	50	15	30.0
3	50	1	2.0
4	26	17	65.4
Total	177	49	27.7

countries, particularly in Thailand. Data from several studies indicated that humans may acquire HEV infections by consuming uncooked or undercooked animal meat or food made with pig organs, such as the liver. A study conducted in France reported HEV contamination in 1.3% of pig liver samples (Berto et al., 2013). Cases of fulminant hepatitis E have been reported in Japan after the consumption of meats and entrails of pigs (Miyashita et al., 2012). In our study, we detected HEV in one of the tested pork product samples (1/214, 0.5%), which was considered to be 2.0% (1/51) among pig liver samples. A higher prevalence of HEV was detected in the excretory products of pigs than in pig products. We also detected HEV (49/177, 27.7%) in pig stool samples. Other studies reported that seven of 10 stool samples were positive for HEV (Siripanyaphinyo et al., 2009). (Fernández -Barredo et al., 2006; Siripanyaphinyo et al., 2009; Vasconcelos et al., 2015) showed that eight of nine pooled stool samples were positive for the HEV ORF1 gene, and Fernández-Barredo et al. (2006) showed that eight of 34 stool samples were positive for HEV in Spain. In our study, the sequence analysis revealed that HEV in all detected samples was classified as genotype 3. All of them clustered to human/swine HEV and were closely related to the samples isolated from Thailand. These data demonstrate a relationship between HEV contamination in pig stools and pork products, especially in pig liver, and potential transmission to humans. This hypothesis was supported by the phylogenetic analysis, which revealed that the HEV detected in both pig liver and stools, all of which were genotype 3, clustered with HEV from humans circulating in 2008 (accession number FJ653660) and pigs in 2012 (JX625217) from Thailand (Keawcharoen et al., 2013; Suwannakarn et al., 2010). More-

over, the sequence data of pig stools from Farm 2 were closely related to the HEV circulating in humans in 2009 (GU947815) (Suwannakarn et al., 2010). The HEV-containing pig stools could contaminate pork products via unsanitary practices of farmworkers or during slaughtering and meat-handling processes and cause foodborne outbreaks. Implementing hygiene policies can help reduce HEV contamination. Consumers should be concerned that pig liver can be contaminated with HEV and carry a risk of infection if it is not well cooked, and this emphasizes that pig liver and pork products must be controlled. The infectivity of HEV in pork products might require further investigation.

5 | CONCLUSION

This study reported one HEV-positive sample in 214 pork products (0.5%), which was 2.0% (1/51) when only pig liver samples were considered, and 49 HEV-positive samples in 177 pig stools (27.7%). Phylogenetic analysis of HEV sequences showed that all these sequences belonged to genotype 3. This study presented important data regarding the risk of HEV transmission from pork products and pig stools in Central Thailand. This information could raise awareness of a lurking threat, calling for actions to address at-risk populations by providing preventive-related health education, ensuring continuous monitoring and quality control of pork products, and conducting routine surveillance for HEV prevalence in pigs and seroprevalence in high-risk populations. Furthermore, this could serve as a basis for public health authorities to implement a holistic approach involving multisectoral

collaborations and community empowerment to tackle the spread of HEV.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

This study did not require ethical approval because the pig stools and pork products were collected from pig farms and fresh food markets, respectively.

AUTHOR CONTRIBUTIONS

Narin Thippornchai: Formal analysis; investigation; methodology; writing – original draft. Pornsawan Leungwutiwong: conceptualization; methodology; project administration; supervision; writing – review and editing. Nathamon Kosoltanapiwat: formal analysis; methodology; visualization; writing – review and editing. Cindy Vuong: investigation; methodology; writing – review and editing. Kellyan Nguyen: investigation; methodology. Tamaki Okabayashi: conceptualization; formal analysis; methodology. Awapuhi Lee: writing – review and editing.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from corresponding author.

PEER REVIEW

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