ระบาดวิทยาเชิงโมเลกุลและการวิเคราะห์ทางพันธุกรรมของเชื้อไวรัสตับอักเสบอีในหมูและผู้บริจาคโลหิต

นางดวงนภา อินทรสงเคราะห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

Molecular epidemiology and genome analysis of hepatitis E virus in swine and blood donors

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2017

Thesis Title	Molecular epidemiology and genome analysis of hepatitis E virus in swine and blood donors
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ดวงนภา อินทรสงเคราะห์ : ระบาดวิทยาเชิงโมเลกุลและการวิเคราะห์ทางพันธุกรรมของเชื้อไวรัสตับอักเสบอีในหมูและผู้บริจาคโลหิต (Molecular epidemiology and genome analysis of hepatitis E virus in swine and blood donors) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. จินตนา จิรถาวร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. ยง ภู่วรวรรณ, หน้า.

จากการรายงานการติดเชื้อไวรัสตับอักเสบ อี ทั่วโลก พบว่ามีการติดเชื้อไวรัสตับอักเสบ อี ประมาณ 20 ล้านคน และเป็นสาเหตุของการเสียชีวิตจากการติดเชื้อมากกว่า 50,000 คนต่อปี ความชุกที่พบมักเกิดในประเทศแถบเอเชียผ่านทางการบริโภคอาหารและน้ำดื่มที่ปนเปื้อน ระบาดวิทยาของเชื้อไวรัสตับอักเสบ อี มีความแตกต่างกันตามจีโนไทป์ที่พบ ไวรัสตับอักเสบ อี จีโนไทป์ 1 ถึง 4 สามารถติดเชื้อในคนได้ ซึ่งจีโนไทป์ 1 และ 2 จะพบในคนเท่านั้นและมักจะระบาดในภาวะน้ำท่วมหรือเกิดภัยพิบัติ ส่วนจีโนไทป์ 3 และ 4 สามารถพบได้ในทั้งคนและสัตว์โดยเฉพาะหมู จากการรายงานการติดเชื้อไวรัสตับอักเสบ อี ในไทย มักพบการติดเชื้อไวรัสตับอักเสบ อี จีโนไทป์ 3 และพบมากในกลุ่มชายสูงอายุ ทั้งนี้การรายงานของสาเหตุของการติดเชื้อยังไม่ชัดเจน หมูเป็นสัตว์เศรษฐกิจที่สำคัญและยังเป็นแหล่งรังโรคที่สำคัญของเชื้อไวรัสตับอักเสบ อี ซึ่งการบริโภคหมูและการสัมผัสกับหมูที่ติดเชื้อจึงมีความเกี่ยวข้องและเสี่ยงต่อการติดเชื้อไวรัสตับอักเสบ อี ได้ นอกจากนี้การติดเชื้อจากการบริโภคหมูยังส่งผลกระทบต่อการบริจาคโลหิตเนื่องจากเชื้อไวรัสตับอักเสบ อี สามารถถ่ายทอดทางการให้เลือดอีกด้วย ดังนั้น การศึกษาครั้งนี้จึงเป็นการศึกษาเกี่ยวกับระบาดวิทยาของเชื้อไวรัสตับอักเสบ อี ผ่านทางการบริโภคเนื้อและส่วนประกอบต่างๆ ของหมู ทั้งนี้ยังได้ทำการศึกษาความชุกของเชื้อไวรัสตับอักเสบ อี ในผู้บริจาคโลหิตด้วย พร้อมทั้งทำการศึกษาความหลากหลายของสายพันธุ์และวิวัฒนาการของเชื้อไวรัสตับอักเสบ อี อีกด้วย สำหรับการศึกษาจะใช้การตรวจหาสารพันธุกรรมของเชื้อไวรัสตับอักเสบ อี (HEV RNA) ด้วยวิธี polymerase chain reaction (PCR) ซึ่งจากผลการศึกษาพบว่า ในตัวอย่างที่เก็บมาทั้งจากตลาดสดและโรงฆ่าสัตว์จำนวน 3,478 ตัวอย่าง พบ HEV RNA ในตัวอย่าง 1.58% ซึ่งเป็นตัวอย่างที่เก็บจากตลาดสด 0.23% และในตัวอย่างที่เก็บจากโรงฆ่าสัตว์พบ 3.93% โดยเชื้อที่พบจัดอยู่ในกลุ่มของไวรัสตับอักเสบ อี จีโนไทป์ 3f ซึ่งสามารถพบได้ในประเทศทางแถบยุโรปและประเทศที่พัฒนาแล้ว จากนั้นได้ทำการศึกษาในผู้บริจาคโลหิต โดยทำการตรวจคัดกรองเชื้อไวรัสตับอักเสบ อี ในผู้บริจาคโลหิต ณ ศูนย์บริการโลหิตแห่งชาติ สภากาชาดไทยจำนวน 30,115 คน พบความชุกของ HEV RNA ประมาณ 0.09% หรือมีความถี่ของการติดเชื้อประมาณ 1 ใน 1,159 ทั้งนี้ความชุกที่พบมีความใกล้เคียงกับที่พบในประเทศจีน ซึ่งตัวอย่างที่พบส่วนใหญ่จัดอยู่ในกลุ่มของไวรัสตับอักเสบ อี จีโนไทป์ 3f เช่นเดียวกับที่พบในเนื้อและส่วนประกอบต่างๆ ของหมู จากผลการศึกษาทั้งสองการศึกษาแสดงให้เห็นว่าเชื้อไวรัสตับอักเสบ อี ที่พบในไทยนั้นมีความชุกและชนิดของสายพันธุ์ใกล้เคียงกับที่พบในประเทศทางแถบยุโรปและที่สำคัญสามารถพบได้ทั้งในคนและหมู สำหรับในการวิเคราะห์ความหลายหลายทางพันธุกรรมและวิวัฒนาการของเชื้อไวรัสตับอักเสบ อี พบว่าสายพันธุ์ที่พบในประเทศไทย คือ ไวรัสตับอักเสบ อี จีโนไทป์ 3f และนอกจากนี้ยังพบจีโนไทป์ 3a และ 3c อีกด้วย ซึ่งยังไม่เคยมีรายงานในประเทศไทยมาก่อน สำหรับวิวัฒนาการของเชื้อไวรัสตับอักเสบ อี ในการศึกษานี้พบว่าไวรัสตับอักเสบ อี มีจุดกำเนิดเมื่อ 1624 ปีก่อน (95% HPD: 1222.40-2110.48) และมี evolution rate 5.63×10^{-4} subs/site/year ซึ่งต่ำกว่าการศึกษาที่ผ่านมา นอกจากนี้เชื้อไวรัสตับอักเสบ อี จีโนไทป์ 3 พบในประเทศไทยตั้งแต่ประมาณ 97-118 ปีก่อนและคาดว่ามีการติดเชื้อในหมูก่อนแล้วถ่ายทอดมาสู่คน สำหรับการศึกษา natural selection พบว่ามี positive selection เกิดขึ้นในยีน ORF2 และ ORF3 ในขณะที่ยีน ORF1 พบเพียง negative selection เท่านั้น ทั้งนี้เนื่องจาก ORF1 เป็นส่วนของยีนที่ทำหน้าที่ในการถอดรหัสเป็นโปรตีนที่สำคัญของเชื้อไวรัส ดังนั้นจึงทำให้พบการเปลี่ยนแปลงที่น้อยกว่า นอกจากนี้ยังได้ทำการศึกษาการกลายพันธุ์ในตัวอย่างที่ได้จากการศึกษาครั้งนี้เทียบกับการศึกษาที่ผ่านมาและมีความเกี่ยวข้องกับลักษณะทางคลินิก พบว่ามีตำแหน่งของการกลายพันธุ์เกิดขึ้น 4 ตำแหน่ง ที่เหมือนกับที่เคยมีรายงานในการศึกษาอื่น แต่อย่างไรก็ตามควรมีการศึกษาการกลายพันธุ์เชิงลึกต่อไป จากข้อมูลที่ได้จากการศึกษาครั้งนี้ สามารถนำไปใช้เพื่อเป็นข้อมูลพื้นฐานทางระบาดวิทยาของเชื้อไวรัสตับอักเสบ อี ของประเทศไทย และสามารถนำไปประยุกต์ใช้สำหรับการศึกษาที่เกี่ยวข้องต่อไปได้อีกด้วย

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5774756030 : MAJOR MEDICAL SCIENCE
KEYWORDS: HEPATITIS E VIRUS / PREVALENCE / SWINE / BLOOD DONOR / GENETIC DIVERSITY / REAL TIME RT-PCR / PATIENT

DUANGNAPA INTHARASONGKROH: Molecular epidemiology and genome analysis of hepatitis E virus in swine and blood donors. ADVISOR: ASSOC. PROF. CHINTANA CHIRATHAWORN, Ph.D., CO-ADVISOR: PROF. YONG POOVORAWAN, M.D., pp.

Hepatitis E virus (HEV) infection affects approximately 20 million individuals and results in more than 50,000 deaths each year. The highest prevalence is in Asia where contaminated food and water are often the sources of infection. There was the difference among the genotype of human Hepatitis E virus which genotype 1 to 4 can infect human. HEV genotypes 1 and 2 are found only in humans and are responsible for viral hepatitis outbreaks, while genotypes 3 and 4 are zoonotic. The HEV infection in Thailand caused by HEV genotype 3f and usually found among adult males. However, the route of HEV transmission remains unclear. Swine is the main reservoir of HEV and economically important livestock, yet pork consumption and close contact with pigs are associated with the risk of hepatitis E virus infection. Thus, HEV infection in the general population may be acquired from the consumption of pork and variety meats. Furthermore, HEV infection of blood donors may concern with the food chain and transmitted to the patients via blood transfusion. To address these concerns, this study aimed to investigate the epidemiology of HEV in Thailand both in pork and variety meats, and in Thai blood donors. Moreover, this study provides the genetic variation and evolutionary of HEV as well. Polymerase chain reaction (PCR) was performed to investigate HEV RNA in samples. For the result of this study, the prevalence of HEV RNA in pork and variety meat from supermarket, bile and feces from slaughter house around 3,478 samples, was 1.58%. The HEV RNA was found in samples collected from fresh market around 0.23% while HEV RNA was found in slaughterhouse around 3.93%. The HEV genotype 3f was circulating in Thai swine from slaughterhouse and swine product from fresh market like in European and developed countries. Then, the screening of HEV in blood donors was performed which the detectable HEV RNA among healthy Thais blood donors at National blood centre, Thai Red Cross society, of 0.09%, or approximately 1 in 1,159 which was closely with China and most of positive samples were HEV genotype 3f. This result demonstrated that the prevalence and genotype of HEV in Thailand was closely with European and developed countries. In addition, HEV genotype 3f was intermixing between human and swine. Finally, the whole genome analysis of the complete genome sequencing isolated from Thailand showed that HEV genotype 3f was mostly predominating in Thailand. Moreover, the other subtype was also found in Thailand including HEV genotype 3a and 3c. The evolutionary analysis was estimated the mean time of the ancestor for HEV genotype 1 to 4 was 1,624 years ago (95% HPD: 1222.40-2110.48) and the substitution rate was $5.63x10^{-4}$ base substitution per site per year which was lower than the previous study. Moreover, HEV genotype 3 in Thailand was most probably originated in Thailand around 97-118 years ago and might be originated with swine infecting ancestor then intermix between human and swine. For the natural selection analysis, there are the positive selection in HEV genome sequence including ORF2 and ORF3 whereas ORF1 found only negative selection. Thus, HEV was the highly conserve in the functional domains. Furthermore, the mutation associated with the clinical manifestation was observed in complete genome sequences in this study. There were 4 mutation sites that were similar to those reported in other studies. However, the other mutation study should be performed in the future. This informative data provided the prevalence of HEV in Thailand. Including the evidence indicates that epidemiology and evolutionary may play a crucial role to help prevention of HEV infections in Thailand and useful for the further study of HEV infection.

Field of Study: Medical Science
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Associated Professor Chintana Chirathaworn, for her competent supervision, guidance, encouragement and criticism which have inspired me to accomplish my study.

I would like to express my heartfelt and appreciation to my co-advisor, Professor Yong Poovorawan, who is my best mentor. I could have asked for better role model, each inspirational, supportive, encouragement, and patient. I could not be prouder of my academic roots and hope that I can in turn pass on the research values that he has given to me.

I would also like to thanks my supervisory examiners, Associate Professor Padet Siriyasatien, Assistant Professor Watcharee Limpanasittikul, Associate Professor Sunchai Payungporn, and Associate Professor Teeraporn Chinchai, who provided constructive feedback. I am grateful for their instructive comments which are helping to shape and guide the direction of my study. I would like to thank my training advisor, Dr.Sompong Vongpunsawad , Dr. Rujipat Wasitthankasem, Thanunrat Thongmee, Dr.Nipaporn Tewawong and every person at Center of excellence in clinical virology for kind support, teaching and all suggestions during the virus discovery training.

I am indebted to Tassanee Sakuldamrongpanich, Sineenart Oota, Kriangsak Chaiwong, Ubonwan Jarunreungrit, Soisa-ang Pikulsod and my colleagues at National Blood Centre, Thai red cross society to support and help me for my Ph.D.study.

I would like to thank for Thai Red Cross Society for giving me a chance to Ph.D. studying and my supporting funding both The Overseas Research Experience Scholarship for Graduate Students of Chulalongkorn University and The 100th Anniversary Chulalongkorn University Fund.

Finally, I would like take this opportunity to express the profound gratitude form the bottom of my heart to my beloved parents and family who instilled within me a love of creative pursuits, and have also been the best of friends along this journey.

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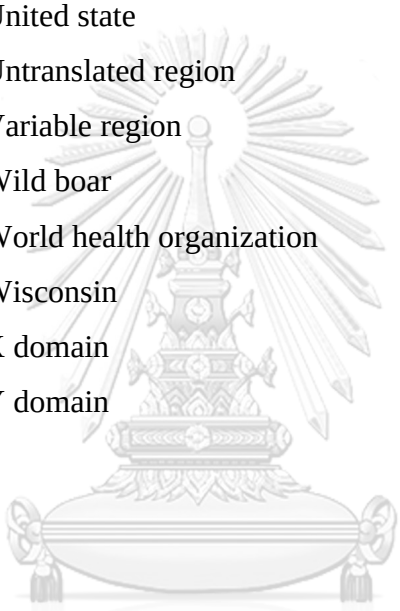
aa	=	Amino acid
ALT	=	Alanine aminotransferase
Anti-HEV	=	Antibodies directed against HEV
Av	=	Avain
bp	=	Base pair
CA	=	California
De	=	Deer
dN	=	non-synonymous
DNA	=	Deoxyribonucleic acid
dN/dS	=	The ratio between nonsynonymous(dN) and synonymous(dS) substitutions
dS	=	Synonymous
eHEV	=	Enveloped HEV
ELISA	=	Enzyme-linked immunosorbent assay
ER	=	Endoplasmic reticulum
ESS	=	Effective sampling size
ET-NANB	=	Enterically-transmitted non- A and non-B
FEL	=	Fixed-effects likelihood model
G	=	Gamma distribution
GTR	=	General Time Reverisible
HAV	=	Hepatitis A virus
HCV	=	Hepatitis C virus
HEV	=	Hepatitis E virus
Hel	=	RNA helicase
HIV	=	Human immunodeficiency virus
HPD	=	Highest posterior probability density
Hsp	=	Heat shock protein
Hu	=	Human
HVR	=	Proline-rich hypervariable region

I	=	Invarience site
ICTV	=	International committee on taxonomy of viruses
IgG	=	Immunoglobulin G
IgM	=	Immunoglobulin M
IRB	=	Institutional review board
LoD	=	Limit of detection
MAPK	=	Mitogen-activated protein kinases
MCMC	=	Markov Chain Monte Carlo method
M domain	=	Middle domain
MeT	=	Methyltransferase
NA	=	Not applicable
NBC	=	National blood centre
Nested RT-PCR	=	Nested reverse transcription polymerase chain reaction
NJ	=	Neighbor-joining
NS	=	Non-synonymous amino acid
nt	=	Nucleotide
ORF	=	Open reading frame
p	=	Probability value
PCP	=	Papain-like cysteine protease
PCR	=	Polymerase chain reaction
P domain	=	Protruding domain
pORF1	=	Nonstructural protein
pORF2	=	Capsid protein
pORF3	=	Hypothetical protein
RAB5	=	Ras-related protein Rab5A
RAB7	=	Ras-related protein Rab7A
Rb	=	Rabbit
RdRp	=	RNA-dependent RNA polymerase
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcription polymerase chain reaction
RT-qPCR	=	Reverse transcription quantitative polymerase chain reaction
Ser	=	Serine

SH	=	Src Homology
Sig	=	Significant
SLAC	=	Single likelihood ancestor counting model
S domain	=	Shell domain
Sw	=	Swine
TMA	=	Transcription-mediated amplification
TMRCA	=	The most common recent ancestor
UK	=	United Kingdom
USA	=	United state
UTR	=	Untranslated region
V	=	Variable region
Wb	=	Wild boar
WHO	=	World health organization
WI	=	Wisconsin
X	=	X domain
Y	=	Y domain



CHAPTER I
GERNERAL INTRODUCTION

Background and rationale

Hepatitis, or the inflammation of the liver, is a major public health problem in Thailand. Hepatitis caused by viral infection is the most reported cause of hepatitis. The main virus pathogens are hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV). Recently, infection by hepatitis D and E viruses were also reported. Hepatitis E virus (HEV) is increasingly recognized serious health risk to pregnant mothers, immunocompromised individuals, and the elderly. Understanding HEV epidemiology and route of transmission is important to minimize or prevent the spread of HEV. HEV can infect pigs raised for food. Consumption of undercooked pork products is suspected to be the cause of HEV infection in industrialized countries. In addition, blood transfusion is another route that may be associated with hepatitis E transmission. A number of prevalence studies reported high rate of asymptomatic HEV infection, which raised concern of blood-borne transmission. However, different assays with vary sensitivity and specificity are not appropriate to compare the different prevalence studies directly. Furthermore, different genotypes of HEV (1 to 4) are found in Southeast Asia, but only genotype 3 is found in Thailand. Given these reasons, knowledge on the prevalence and genome studies of HEV infection in Thailand is needed.

Objectives and significance of this study

Presently, the HEV infection has occurred in developed countries and the number of sporadic case is increasing in which the route of infection is unclear. Moreover, the HEV is spread by the fecal-oral route similar to hepatitis A virus so the number of hepatitis E infection is underestimated. Additional, the fecal–oral route is the primary and most well-documented mode of HEV transmission but now it was reported the other routes including zoonotic transmission, food-borne transmission, and blood-borne transmission. Besides humans, HEV have been isolated from various animal such as domestic swine, wild boar, deer, chicken, rat, ferret, rabbit, and camel.

In Thailand, the number of HEV infection is reported over the world led to Thai researcher to interest in HEV. Nevertheless, the prevalence and the mode of HEV transmission are still unclear. Hence, this study aims to investigate the prevalence of HEV infection in Thailand by followed the possible route that usually found in Thailand. Moreover, the relationship between genomic of HEV in human and reservoir is performed to support the source of transmission as well. Because HEV is concern to daily life so the benefit of this study is assisting awareness and facilitating disease prevention and control.

In this thesis, I am interested the investigating HEV because many studies in other countries have reported HEV transmission and zoonotic reservoir, but the knowledge of HEV in Thailand is very limited. Thus, this study focuses on the epidemiology of the virus, the predominant genotype, and the possible origin of HEV that found in Thailand.

objectives of this study were:

(i) To investigate the prevalence of HEV in pork and variety meats from markets and pig tissues from slaughterhouse in Bangkok.

(ii) To determine the prevalence of HEV among Thai blood donors to assess the potential risk of HEV infection from blood transfusion.

(iii) To determine the whole genome sequencing and analyze the HEV strains present in Thailand.

Hypothesis

1. Domestic swine is one major source of HEV infection because HEV is present in pork and variety meats.
2. Blood product from healthy donors can harbor HEV which can be transmitted to hospital patients.
3. HEV genotype 3 present in human and swine is genetically similar.
4. HEV strains present in Thailand are genetically similar to those found in developed countries.

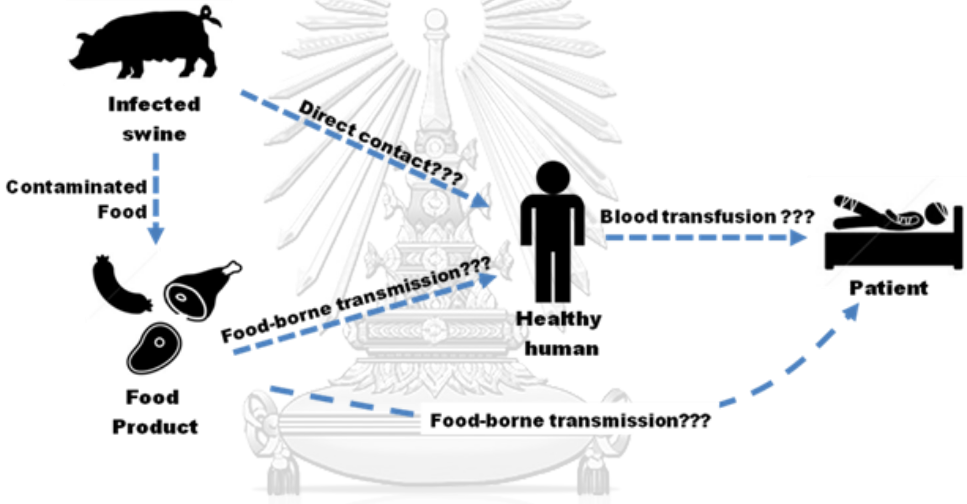


Figure 1 Hypothesis of this study

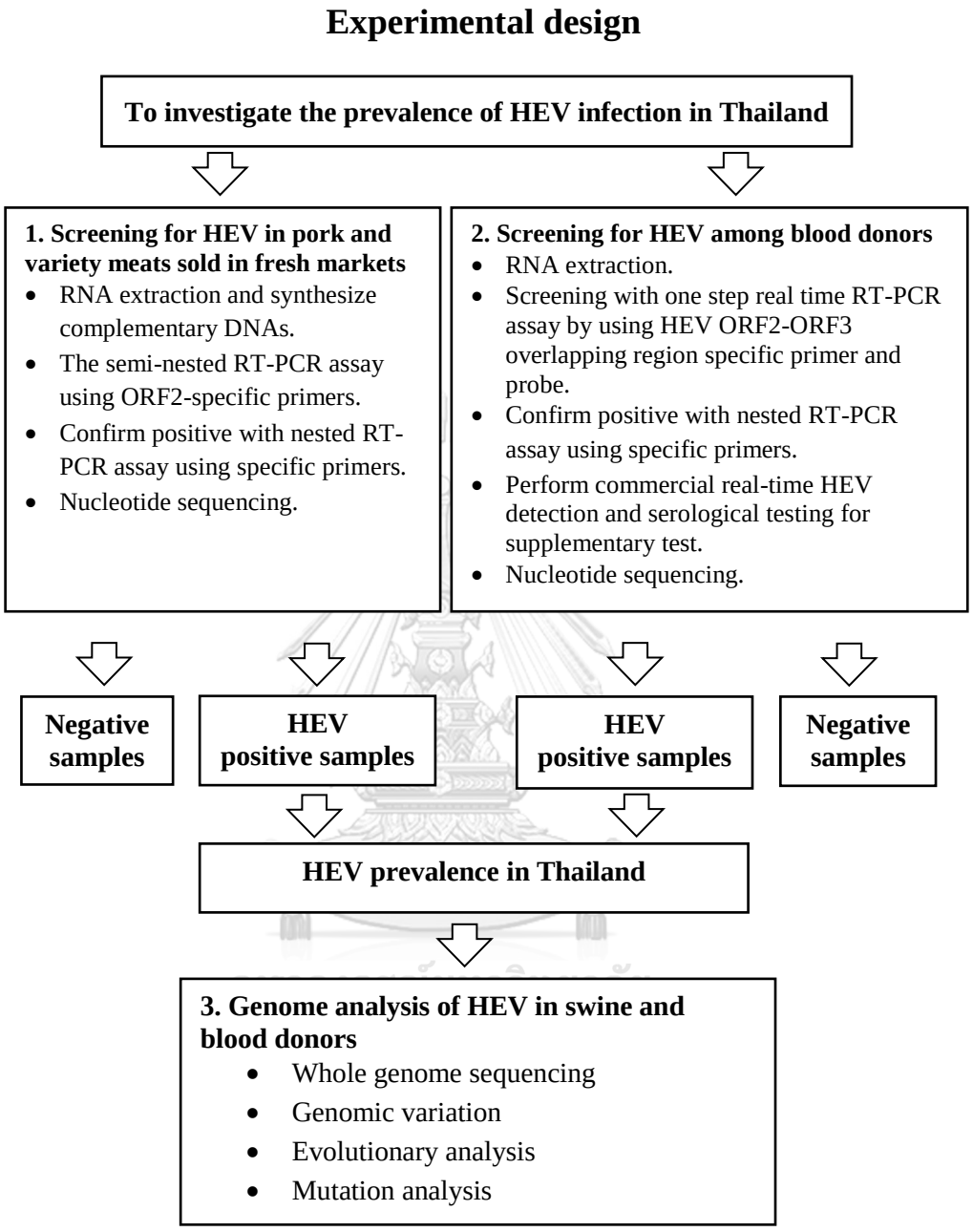


Figure 2 Research Design and Methods

CHAPTER II
LITERATURE REVIEW

2.1 Introduction

History

HEV is one of known pathogen that is the causative of human hepatitis and responsible for hepatitis infection across the world. In 1957, the first outbreak of enterically transmitted, non-A, non-B hepatitis virus (ET-NANB) was in New Delhi, India around 29,000 cases by fecal contamination of drinking water. This evidence was thought to be caused by hepatitis A virus as the mode of transmission, length of incubation, clinical manifestation and biochemical test result similar to HAV (1). But the retrospective serological study of this sample and other outbreak from ET-NANBH were not the same neither HAV nor HBV (2, 3). Soon thereafter, an outbreak occurred again in the Kashmir valley, India, with acute viral hepatitis 275 cases. Most of the cases were 11-40 years old and high disease attack rate among pregnant women which the finding suggested that it was occurred because of new hepatitis virus (4). Other outbreaks of ET-NANBH were reported form Nepal, Burma, Africa, and small outbreaks from Mexico (5-7) In 1983, the demonstration of immune electron microscopy was spherical virus-like particles in stool samples collected from acute hepatitis patient in Afghanistan (1). After that this virus was isolated for genome sequencing and molecular cloning from several geographical regions then it was recorded as hepatitis E virus (HEV) after four hepatitis viruses known already which the disease's proclivity to be a cause of epidemic and endemic disease (8, 9). In addition, the alphabet "E" was stand for epidemic, enteric, or endemic to describe the epidemiology of HEV as well.

Classification of HEV

In the past, HEV is classified in the family *Caliciviridae* because of the size of virion, morphology, the sedimentation of viral particle but it was removed because HEV does not share sequence homology with this family. Additionally, the putative enzyme and the cap at the 5' end of genome are different (10). The analysis of genomic

sequencing based on ORF1 region compared between HEV and “alpha-like” viruses that showed significant similarity. However, “alpha-like” viruses have enveloped particles whereas HEV is non-enveloped particle (11). Until 2004, *Hepeviridae* family was designated that HEV was member placed in this family (12). Presently, A new consensus classification of *Hepeviridae* family was presented for resolving current confusion that HEV infected human was placed in *Orthohepevirus* A species. (Table 1)

Table 1 Classification of *Hepeviridae* family
(Modified from Smith, DB. et al., 2014 (13))

Family	Genus	Species	Genotype	Reference strain	Predominate host
Hepaviridae	***Orthohepevirus***	*Orthohepevirus* A	HEV-1	Burma	Human
			HEV-2	Mexico	Human
			HEV-3	Meng	Human, swine, wild boar
			HEV-4	T1	Human, pig
			HEV-5	JBOAR135-Shizs09	Wild boar
			HEV-6	wbJoy_06	Wild boar
			HEV-7	DcHEV-178C	Camel
		Orthohepevirus B			Chicken
		Orthohepevirus C	HEV-C1	R63	Rat
			HEV-C2	FRHEV4	Ferret
		Orthohepevirus D			Bat
	Piscihepevirus	*Piscihepevirus* A			Trout

The genus of *Orthohepevirus* has four species including *Orthohepevirus* A, *Orthohepevirus* B, *Orthohepevirus* C, and *Orthohepevirus* D. *Orthohepevirus* A is very important because its concerned to infect in human and had more genetic variation among HEV genotypes which comprising seven genotypes as shown in Table 1. The analysis of nucleotide *p*-distances is the base of the assignment and identification of reference sequences for each subtype which the value of *p*-distance threshold of HEV genotype discrimination is 0.088 for amino acid distance of HEV genome except hypervariable regions (13). For the difference among HEV genotype in *Orthohepevirus*

A, the nucleotide difference at genotype level of genotype 1 and 2 were 19.1% whereas genotype 3 and 4 were 18.8% -23.6%. At subtype level, the nucleotide difference among subtype namely genotype 1 and 2 were 5.6% and 8.5% while genotype 3 and 4 were 11.5-20.0% (14). For *Orthohepevirus B, Orthohepevirus C*, and *Orthohepevirus D.*, the identity of each genus was reported 50%, 55-59%, and 74-79% when compared with HEV genotype 1-4, respectively (15-17) Nevertheless, the criteria of HEV genotype and subtype classification is remain less consistent and confusing so now the International committee on Taxonomy of Viruses (ICTV) adopt the classification of the *Hepeviridae* family and recommend to follow the criteria proposing by Smith et al. (2016) that was consensus to be the standard reference for interpretation of HEV genotype and sub-genotype (13, 18).

2.2 Structural and Morphology of HEV

The structure of Hepatitis E is a small positive sense single strand RNA virus with a nearly 7.2 kb. in size and non-enveloped. The virions are spherical particles generally ranged from 27-34 nm. in diameter and have protrusions on surface. In 2016, HEV was declared to be a quasi-enveloped virus which it has two forms including enveloped HEV and non-enveloped HEV. Enveloped HEV (eHEV) produced in cell culture has some lipid on capsid of virion while non-enveloped HEV was shed in stool and no lipid on its capsid, like Hepatitis A virus (19). It demonstrated that the structure of HEV virion was difference in different source which the virions from cell culture have more lipid envelope and ORF3 protein than virions from feces (20). The HEV genome consist of a short 5' untranslated region (UTR) about 26 nucleotides in length that was not reported in genotype 2 (Mexican strain), 3' untranslated region (UTR) about 65 nucleotides in length that various among isolates, and 3 open reading frames as ORF1, ORF2, and ORF3 which begin at the 5'end (21). The region of ORF3 overlaps with ORF1 and ORF2 whereas ORF2 is not overlap with ORF1. Recently, there was reported of ORF4 that was identified from genotype 1only (22). Each of ORFs has unique functions which ORF1 encodes the polyprotein (non-structural proteins), ORF2 encodes the structural protein (capsid protein), and ORF3 encodes a multifuntional protein. In the target cell, Only ORF1 is translated from genomic RNA while ORF2 and ORF3 are translated from bi-cistronic sub-genomic mRNA.

Moreover, the additional untranslated region (UTR) is presence in the intergenic region with cis-element may responsible for sub-genomic promoter as well (23)(Figure 3).

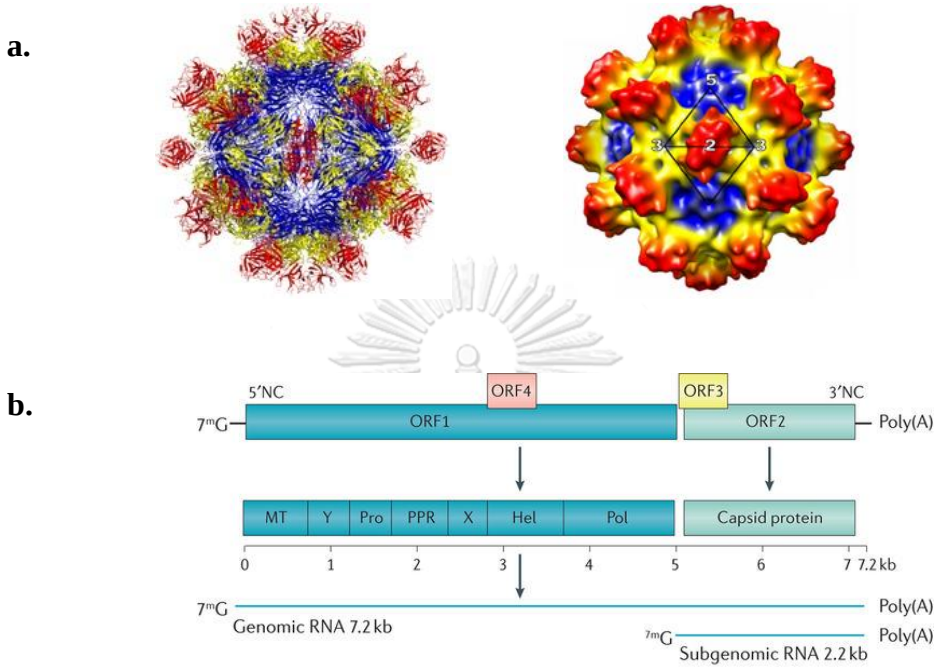


Figure 3 Schematic diagram of the HEV structure and HEV RNA genomic.
(a.) The crytal structure of HEV-like particle and the 14-Å cryo-EM map of HEV particle. (b.) The genomic and subgenomic of the HEV genome
(Modified from Cao, D. et al., 2012 and Guu, TS. et al., 2009 (22), (24), (25)).

Open reading frame 1

First, ORF1 is the largest ORF which located at the end of 5' NCR. It extends around 5,082 bp with a molecular mass of 186 kDa which contains several functional domains and responsible for the encoding of nonstructural protein (1693 amino acid polypeptide). These include the methyltransferase (56-240 aa), Y domain (216-442 aa), papain-like cysteine protease (433-592 aa), proline rich hypervariable region (712-778 aa), X domain (785-942 aa), helicase (960-1204 aa), and RNA dependent RNA polymerase (1207-1693 aa) (11), shown in Figure 4.

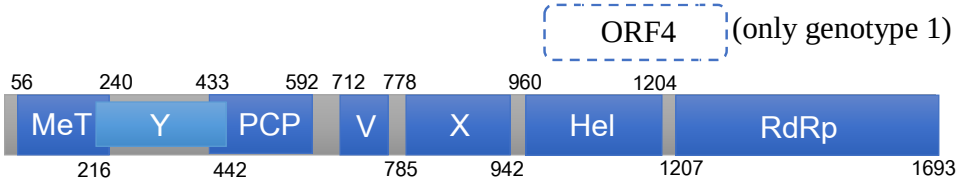


Figure 4 Schematic diagram of HEV ORF1 protein domains (Modified from Ahmad, I. et al., 2011 (26)).

In addition, there are Y domain and X domain which now called the macro domain, are remain unknown function, and hypervariable region shows high variation of amino acid among HEV isolates as well (24). The function of HEV ORF1 product are conflicting that the function are processed by polyprotein or smaller units. The recent study reported the detection of the cleaved ORF1 product inside the HEV infected cells by using specific antibodies against the HEV ORF1 domains. In addition, the proteolysis of ORF1 polyprotein was demonstrated intraviral interactome within ORF1 domains by using yeast two hybrid (27). Futhermore, the proline rich hypervariable region was reported that it possible to involve the HEV host tropism because this domain is not conseved among HEV genotype. Thus, the function and processing of ORF1 product during infected cell are still lack and the resposibility of some domains are unknown. However, ORF1 is a crucial for the replication of the HEV, shown in Table 2.

Table 2 Characteristics and functions of HEV ORF1 region (11, 28).

Domain	Protein	Function
Methyltransferase domain (MeT)	methyltransferase	Catalyzes the capping of viral RNA
Y domain (Y)	unknown	No information for the function.
Papain-like cysteine protease (PCP)	Protease	Cleave the HEV ORF1 in polyprotein processing.

Domain	Protein	Function
Hypervariable region proline-rich domain (HYR)	hypervariable region and a proline-rich region	Genetic heterogeneity of HEV (may concern the host range in HEV)
X domain (X)	unknown	No information for the function.
Helicase (Hel)	Helicase	Essential for their replication.
RNA-dependent RNA polymerase domain (RdRp)	Polymerase	Essential for their replication

Open reading frame 2

Second, ORF2 was located at 3'end of the coding region with 660 amino acid in length and responsible to encode the viral capsid protein that has arginine-rich region signal peptide sequence and potential N-linked glycosylated sites, that protein product seem to be a glycoprotein.(21), (24) The structural features of ORF2 consist of three subdomains including the S domain forms the capsid, P1 domain supports the two-fold, three-fold, and five-fold icosahedral symmetries of the capsid. The P2 domain works with P1 domain to build protrusion spikes from the shell to be changeable for polysaccharide-binding and antigenicity determination (Figure 5)(25, 29). ORF2 capsid protein is immunogenic for binding with neutralizing antibody both linear and conformational epitopes that are amino acid residue at C-terminal region of ORF2 (30). In addition, ORF2 has some minor structural difference for four HEV genotype but it has only single serotype of all (29). Hence, ORF2 is the main region which encodes the structural protein to involve the assembly of HEV virus particle, interaction with host cell by ER localization signal and has several epitopes to induce immunity due to vaccine development (31).

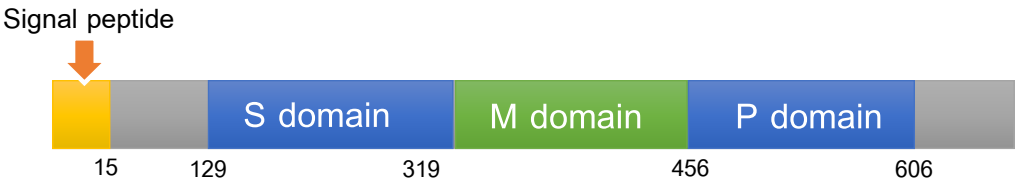


Figure 5 Schematic diagram of HEV ORF2 protein domains
(Modified from Nan, Y. et al., 2016 (32)).

Open reading frame 3

Third, ORF3 is the smallest open reading frame in HEV genomic RNA around 112 amino acid in length that encode a phosphoprotein that is intracellular expression and relate with the cytoskeleton (33). ORF3 overlap with ORF2 in a different frame about 300 nucletides and overlap with ORF1 (23). The third in-frame AUG is the initiation site of ORF3 and it consists of 2 hydrophobic domains (D1 and D2) at the N-terminal and 2 proline-rich domains (P1 and P2) at the C-terminal portion (Figure 6) (34). The biological function of ORF3 protein is unclear. However, the previous study showed the The D1 domain of ORF3 is cysteine-rich and colocalizes with cytoskeleton to bind microtubule and a MAPK pathway phosphatase (35). The D2 domain is binding site for hemopexin (36). P1 domain contain with phosphorylation kinase motif that is phosphorylated by MAPK at Ser-71 during HEV infection (37). The P2 has been reported that contains a proline-rich PxxP motif, bind the Src homology3 (SH3) domains that is a main function in signal transduction pathways, lead to promotion of cell survival (37). Furthermore, it has been showed the study of tumor susceptibility gene 101 (Tsg101) interact with the PSAP motifs of ORF3 in infected cells led to involve in HEV release from infected cell also (38). Consequently, ORF3 protein plays a role in HEV replication, pathogenicity, and viral egress.

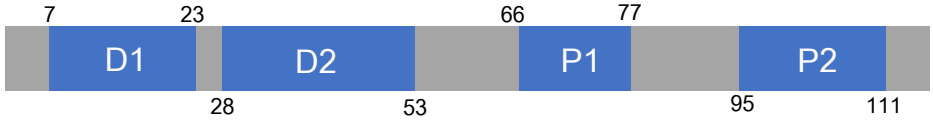


Figure 6 Schematic diagram of HEV ORF3 protein domains
(Modified from Ahmad, I. et al., 2011 (26)).

Open reading frame 4

Recent study demonstrated the new open reading frame in ORF1 region which it is found only in genotype 1. ORF4 was a new finding of viral product that it expressed after endoplasmic reticulum stress inducer in the viral replication process. ORF4 is responsible for interaction with several protein and host proteins for stimulation of viral RdRp activity (22)(Figure 4).

2.3 Replication cycle

Life cycle of HEV is largely unknown as the cell culture system HEV is limited and ineffective. Because HEV is a quasi-enveloped virus lead to enter the target cells by using the different ways. For enveloped HEV, it attached to the cell through clathrin-dependent and dynamin-dependent as receptor-mediated endocytosis. While non-enveloped HEV required the GTPases Ras related protein Rab5A (RAB5) and Ras-related protein Rab7A (RAB7), small GTPases involved in endosomal trafficking, and lysosomal lipid degradation (39, 40). Then the viral enter the cells and believe that the lysosomal lipid is degrade to expose the capsid protein (39). In addition, It has been reported that Hsp90 and Grp78 may be a crucial role in the cellular transportation of HEV capsid trafficking (41). After virus penetrates into the cell, the virion uncoats and positive-sense genomic RNA is released into the cytoplasm then ORF1 translation start to produce non structural protein. The nonstructural protein will be translated to negative sense RNA act as the templates to produce the positive sense subgenomic RNA. This sub-genomic RNA will be translated to produce structural proteins and small multifunctional proteins (11, 24). After that, ORF2 protein packages by binding to the end of the HEV genome may play a role in viral encapsidation (42). Meanwhile, ORF3 is believed to be a crucial role in viral egress by using PSAP motif of ORF3 protein and facilittates the transportation of the HEV virion from infected cell (Figure 7)(43).

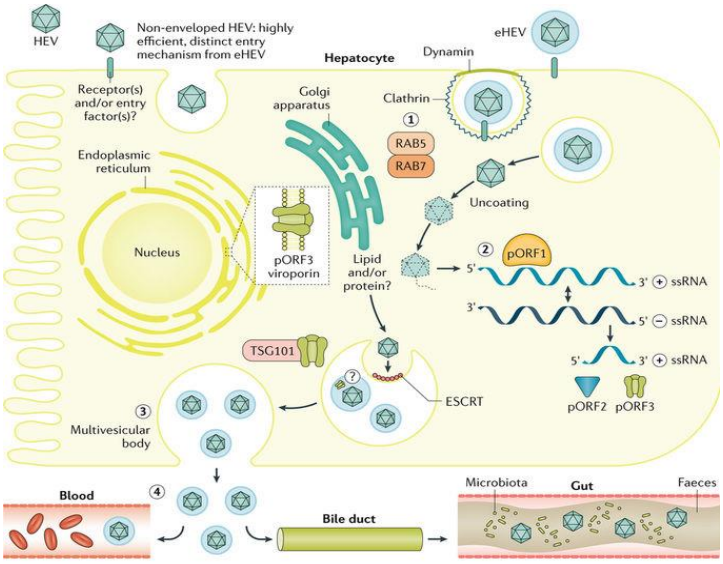


Figure 7 Life cycle of HEV.
(Nimgaonkar, I. et al., 2017 (44)).

2.4 Mode of transmission

HEV is generally transmitted via fecal-oral route as same as the Hepatitis A virus. In addition, HEV could be transmitted by four main routes including water-borne transmission, vertical transmission, blood borne transmission and zoonotic transmission (45). There are the distinct transmission and epidemiology pattern among the geographic regions. In developing countries where have a public health problem, there are mainly prevalent with genotype 1 and 2 by contamination in water and food. HEV genotype 1 is mostly found in Asia, while genotype 2 is more common in Africa and Mexico (46). In case in endemic area, vertical transmission is usually occurred during epidemic or the outbreak of HEV led to increase incidence and severity in pregnant women in third trimester. Additional, HEV genotype 1 causes outbreaks in non-industrialized countries as a result of contaminated drinking water (47). HEV genotypes 1 and 2 have been identified from human cases only.

In developed countries, hepatitis E genotype 3 and 4 are considered a prevalent such as Western Europe, North America, and Japan. The outbreaks have not been reported but there have been only sporadic reported cases. The mode of transmission is mainly through the consumption of contaminated meat or direct contact with wild

animals or domestic swine is the main reservoir (45). In addition, shell fish was considered to be a reservoir of this virus (48). Moreover, blood-borne transfusion and organ transplantation have been reported in recently. There were the reported cases of patients who received blood product from infected donor in Japan, France, Saudi Arabia, etc. (49-51). The risk of HEV infection by blood transfusion is varies according to recipients or patients who received blood product. Although, HEV is usually self-limiting and less virulent in healthy human but it may be cause of chronic hepatitis in pregnancy, immunocompromise and immunosuppressive patients (52, 53). However, there is no evidence of morbidity in recipients HEV in blood transfusion and transplantation have raised the question whether it should be investigated for HEV before give blood or organ to recipients or not.

2.5 Course of infection and clinical presentation

The infection of HEV is initiated at gastrointestinal mucosa absorbed HEV into the circulation to the liver for viral replication. HEV generally replicate in cytoplasm of hepatocyte without causing direct cytolytic damage that the pathogenesis may occur due to immune system rather than viral itself (54, 55). In the early infection, the level of alanine transaminase (ALT) increased similar with HEV RNA can be detectable in blood and stool for 4- 6 weeks (4 weeks for blood and 6 weeks for feces). Then the titer of anti-HEV IgM rose rapidly after infection during symptomatic phase. Anti-HEV IgG gradually rose beginning 2 weeks post-infection. Anti-HEV IgM decline to normal level within 3-6 months similar to ALT but anti-HEV IgG titers remain detectable up to 15 years (Figure 8)(55-57).

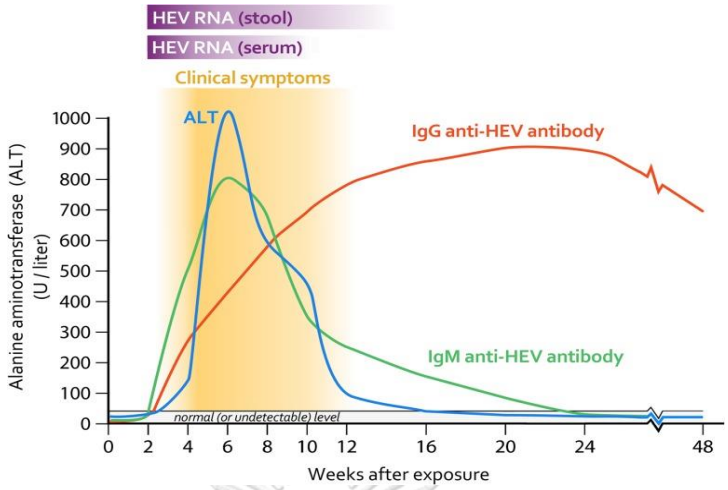


Figure 8 Course of acute HEV infection
(Krain, LJ. et al., 2014 (55)).

The most common manifestations are self-limited acute hepatitis. In case of the patients who were infected with HEV, symptoms could not be observed within the first month after infection, but after 4-5 weeks (incubation period) patients showed signs of the symptoms, like anorexia, fever, abdominal pain, jaundice and finally, acute icteric viral hepatitis (58). On the other hand, if it occurs in immunocompromised and immunosuppressive patients, such as human immunodeficiency virus (HIV) infection, malignancy and organ transplant patients, they could develop into a chronic hepatitis E infection with mortality rate around 1-2% (59, 60). Also, the mortality rate during pregnancy was higher and associated with hormonal and immunological changes (53). Moreover, there are many reports that showed the extrahepatic symptoms including pancreatitis, neurological system, haematological disorders etc.(61). However, these findings are complicate and still unclear.

2.6 Epidemiological of HEV and outbreaks

Outbreaks

The HEV was thought to be cause of the outbreak in only the developing countries but nowadays the industrialize countries were recognized to have the HEV infection in these countries (62). The outbreaks of HEV infection were reported in several areas that it usually occurred in the resource limited countries including Asia,

Africa, America which attributed to genotype 1 and 2. And the size of outbreak is associated with the quality of sanitation and density of the population.

The first outbreak was described in New Delhi, India (1957) concerned with the contaminated drinking water lead to have 29,000 icteric patients and ET-NANB hepatitis virus seem to be the cause of infection (63, 64). Then, a large-scale of outbreak was occurred in 1970s in the Kashmir Valley of India which spread through 200 village and the cause of infection was ET-NANB hepatitis virus by drinking water. Since then, several serologically confirmed outbreaks and sporadic cases and ET-NANB hepatitis virus remained to be the etiological agent of acute hepatitis infection in several times in Nepal, Burma, Mexico, and China. In 1983, the outbreak was occurred in the Afghanistan and discovered that the ET-NANB hepatitis virus could infected by faecal-oral route and the immune microscopy was reported the result as the spherical virus-like particles (1). In 1989, the ET-NANB virus was ordinally specified as HEV. After that, it was circulated and usually found contamination in water supply and low quality of sanitation system and HEV was suspect to be the cause of acute viral hepatitis.

In Africa, the outbreak of HEV was occurred in refugee camps, Tusnisia which spread to 11 countries in Africa (65). Then, a half of African countries have been reported the outbreaks since the HEV diagnosis assays became available for routine surveillance (66). In North America, only two outbreaks were reported in Mexico with many icteric cases and death reports. Then, HEV genotype 2 was recognized in this area.

The outbreak in Asia was reported especially in Central and Southern Asia with large outbreaks and sporadic cases. The largest outbreak was occurred in India, 1990 which an attack rate was 3.76% with suspected cases around 79,000 cases (67). For the outbreak, the adult male are more likely to be infected with HEV more than female and often occurred in rural areas (68).

Epidemiological

The global burden was reported the HEV infection around 3.3 million symptomatic cases and estimate 44,000 deaths in 2015 (60). The epidemiological of HEV has two distinct patterns in different geographical areas which the prevalence of

HEV genotype was different and these may lead to determine the difference of the epidemiological in each area (Table 3 and Figure 9) (69).

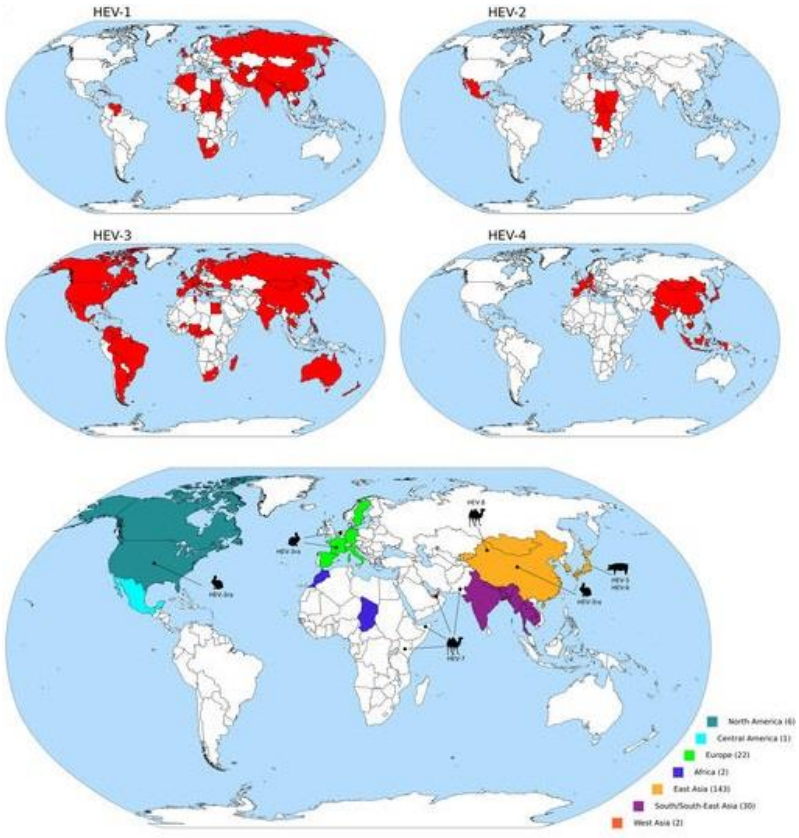


Figure 9 Geographical distribution of HEV genotypes 1 to 4 and the geographic origin of *Orthohepevirus* A genome.
(Modified from Forni, D. et al., 2018 (70)).

Hyperendemic regions

The outbreak of HEV infection in these regions often related to consumption of HEV contaminated drinking water especially follow the heavy rainfall and floods or the drinking the water from the river that was contaminated with the disposal of human excreta into the rivers (71). In hyperendemic area has a high attack rate and mortality among pregnant women with fulminant liver disease. Moreover, acute sporadic hepatitis was found in all age groups which the reason is remain unclear (72). For the hyperendemic regions, 50% of the deaths of hepatitis was used to indicate the highly endemic which in East and South Asia were estimated 160,000 deaths every year. The attack rates in these areas have ranged from 1% to 15% during hepatitis E outbreaks

which genotype 1 or 2 were the main cause of the outbreaks and genotype 4 was suspected to infect in swine lead to the reservoir of the virus (45). The hyperendemic of HEV was considered in several developing countries including India, Bangladesh, Egypt, Mexico, and China. Moreover, Western Europe, New Zealand, South America and some of Asia and the Middle East were include to be the endemic regions shown in Figure 9 (73).

Low endemic regions

In these regions, the outbreak never occurred and the cases were the autochthonous cases which related to travel in the hyperendemic areas. These areas include the USA, Europe (UK, France, the Netherland, Austria, Spain, Greece and German) and Asian-Pacific countries (Japan, Taiwan, Korea, Hong Kong, Australia and New Zealand) (69). The prevalent of HEV infection was reported that genotype 3 and 4 were cause of infection and only limited to sporadic cases. The virulent of genotype 3 and 4 seem to be less than genotype 1 and 2 which genotype 3 and 4 related to zoonotic transmission and blood borne transmission (Figure 9). Genotype 3 and 4 are mainly transmitted by consumption of HEV contaminated food products which swine was the major reservoir of HEV (74). In addition, the transfusion of HEV contaminated blood products was suggested a potential risk of HEV transmission (75). Recently, the reservoirs of HEV genotype 3 and 4 were reported and showed the evidence linking the onset of hepatitis E to the consumption of contaminated food such as wild boar and deer (76, 77). In these areas, the incidence of hepatitis A and B declined while the incidence of hepatitis E was increasing that hepatitis E may be the common cause of hepatitis in future.

Table 3 The difference of HEV epidemiological between hyperendemic regions and endemic regions
(Aggarwal, R. et al., 2009 (69)).

Feature	Endemic regions	Non-endemic regions
Geographical locations	Underdeveloped countries mostly in Asia and Africa	Developed countries in Europe, North America, parts of Asia, Australia
Epidemiological patterns	Large epidemics, small outbreaks and sporadic cases	Only sporadic cases
Water-borne transmission	Well known, most common route	Unknown
Animal to human transmission	Not reported	Shown; a very likely mode of transmission
Animal reservoir	No	Yes
Virus genotype	Almost entirely genotypes 1 and 2, a few cases of genotype 4 in China	Genotype 3; occasional cases of genotype 4 in Taiwan
Age group	Young men most commonly affected	Usually elderly
Chronic infection	Not known	Reported in transplant recipients receiving immunosuppressive drugs
Severity	Variable severity, including fulminant hepatic failure	Severity and poor outcome is related to coexistent disease conditions
Relationship with pregnancy	Particularly high rates of symptomatic disease and of more severe disease in pregnant women than in men and non-pregnant women	No data on pregnant women

HEV in Thailand

In Thailand, the data on HEV infection prevelance is limitted. However, the trend to conduct studies on this matter is increasing. One study showed that occurrence of the infection in acute hepatitis patients was 4.2%, especially in the elder group (78). In the same way, in another study examining high risk groups, like patients with immunocompromise, liver cirrhosis and elderly patients, they found that these subjects have a higher mortality rate. Similary, the 90-day motality rate in very elderly, cirrhotic and immune-compromised patients and longer duration of hospital stay are higher motality rate than the low risk group (79). In addition, a slight seroprevalence in Thailand has been reported since 1996. The first report by our group showed a prevalence of anti-HEV IgG, which was 9%-22% in several adult subjects and that prevalance increased with age (79). Another group examined, the Hmong people, showed a seroprevalence rate of 6.5% (80). Similarly, the healthy adults who lived in the central part of Thailand whom were investigated for anti- HEV IgG, showed a prevalence of 23.3% (81). So, by conducting our research, we try to find the possible source of HEV transmittion to human in Thailand (82).

2.7 Genetic evolution and mutation of HEV

Hepatitis E virus is an RNA virus and the its genome sequence around 7,000 nt. The mutation rate of HEV is nearly similar to hepatitis C virus which pose at a high mutation rate around 1.5 base substitutions per site per year (83). The genomic sequence of HEV that isolated from various host, has genomic diversity among animal species. In addition, the sequence from chronic hepatitis E infection has shown the viral strains carrying gene insertion from human genes which take advantage for adaptive in culture and might be the reason to expands the host range and tropism of virus (84). Moreover, the recombination and genomic intermixing of HEV were reported that it is high level to take place in patients and animal (85).

ORF1 variation and mutation

For ORF1 genome, 80-86% identity of ORF1 protein was reported among HEV genotype 1 to 4. And there are the sequence variation of genotype 3 and 4 have more sequence variation and longer than genotype 1 and 2 (14). Additionally, each of the functional domain in ORF1 region has a difference of an amino acid sequence which MeT and Y domain are highly conserved more than PCP, macro domain, Hel, and RdRp, the viral RdRp of HEV lacks the proof-reading capacity lead to have the high mutation rate as well (86). For the hypervariable region (HVR), the structural and functional of HVR are more flexibility which this region has various size and amino acid mutation both insertion and deletion among HEV genotypes. In addition, the interaction between host factor and viral were reported in HVRs region to modulate the HEV replication in cell culture (87, 88). Nevertheless, the key functional domain sites remain conserved. For the mutation, the transcription process is a cause of the high variability and the selection of mutation in the HEV genome as shown in Table 4. Moreover, the selection pressure from antiviral drug especially ribavirin and the immune response of host may increase the variability of HEV genome (86).

ORF2 variation and mutation

ORF2 encodes the structural protein of virion around 660 amino acid that is the most highly conserved among ORFs protein in HEV. The sequence identity of HEV genotype shares 92-93%, 90-91%, 89-92% when compared HEV genotype 1 with HEV

genotype 2, 3, and 4, respectively (89). Three point mutations under selective immune pressure was reported including T5338C, A5362G, and C6356T which were a cause of amino acid change, may affect to the viral protein function (90). In addition, the non-synonymous substitution of ORF2 region was shown several positions that some of mutation points affect to the dimerization of ORF2 protein structure shown in Table 4. These may involve the adaptive of HEV to escape from host immune response, viral replication, and infectivity of HEV (91). Moreover, the mutation concerned with HEV-neutralizing epitope may affect to the vaccine that constructed based on ORF2 fragment as well (92).

ORF3 variation and mutation

The ORF3 protein is multifunctional that crucial to promote cell survival, regulation of HEV replication and infectivity, interacts with host cell, and proliferation of HEV so the expression of ORF3 protein may concern the HEV pathogenicity (24). In the same genotype of HEV, the ORF3 amino acid sequence is highly conserved and the identities of among HEV genotype 1, 2, and 3 ranged from 72-79% to 83-86%, while the amino acid sequences within genotype were 96.5-99.6%, 92-100%, and 83-100%, respectively (89) Although ORF3 was reported conserve region, other finding showed that the ORF2-ORF3 overlapping region was more strong evidence of variation in selective pressure (93). The ORF3 almost entirely overlaps ORF2 that the mutation of ORF3 associates with ORF2 due to the overlapping of ORF2 and ORF3 so some of mutation in ORF3 region also affect to ORF2 protein; example the mutation S80A also affect to the V66G mutation in the ORF2 that related with the HEV assembly (94). Furthermore, there are the mutation of ORF3 and overlapping regions are concerned with ORF2 and ORF3 protein production result in the HEV infectivity shown in Table 4 (94) The mutation in the interaction region between ORF3 protein and host factors may stimulate and enhance the immune response of host that may be the explanation of the self-limitation in the HEV infection. However, the data still unclear.

Table 4 HEV mutations and functional significance.
(van Tong H, et al., 2016 (95)).

. Substitution/mutation	Amino acid change	Domain/region	Functional significance	References
NA	H443L; C457A; C459A; C471A; C472A; C481A; C483A; H497L; H590L	PCP/OFR1	Completely abolish HEV replication by modifying the enzyme structure	(96, 97)
Insertion/deletion	NA	HVR/ORF1	Associated with HEV attenuation	(87)
Complete deletion	NA	HVR/ORF1	Abolish HEV infectivity but not influence HEV replication	(88)
Insertion/deletion of a 24 bp RdRp-derived fragment	NA	HVR/ORF1	Decrease HEV replication	(98)
NA	N809A; H812L; G816A/V; G817A/V	X/OFR1	Completely abolish HEV replication	(96, 99)
NA	L1110F; V1120I	Hel/ORF1	Decrease HEV replication by affecting the ATPase activity but not the RNA duplex unwinding activity of helicase enzyme	(100)
Deletion	NA	Hel/ORF1	Decrease HEV replication impairing the ATPase and unwinding activities of helicase enzyme	(101)
NA	K1383N	RdRp/ORF1	Reduces viral replication and increases ribavirin sensitivity	(98)
NA	Y1320H; G1634R/K	RdRp/ORF1	Increased efficiency of viral replication and infectivity	(91, 101, 102)
T5338C	F51L	ORF2	Decrease HEV replication and infectivity by affecting viral genomic RNA packaging	(103, 104)
A5362G	T59A	ORF2	Decrease HEV replication and infectivity by affecting viral genomic RNA packaging	(90, 105)
C6356T	S390L	ORF2	Decrease HEV replication and infectivity by preventing host virus interaction	(103, 104)
NA	N137Q; N310Q; N311Q	ORF2	Prevent glycosylation of capsid protein and formation of HEV particles	(106)
NA	N562Q/D/P/Y	ORF2	Affect the dimerization of ORF2 protein and HEV infectivity	(106)

Substitution/mutation	Amino acid change	Domain/region	Functional significance	References
NA	L477T; L613T	ORF2	Influence immunoreactivity of HEV by affecting the neutralization epitope	(107, 108)
NA	V606A	ORF2		(94)
A5145C; A5178C; A5190C; G5676T; T5690G	NA	ORF2–ORF3	Abolish the ORF2 production (but not ORF3)	(94)
CGC5148–5150AGA	NA	ORF2–ORF3	Abolish ORF3 production (but not ORF2)	(94)
A5108Δ; T5109C; C5112U; TCT5116–5118AGC; T5121C	NA	ORF2–ORF3	Abolish both production of both ORF2 and ORF3	(94)
NA	S80A (V66G)	ORF3 (ORF2)	May affect the regulatory role of ORF3 protein in HEV assembly, influence ORF2–ORF3 interaction	(94, 109)
G5101U; U5100C; C5117G; U5118G	NA	CRE/ORF3	Affect HEV replication and infectivity by modifying the CRE structure	(110)
G6574C; C6570G; G7106T/A; G7097A; C7144A	NA	CRE/ORF2	Affect HEV replication and infectivity by modifying the CRE structure	(111-113)

2.8 Treatment and Prevention of HEV

Generally, Hepatitis E is usually self-limiting so the drug for treatment is not need. However, it still has not got specific therapy but in cases of chronic infection were chosen ribavirin and pegylated interferon for the first line drug, could not use for all patient especially organ transplantation (114). For prevention, the providing of good sanitary in developing countries is important and decreased the viral spreading such as clean drinking water, good sanitation, and proper personal hygiene, etc. (46). In developed countries, there are many possible routes of infection which the data are insufficient led to the prevention of these countries is not cleared. Besides, vaccine was performed but it is used in limited and not approved to use worldwide (45).

Summary and significance of this study

Presently, the HEV infection has occurred in developed countries and the number of sporadic cases is increasing in which the route of infection is impossible. Moreover, the HEV is spread by the fecal-oral route similar to hepatitis A virus so the numbers of hepatitis E infection is underestimated. Additional, the fecal–oral route is the primary and most well-documented mode of HEV transmission but currently there were the report of the other routes including zoonotic transmission, food-borne transmission, and blood-borne transmission. Besides humans, HEV have been isolated from various animal such as domestic swine, wild boar, deer, chicken, rat, ferret, rabbit, and camel. In Thailand, the number of HEV infection is reported over the world led to Thai researcher to interests in HEV. Nevertheless, the prevalence and the mode of HEV transmission is still unclear. Hence, this study aimed to investigate the prevalence of HEV infection in Thailand by followed the possible route that usually found in Thailand. Moreover, the relationship between genomic of HEV in human and reservoir is performed to support the source of transmission also. Because of HEV is concern to daily life so the benefit of this study is assisting awareness and facilitating disease prevention and control.

CHAPTER III
EXPERIMENTAL RESULTS

Part I: Hepatitis E virus in pork and variety meats sold in fresh markets in Thailand

(Publish in: Food and Environmental Virology. 2017 Mar; 9(1):45-53)

Summary

Swine is an economically important livestock, yet pork consumption and close contact with pigs are associated with hepatitis E virus (HEV) transmission. Due to the limited data on the prevalence of HEV in pigs in Southeast Asia, we examined HEV in porcine tissues obtained from fresh markets and slaughterhouse in the Bangkok metropolitan area. A total of 1,090 liver, 559 pork meat and 556 intestines from fresh markets; 720 bile and 553 fecal samples from slaughterhouse were collected from November 2014 to February 2015. Amplification of the HEV ORF2 gene using nested PCR found 55 positive samples (1.58%). Fecal and bile samples were more likely to test positive compared to liver, pork meat, and intestine samples ($p < 0.001$). Characterization of HEV by phylogenetic analysis showed that all sequences clustered in a distinct group closely related to the 3f sub-genotype. Interestingly, HEV from 1 liver and 2 fecal samples possessed ORF1 of genotype 3i, but ORF2 of genotype 3f. Pork and variety meats derived from pigs sold in fresh markets are common throughout Southeast Asia. Therefore, better public health awareness is required towards reducing HEV transmission. In conclusion, this study had found the contamination of HEV RNA in the swine samples collected from the open market and slaughterhouse in a low level of prevalence. However, there is a possibility that HEV could be transmitted from swine to man through consumption or contact. It is suggested that there should be public health measure to have a standard action for the prevention of the disease and public education to prevent the infection from the diseases.

Background and Rationale

The global burden of HEV infection is estimated at >20 million, which results in 3.4 million symptomatic illness and 70,000 deaths annually (60). Although most HEV infection is self-limiting and spontaneously resolves (57), some individuals may experience symptoms such as anorexia, fever, abdominal pain, jaundice, and acute icteric viral hepatitis (58). Individuals who are immunocompromised, elderly, HIV-positive, as well as those with cancer, organ transplants, or liver cirrhosis are more susceptible to complications from HEV. Chronic hepatitis E infection is associated with higher mortality rate and longer hospital stay (59, 79), while HEV infection during pregnancy is especially critical for the well-being of the mother and the baby (53). There are 4 genotypes described that infect humans and all genotype represent only one serotype (47). Human-restricted HEV genotypes 1 and 2 are found mainly as outbreaks in low-income countries of Africa, the Americas, and parts of Asia (45). In contrast, genotypes 3 and 4 are often found in sporadic cases of autochthonous HEV in both developing and industrialized countries (115). To date, the presence of HEV in pigs, wild boars, deer, rats, rabbits, and chicken has been reported (116-121). HEV isolated from humans have shown high resemblance to the strains isolated in swine, particularly genotype 3 (82, 122). A strong zoonotic potential for HEV transmission to human includes an association between the consumption of contaminated pork products, variety meats, undercooked sausage and offal in France (123, 124) and in Japan (125). In addition, the prevalence of HEV in swine samples in other countries is shown in Table 5.

Evidence-based data suggest that HEV infection represents approximately 4.2% of suspected acute hepatitis patients in Thailand (78). Past exposures to HEV among Thais have examined the prevalence of anti-HEV IgG, which was 15.7% in the general population (126) and 14% among young men of military age (127). As the sero-prevalence appears to increase with age, risk of exposure to HEV may be cumulative over time. HEV found in Thailand thus far has been genotype 3 and a possible source of human infection may come from swine (82, 128-130). Fecal samples from Thai wild boars have also tested positive for HEV genotype 3 (131). Since there are limited data on the potential for HEV zoonosis from pork consumption, we will assess the apparent prevalence of HEV in pork and variety meats sold in fresh markets in the Bangkok

metropolitan area in comparison to bile and feces samples obtained from a slaughterhouse.

Table 5 Incidence of detectable HEV in swine samples in other countries.

Region	Year of study	Countries	Sample Type	Sample size	% of positive detections	Genotype	Ref.
Europe	2001	Netherlands	F	115	22.0	NA	(132)
	2005-2009	Hungary	F,L	248, 45	21.0, 31.0	3a, 3e, 3h	(133)
	2006	Italy	BI	137	29.9	3c ,3e, 3f	(134)
	2007	France	F,SE	207,215	65.0, 22.0	3e,3f	(135)
	2009-2010	Croatia	BL,SP,L	848	24.5	NA	(136)
	2010-2011	Portugal	F	200	10.0-30.0	3c	(137)
	2012-2014	Italy	F	242	18.6	3c,3e,3f,3h	(138)
	2012	Italy	F	15	73.3	3e, 3f	(139)
	2013	UK	P, C	629	15.0, 3.0	3c	(140)
	2014	Germany	F	120	2.5	3i	(141)
Asia	2003	Japan	L	363	1.9	3 and 4	(125)
	2005	Thailand	SE	76	13.1	3	(130)
	2007	India	L	240	0.83	4	(142)
	2007	China	F	65	23.0	3,4	(143)
	2009	Thailand	SE, F	258, 10	7.75, 70	3	(129)
	2009	China	L	114	3.5	4	(144)
	2011-2012	Thailand	F	237	1.27	3f	(128)
	2011-2013	China	BI	228	6.5-22.7	4	(145)
	2013	Thailand	F	875	2.9	3	(146)
Africa	2009	Congo	F	40	2.5	3c	(147)
	2011-2012	Nigeria	SE	90	76.7	3	(148)
	2013	Madagascar	L	250	1.2	3	(122)
	2013	Faso	L	157	1.0	3	(149)
America	2001	U.S.	F, SE	96	35.0	US.strain	(150)
	2010-2013	Mexico	F	40	0	NA	(151)
	2014	Canada	L	283	4.9	NA	(152)

F = feces, L = liver, BI = bile, BL = blood, SP = spleen, P = plasma, C = cecal content, SE = serum, M = meat, NA = not applicable

Research Questions

1. Is the domestic swine the source of HEV infection in Thailand?
2. What is the prevalence of HEV in pig samples collected from markets and slaughterhouses in Bangkok?

Objective

To investigate HEV in pork and variety meats from fresh markets and bile and feces from a slaughterhouse in Bangkok.

Expected benefits and applications

Potential zoonotic transmission of HEV especially from autochthonous infection in industrialized countries is generally recognized from studies in experimental pig model and foodstuff containing pork products. As pigs comprise an economically important food animal, it is necessary to quantify the HEV risk in order to evaluate the zoonotic potential. In Thailand, limited data on possible contamination of HEV in pork and variety meats sold to consumers led us to analyze HEV in swine samples. Although most meats consumed in Thailand are prepared cooked, inadequate cooking and cross-contamination from affected pork products may allow inadvertent transmission of HEV. This informative data will provide the prevalence of HEV in Thailand. Including the evidence indicates that epidemiology may play a crucial role to help prevention of HEV infections in Thailand.

Materials and Methods

Swine samples

The age at slaughter for most pigs destined for the market is typically 6 to 8 months. A total of 3,478 random samples comprising liver, pork, and intestine from six fresh markets (Khlong Toei, Suanplu, Bang Kruai, Bang Po, Bangson and Taopoon) located in the Bangkok metropolitan area. In addition, random samples comprising bile and feces were obtained from the nearest local slaughterhouse in Khlong Toei district. To avoid cross-contamination, samples were collected in individual packed and always processed on the same day. Sample types were process separately (e.g. RNA isolation from pork samples was completed before handling liver samples) and the biosafety cabinets were UV-irradiated daily.

Figure 10 A typical fresh market**.** Meats including pork and offal are sold in open-aired markets typically found in Thailand.

Sample size estimation

The HEV prevalence values have been reported by many countries. However, the prevalence of HEV in Thailand and other countries were shown in previous study that I calculate the sample size by using average value in each type of sample as follows: in liver 6.3%, intestine 3.0%, pork 15.0%, bile 26.3%, and feces 25.5%**.** Then, the calculation used the following formula for the sample size n:

$$n = \frac{Z\alpha^2 \, P \, (1\text{-}P)}{e^2}$$

n = required a sample size

$Z\alpha$ = confidence level at 95% (standard Z value of 1.96 (two-tail))

P = incidence proportion HEV infection estimated from previous study

e = acceptable margin of error at 5% (standard value of 0.05)

Therefore, samples should be collected including liver 87 samples, pork 196 samples, intestine 45 samples, bile 296 samples, and 288 feces samples. Each type of samples was minimum required for the HEV screening to achieve the statistically supported results with 5% acceptable error.

Research Methodology

Ethical Considerations Specimens collected in this study are from non-human subjects and obtained from fresh markets (for retail sale to consumers) and local slaughterhouse. Therefore, the IRB for this study is waived. All the specimens were treated as infectious materials, handled under the laboratory biohazard safety recommendation, and process in the bio-safety level 2 environments.

Detection of HEV nucleic acid

Liver, pork, and intestine samples were minced into small pieces (~1 mm^3) and digested with proteinase K solution for 30 minutes at 50°C. Fecal samples were suspended in PBS (pH 7.4) and bile samples are ready to use. 200 µl was for RNA extraction with Ribospin vRD II (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Complementary DNAs was synthesized using ImProm-II reverse transcriptase (Promega, Madison, WI). Briefly, the RNA template and random hexamers were incubated at 70ºC for 5 minutes and chilled on ice at 4ºC for 5 minutes. Reverse transcription mix was added and the reaction was incubated at 25ºC for 5 minutes. The extension was performed at 42ºC for 2 hours and the reaction was inactivated at 70ºC for 15 minutes.

The presence of HEV was determined by semi-nested PCR for HEV ORF2 using published primers known to detect all HEV genotypes (153). In the first round of PCR, amplification using the forward primer HE040 and reverse primer HE044 yielded ~500 bp products. In the second round of PCR, HE040 and another reverse primer HE041 produced a 467 bp product (Table 6). Amplification of the housekeeping β-actin gene was served as an internal control. Samples initially tested positive was verified by ORF1 amplification using the forward primer HE_1 and a mixture of reverse primers HE_4 and HE_5 to yield 542 bp amplicon, followed by a nested PCR using the forward primer HE_2 and reverse primers HE_3 and HE_6 to yield ~370 bp (Table 6) (154). PCR products were resolved by 2% agarose gel electrophoresis, purified, and sequenced by First BASE Laboratories (Seri Kembangan, Selangor, Malaysia). Sequences were subjected to BLAST analysis tool in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 6 Sequence of HEV primers for nested PCR and semi-nested PCR. (153, 154)

Primer	Sequence 5'- 3'	Position	Size (bp)
ORF1			
Outer primer			
HE5-1	TCG ATG CCA TGG AGG CCC A	2-20	541
HE5-4	CAT AGC CTC SGC RAC ATC AG	524-543	
HE5-5	CAT YGC CTC SGC AAC ATC GG	524-543	
Inner primer			
HE5-2	GCC YTK GCG AAT GCT GTG G	88-106	365
HE5-3	TCR AAR CAG TAR GTG CGG TC	433-452	
HE5-6	TYA AAA CAG TAG GTT CGA TC	433-452	
ORF2			
HE044	CAA GGH TGG CGY TCK GTT GAG AC	5924-5946	506
HE040	CCC TTR TCC TGC TGA GCR TTC TC	5327-5345	467
HE041	TTM ACW GTC RGC TCG CCA TTG GC		
House keeping gene			
Beta-actin/F	AGA AGA TGA CCC AGA TCA TG	483-502	577
Beta-actin/R	CTT GCT GAT CCA CAT CTG CT	1040-1059	

Phylogenetic analysis

All sequences were analyzed using Chromas LITE program (v2.1.1), aligned using BioEdit program (v7.2.5), and compared to known HEV sequences available in GenBank. Phylogenetic trees were constructed from Clustal W alignments of partial nucleotide sequences using the neighbor-joining method implemented in MEGA (v5.2). Bootstrapping was applied with 1,000 replicates to support tree topology.

Statistical Analysis

Descriptive statistics analysis was performed using SPSS software for Windows 22.0 (SPSS Inc., Chicago, IL). For the comparison of variables between different sources and types of sample, χ^2-test was used. Data was considered statistically significant at p-value ≤ 0.05.

RESULTS

Detection of HEV nucleic acid

We obtained liver, pork, and intestine samples from all markets except market 6 in which only liver samples were available (Table 7).

Table 7 Number and source of swine samples.

Source	Sample Type	No. of sample	Market 1	Market 2	Market 3	Market 4	Others
Markets	Liver	1,090	179	190	189	521	11
	Pork	559	182	189	186	1	1
	Intestine	556	178	187	189	1	1
Slaughter	Bile	720	720	-	-	-	-
	Feces	553	553	-	-	-	-

Market 1 (Khlong Toei), Market 2 (Suanplu), Market 3 (Bang Kruai), Market 4 (Bang Po) and Others (Bangson and Taopoon).

In all, HEV nucleic acid was found in 1.58% (55/3,478) of samples (Table 8). Among the positive samples, 5.2% of feces (29/553), 2.9% of bile (21/720), 0.28% of liver (3/1090), and 0.36% of pork (2/559) samples were tested positive for HEV by using ORF2 regions. None of the intestine samples was tested positive for HEV. In addition, a significant difference was observed between each type that we found the number of positive in Feces and bile more than from liver, pork, and intestine.

Table 8 Prevalence of detectable HEV in Thai swine samples.

Source	Type of sample	Total	No. RNA Positive	Prevalence (%)	*p*-value
Market	Liver	1,090	3	0.28	0.406[a]
	Pork	559	2	0.36	
	Intestine	556	0	0	
	Total	2,205	5	0.23	
Slaughterhouse	Bile	720	21	2.92	0.034[b]
	Feces	553	29	5.24	
	Total	1,273	50	3.93	

[a] Denote no significant difference
[b] Denote significant difference

Sequence analysis of swine ORF1 and ORF2

All positive samples were not sent to sequence because some sequences had low viral load. So, we had 49/54 sequences from ORF1 and 49/54 sequences from ORF2 which 42 sequences were the same samples. Furthermore, either ORF1 or ORF2 sequences were available from an additional 13 samples. Phylogenetic analysis of the HEV ORF1 suggested that the strains belonged to genotype 3 as they clustered with reference sequences previously isolated from swine and HEV-infected patients in Thailand (Figure 11). Analysis based on 320 bps from ORF1 showed approximately 91-94% nucleotide identity with reference sequences. Genotype 3 is subdivided into 10 subtypes (3a-3j) (14, 155, 156). Most HEV strains isolated from swine were closely related to FJ653660 and GU947815 strain from a human. However, some samples clustered with another reference sequence such as EU495148, EU375463 and JN671918 isolated from France and Thailand. In particular, 3 strains appeared closely related to the KR362698 isolated from patient in Netherlands and FJ705359 isolated from a wild boar in Germany and demonstrated 96-98% nucleotide identity. Analysis of ORF2 showed 92-96% sequence identity with the reference strains and clustered with genotype 3f (Figure 12).

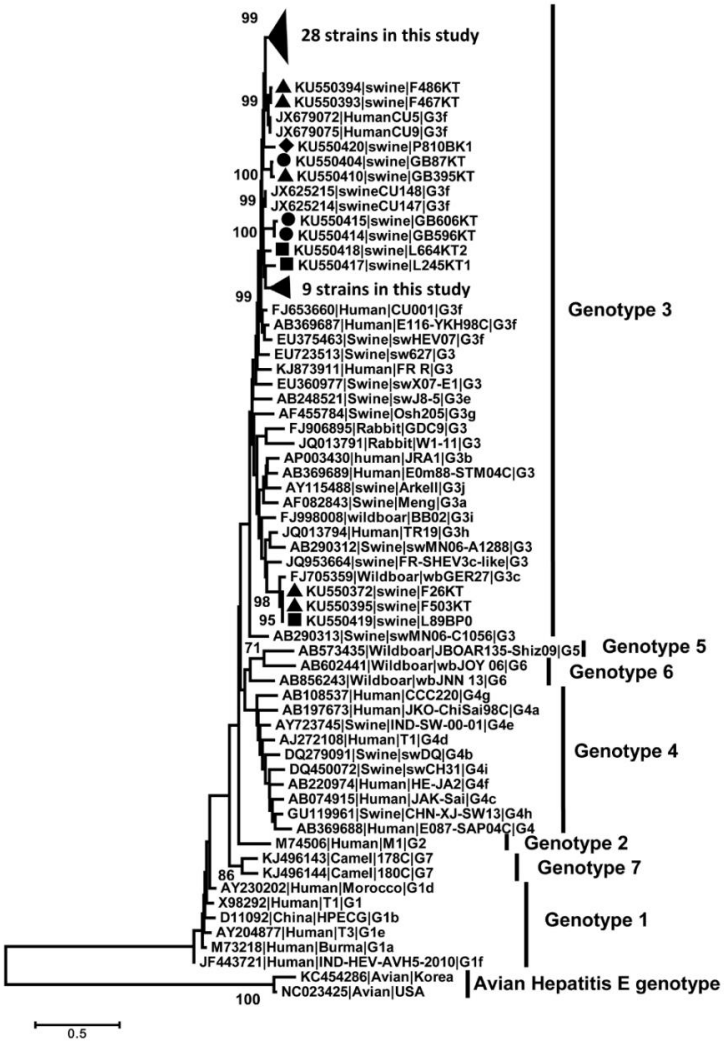


Figure 11 The phylogenetic analysis of the HEV ORF1 region.

Forty-nine partial nucleotide sequences obtained from this study were compared to those available in the GenBank database. Phylogenetic tree was constructed by the neighbor-joining method with bootstrap consensus inferred from 1,000 replicates. Only bootstrap values >70% are shown. Samples from bile (●), feces (▲), pork (◆), and liver (■) are noted. Reference sequences for HEV subtypes (155, 156) are bolded. Two avian HEV sequences represented the outgroup.

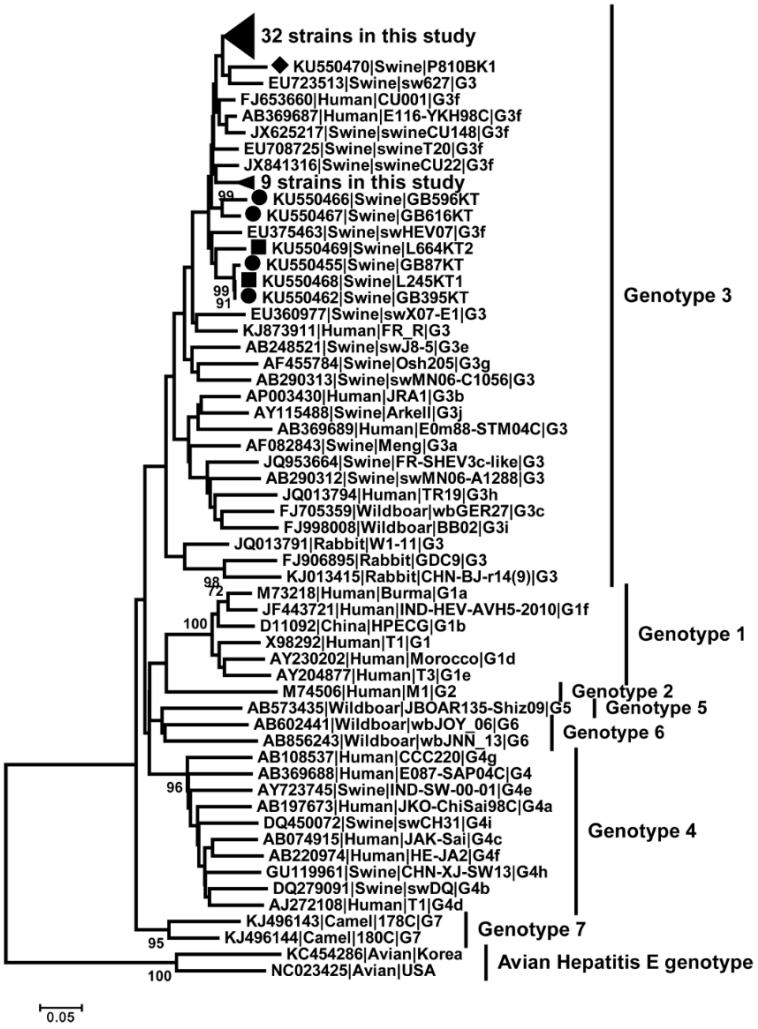


Figure 12 The phylogenetic tree of the partial HEV ORF2 region.
Tree was constructed by the neighbor-joining method with bootstrap values denoted at the nodes. Forty-eight HEV sequences identified from porcine samples were from bile (●), feces (▲), pork (◆), and liver (■).

Incidence of HEV found in swine samples

To determine how frequently HEV is detected in samples derived from pigs, we compiled a list of studies reporting the presence of HEV RNA in porcine-derived specimens (Table 7). As early as 2001, the incidence of HEV infection was reported in both developing and industrialized countries. Overall, the prevalence of porcine-associated HEV ranged from 1.9% to 65% depending on the samples, regions, and

methodologies used. In developed countries, the majority of documented HEV infections were autochthonous and genotype 3 was the most common strain found in both human and swine. In Europe, HEV RNA has been detected in as high as 65% in France and as low as 2.5% in Germany in swine feces. In addition to being found in pork, many studies also showed HEV contamination in food products from wild boar and deer. Among Asian countries, both genotypes 3 and 4 have been characterized and the rates varied by countries and samples analyzed. Although fewer countries were represented, HEV RNA has been detected in as high as 66.7% and as low as 1.27% of swine feces in China and Thailand, respectively. Thus, it is increasingly recognized that HEV transmission may be zoonotic.

Part II: Hepatitis E virus in Thai blood donors in Thailand

(2nd revised of Transfusion journal)

Summary

Hepatitis E virus (HEV) infection in industrialized and some developing countries is associated with the consumption of pork and other meat products. We aimed to evaluate the prevalence of HEV in plasma from healthy blood donors in Thailand. We screened blood samples collected between October and December 2015, from 30,115 individual blood donors in 5,020 pools of 6, for HEV RNA using one step real-time reverse-transcription polymerase chain reaction (real-time RT-PCR). HEV RNA-reactive samples were re-tested using a commercial real-time RT-PCR (cobas HEV test) and evaluated for anti-HEV IgM and IgG antibodies. Genotyping using nested RT-PCR, nucleotide sequencing, and phylogenetic analysis was performed.

HEV RNA was consistently detected, 26 individual donor samples were detected using one step real-time RT-PCR, and within this 26 only 9 were reactive using cobas HEV test. The serological assays used for supplementary of HEV infection showed that of those reactive HEV RNA samples, no anti-HEV IgM reactive, while 9 samples were reactive for anti-HEV IgG antibodies. Six samples were successfully genotyped and found to be HEV genotype 3. The frequency of detectable HEV RNA among healthy Thai blood donors was approximately 1 in 1,158. This largest data set to date on HEV infection in Thailand showed the rate of HEV RNA in Thai blood donors (0.09%) was comparable to those found in western European countries but higher than in North America and Australia.

Background and Rationale

Hepatitis E genotype 3 was found sporadic cases in Thailand which usually led to be asymptomatic or self-limiting illness (82). The annual officially report showed the sporadic case in Thailand around less than 100 cases and no reported of death (http://www.boe.moph.go.th/boedb/surdata/disease.php?dcontent=old&ds=70). A common route of HEV transmission in Thailand is a foodborne transmission that was the same as Europe countries especially the consumption of undercooked pork products

(127). Recently, the studies of HEV RNA in pork and variety meats sold in Bangkok fresh market was low prevalence (157). However, the nucleotide sequence identity that found between swine and human was high (82). Additionally, the serology studies which investigated in two different area followed by swine farm densities and local norms showed high sero-prevalence in the area of most residents abstained from pork and fewer swine farms (158). Also in the past study of young Thai soldier that showed low sero-prevalence in the man who lived in the Islam religion area when compared with other provinces (127). These were partly of all studies in Thailand that tried to find the possibility route of HEV infection especially zoonotic foodborne transmission led to HEV infection by the other route including blood borne transmission as the studies in Japan (159).

The first case of HEV infection from a blood transfusion that occurred in Hokkaido, Japan in 2004 who received massive blood product during heart surgery then developed to be acute HEV infection with normal ALT (49). Additional, the case of the HEV-infected case from zoonotic foodborne was reported in Japan again. The patient received blood from a donor who was HEV-infected by consumption of grilled pork meats then the patient developed acute hepatitis E infection (159). After that, there was increasing interested and reported about HEV in blood transfusion and organ transplantation. Many instituted that concern with transfusion and transplantation attempt to propose the guidelines of hepatitis E infection among blood donor and required to assess the risk of hepatitis E infection over the world especially endemic countries (51). Thus, transfusion transmitted HEV infection is interesting, and many countries tried to assess the risk of infection in their countries (Table 9). The United states and Europe countries showed the rate of infection ranged 1 in 500 to 1 in 10,000 and HEV sequencing were genotype 3. While Asia countries were showed lower prevalence rate than that and mostly found genotype 4 but only 3 countries were reported. Additional, some countries start to investigate the HEV infection in blood product before make discussion for using in immunocompromised patients also (160).

Thus, HEV is the new pathogen that several countries are interesting especially in blood donor screening. In Thailand, the risk of HEV infection in blood transfusion was unknown. There was only one study that reported sero-prevalence in blood donor around 15.7% led to the aim of this study is to determine the prevalence of HEV among

Thai blood donors for investigating the risk of HEV infection in Thai blood transfusion (126). We performed by using one-step real-time RT-PCR for HEV RNA screening in all pooling samples. So, the knowledge of prevalence and extent of HEV infection among Thais will assist in assessing the risk of infection.

Table 9 Incidence of detectable HEV in blood donor samples in other countries.

Countries	Year	Laboratory techniques	No. test	No. positive	Positive ratio	Genotype	Ref.
Europe							
Spain	2013	TMA	9,998	3	1:3,333	3f	(161)
Denmark	2015	TMA	25,637	11	1:2,331	3	(162)
France	2012	RT-PCR	53,234	22	1:2,218	3f, 3c	(50)
Germany	2011	RT-PCR	16,125	13	1:1,240	3	(163)
Germany	2012	Real-time RT-PCR	18,100	4	1:4,525	3	(164)
Ireland	2014	TMA	24,985	5	1:4,997	3	(165)
Netherland	2011-2012	Real-time RT-PCR	40,176	13	1:2,671	3	(166)
Netherland	2013-2014	Real-time RT-PCR	59,474	41	1:762	3	(167)
Scotland	2012	Nested RT-PCR	43,560		1:14,520	3	(168)
England	2012-2013	RT-PCR	225,000	79	1:2,848	3	(75)
Sweden	2012	Real-time RT-PCR	95,835	12	1:7,986	3	(164)
Austria	2013-2014	RT-PCR	58,915	7	1:8,416	3	(169)
Asia							
Cambodia	2014	RT-PCR	301	1	1:301	3	(170)
China	2002-2008	RT-qPCR	44,816	30	1:1,493	NA	(171)
China	2012	RT-qPCR	450	0	0	NA	(172)
Japan	2004	Real-time RT-PCR	620,140	36	1:15,075	3	(173)
America							
US	2012	Real-time RT-PCR	51,075	0	0	NA	(164)
US	2013	TMA	18,829	2	1:9,500	NA	(174)

Research Questions

1. Can blood product be the source of HEV infection in Thailand?
2. What is the prevalence of HEV infection among Thai blood donors?

Objectives

To determine the prevalence of HEV among Thai blood donors.

Expected benefits and applications

To date, the prevalence of HEV infection in Thai blood donors is unknown. So, this study will support the possible relation between potential transmission due to foodborne transmission. Additionally, HEV infection in Thai blood donors should be deserved to consideration as the emerging pathogen in organ transplantation and blood transfusion. For HEV screening in blood donor should be developed and introduced in the wide scale implementation with reasonable and cost effectiveness for the blood bank. Therefore, this study should be useful for determination and consideration to change the policies for management the transfusion transmitted HEV infection in Thailand.

Materials and Methods

Ethical considerations

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 435/58) and by the Research Ethics Committee of The National Blood Center, The Thai Red Cross (No. 10/2558). The IRB waived the need for consent because the samples were de-identified and anonymous. Because we have not got back-up samples system from the previous donation and our ethics did not cover the patient studies, so the look-back studies and follow-up patients did not involve in this study.

Blood Donor samples

All EDTA-treated blood samples were collected by anonymous from National Blood Center, Thai Red Cross Society, Bangkok, Thailand. Overall samples were tested for hepatitis B virus (HBV), and hepatitis C virus (HCV) and human immunodeficiency virus (HIV) by serological tests, and negative samples were performed in a mini pool of 6 (each pool contained blood from 6 donors) by using automated specimen pooling (HAMILTON Microlab STAR/STARlet IVD Pipettor, Company, City, Country). Hence, pooled samples that were processed in this study were kept at -70 ºC until analysis. Finally, the patients who received the HEV positive blood product and HEV positive blood donors were not followed up (Figure 13).

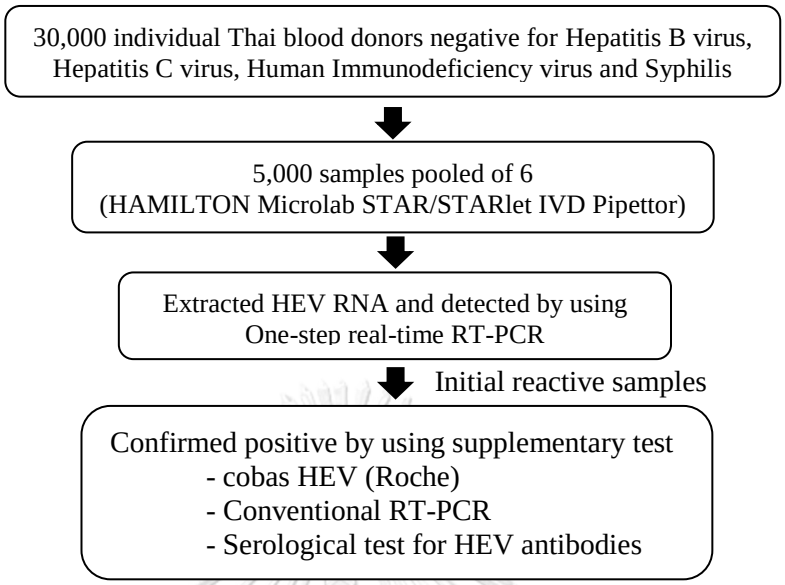


Figure 13 Algorithm of HEV in Thai blood donors.

Sample size estimation

The HEV prevalence values have been reported by many countries that we estimate the prevalence as 1 per 3,000 followed Europe countries because their prevalent with HEV genotype 3 like previous study in Thailand. This calculator uses the following formula for the sample size n:

$$n = \frac{Z\alpha^2 \, P \, (1-P)}{e^2}$$

n = required a sample size

$Z\alpha$ = confidence level at 95% (standard Z value of 1.96 (two-tail))

P = incidence proportion HEV infection estimated from previous study (average value of 0.03%)

e = acceptable margin of error at 5% (standard value of 0.05)

Therefore, a total of 45 samples were required for the HEV screening to achieve the statistically supported results with 5% acceptable error. However, we collected specimens 30,115 samples because of the reliability of prevalence. All samples were

determined positive by one-step real-time RT-PCR assay in ORF2-ORF3 overlapping region.

Detection of HEV nucleic acid

Construction of plasmids as a positive control

Positive control was constructed by using HEV RNA positive samples which known the sequence. Then, we processed using RNA extraction and cDNA synthesis as described above. HEV cDNAs was cloned into pGEM-T Easy Vector System (Promega, CA, USA). The resulting plasmid constructs were confirmed by PCR and sequence analysis by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia).

RNA standard and sensitivity test

The recombinant plasmid was transformed into electrocompetent *Escherichia coli* (Invitrogen, NY, USA) according to the manufacturer's instructions. Agar containing lactose and ampicillin (100 µg/ml) were used for culture then it was incubated and grown at 37°C overnight. White colonies were picked, and confirmed successful transformation by sequence analysis. Then, products were purified using Fast Plasmid Mini kit (Eppendorf, Germany). HEV-RNA transcript purity and concentration were calculated the by measuring ultraviolet absorbance at 260 nm. Finally, the sensitivities and limit of detection were established for each HEV by testing transcripts of known concentrations serially diluted 10-fold (ranging from 10^8 to1 copies/µl) which the result shown the final dilution that it was remained positive at 10^2 and the Ct value around 36-38.

Furthermore, this sensitivity test was performed by using various concentrations from serial dilutions of the first WHO international HEV RNA standard (6329/10) as well, the 95% limit of detection determined by Probit was analyzed by using SPSS software for Windows 22.0 (SPSS Inc., Chicago, IL) and the 95% limit of detection determined by probit analysis was the 50% and 95% limit of detection determined by probit analysis were 7.4 IU/ml (confidence interval 2.4-11.8) and 53.5 IU/ml (confidence interval 34.3-144), respectively. For specificity test, we tested by using HEV negative samples including HBV, HCV and HIV which transmitted by blood

transfusion. In addition, we used RNA from hepatitis A virus, Norovirus, Rhinovirus, Echovirus, Adenovirus, Coxackiesvirus, and Rotavirus as well. The specificity of this testing was 100%.

One-step real-time RT-PCR

Pooled 6 samples were thawed at 4ºC and 200 µl volume of pool plasma was used for RNA extraction with Ribospin vRD II (GeneAll Biotechnology, Seoul, Korea) following the manufacturer's instructions. Then, HEV RNA was screened with one-step real-time RT-PCR assay by using published primers known to detect HEV genotypes based on GenBank accession no. M73218 (175). RNA templates were detected for the HEV ORF2-ORF3 overlapping region (Table 10).

The 20 µl real-time RT-PCR mixture is consisted of 10 µl 2x SensiFAST Probe No-ROX One-Step Mix (Bioline Reagents Ltd., London, UK), 0.8 µl each of HEV ORF2-ORF3 overlapping region forward and reverse primers (10 µM), 0.2 µl Probe (10 µM), 0.2 µl Reverse transcriptase, 0.4 µl RiboSafe RNase inhibitor and 5.6 µl RNase free water. Finally, 4 µl of RNA template was added. Real-time RT-PCR will be performed with ABI ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Wilmington, DE) PCR condition was as follows: reverse transcription at 45°C for 10 min and preliminary denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 54°C for 20 s, and extension at 72°C for 30 s, with a single fluorescence acquisition step at the end of the annealing step. One-step real-time RT-PCR was used for initial screening and individual discrimination.

Table 10 Sequence of HEV primers for one step real-time RT-PCR (175).

Primer	Sequence 5'- 3'	Target region	Position
JVHEVF	GGTGGTTTCTGGGGTGAC	ORF2-ORF3 overlapping	5261–5330
JVHEVR	AGGGGTTGGTTGGATGAA	ORF2-ORF3 overlapping	5261–5330
JVHEVP	TGATTCTCAGCCCTTCGC	ORF2-ORF3 overlapping	5261–5330

Conventional RT-PCR for HEV detection

Positive samples were extracted RNA by using phenol-guanidinium thiocyanate-chloroform extraction. Then, cDNA was synthesized using Improm-II reverse transcriptase (Promega, Madison, WI). Briefly, the RNA template and random hexamers were incubated at 70ºC for 5 minutes and chilled on ice at 4ºC for 5 minutes. Reverse transcription mix was added and incubated at 25º C for 5 minutes. The extension was performed at 42º C for 2 hours and the reaction was inactivated at 70º C for 15 minutes.

The presence of HEV-RNA was determined by semi-nested RT-PCR for HEV ORF2 using published primers known to detect all HEV genotypes (153). In the first round of PCR, amplification using the forward primer HE040 and reverse primer HE044 yielded ~500 bp product. In the second round of PCR, HE040 and another reverse primer HE041 were produced a 467 bp product. Amplification of the housekeeping β-actin gene served as an internal control. Moreover, Samples initially were tested positive by using ORF1 amplification with forward primer HE_1 and a mixture of reverse primers HE_4 and HE_5 to yield 542 bp amplicons, followed by a nested PCR using the forward primer HE_2 and reverse primers HE_3 and HE_6 to yield ~370 bps (Table 6) (154). All PCR products from ORF1 and 2 regions were resolved by 2% agarose gel electrophoresis, purified, and sequenced by First BASE Laboratories (Seri Kembangan, Selangor, Malaysia). Sequences were subjected to BLAST analysis tool in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Commercial HEV detection (cobas HEV)

The cobas HEV test was run on the cobas 6800 System for detection of HEV RNA (Roche Molecular Diagnostics, Pleasanton, CA). The cobas HEV test is real-time RT-PCR technique that based on fully automated systems including HEV RNA extraction followed by RT-PCR amplification and detection. Limit of detection (LoD) of HEV RNA was 18.6 IU/ml (95%CI 15.9-22.6%) by using the WHO 1st international standard (PEI code 6329/10).

HEV serological test

Positive samples were tested for the presence of HEV antibodies both IgM and IgG by using two commercial ELISA kits: (i) of anti-HEV IgM and IgG ELISA

(Euroimmun, Lübeck, Germany) (ii) HEV IgM/IgG ELISA (Beijing Wantai Biological Pharmacy, Beijing, China) and following the manufacturer's instructions. For Euroimmun ELISA kit, the result of the HEV IgM was evaluated semi-quantitatively by calculating the ratio of the extinction value of the sample over that of the calibrator. The interpretation of anti-HEV ELISA IgG test was used point-to-point of the standard curve for calculation and determination of HEV IgG antibodies in patient samples. Both tests were used the interpretation results as followed: no antibodies detectable (ratio less than 0.8: negative), evidence for the presence of antibodies (ratio between 0.8 and 1.1: borderline) and antibodies detectable (ratio more than 1.1: positive). For Wantai ELISA kit, the result of the HEV IgM and IgG were evaluated qualitatively by calculating the ratio of the absorbance value of the sample over the cut-off. Results were interpreted as negative (<1.0), borderline (≥0.9 to <1.1), and positive (≥1.0).

Phylogenetic analyses

All sequences were analyzed using Chromas LITE program version 2.1.1 and aligned using BioEdit program version 7.0.4.1. Sequences were compared with known HEV strains available in the GenBank database. Phylogenetic tree was constructed from ClustalW alignments of partial nucleotide sequences using the neighbor-joining method implemented in MEGA version 5.2. Bootstrapping was applied with 1,000 replicates to support tree topology.

Statistical Analysis

Descriptive statistics analysis was performed using SPSS software for Windows 22.0 (SPSS Inc., Chicago, IL). For the comparison of variables between different sources and type of samples, χ^2-test was used. Data was considered statistically significant at p-value ≤ 0.05.

RESULTS

Real-time RT-PCR for HEV RNA

The 30,115 samples from blood donors obtained within a 3-month period in 2015 comprised roughly equal numbers of men and women, most of whom were repeat donors (Table 11). The average age of the donors was 36 years (range 18-65 years) and significantly different compared to the mean age of all donors. A total of 5,020 residual plasma pools were tested by in-house real-time RT-PCR for HEV RNA, of which 108 pools tested reactive. Next, individual donor plasma samples constituting these 108 pools were tested. From 648 donor samples, 65 donors tested reactive for HEV RNA (Ct values range ~32-38). Real-time RT-PCR was repeated twice more on these 65 samples, of which 26 samples (18 men and 8 women) repeat reactive (0.09% or approximately 1 in 1,158) (Table 12 and Figure 14).

We next asked if the in-house HEV-positive results were reproducible using a different real-time RT-PCR platform. Among the original 65 donors who tested positive by the in-house assay, 9 samples tested positive by the cobas HEV test. These were the very same samples which yielded three-positive by the in-house assay. Meanwhile, randomly selected individual donor samples (n = 25) from the HEV-negative pools all tested negative using the cobas HEV test. Therefore, 26 out of 30,115 donor samples demonstrated consistently detectable levels of HEV RNA using two different assays (Figure 14). This yields the frequency of detectable HEV RNA among healthy blood donors of 0.09%, or approximately 1 in 1,159.

Table 11 Characteristics of the Thai blood donors and results of HEV RNA- reactive donors.

Characteristics	Total	In-house real-time RT-PCR		Cobas HEV test	
		HEV RNA reactive (%)	P-value	HEV RNA reactive (%)	P-value
Gender[†]			0.040*		0.750
Male	14,788	18 (0.12)		5 (0.03)	
Female	15,324	8 (0.05)		4 (0.03)	
Age[‡]			0.923		0.320
<25	5,790	6 (0.10)		3(0.05)	
26-35	9,308	8 (0.09)		4(0.04)	
36-45	8,533	6 (0.07)		2(0.02)	
>45	6,468	6 (0.09)		0(0.00)	
Donation			0.518		0.610
First-time	3,225	1 (0.03)		0(0.00)	
Returned	26,890	25 (0.09)		9(0.03)	
Occupation[§]			0.931		0.308
Student	3,525	4 (0.11)		3(0.09)	
Monkhood	182	0 (0.00)		0(0.00)	
Government	3,837	3 (0.08)		1(0.03)	
Private sector	19,064	17 (0.09)		5(0.03)	
Others	3,506	2 (0.06)		0(0.00)	

* denotes statistical significance.
[†] no gender information (n = 3).
[‡] no age information (n = 16).
[§] no occupation information (n = 1).

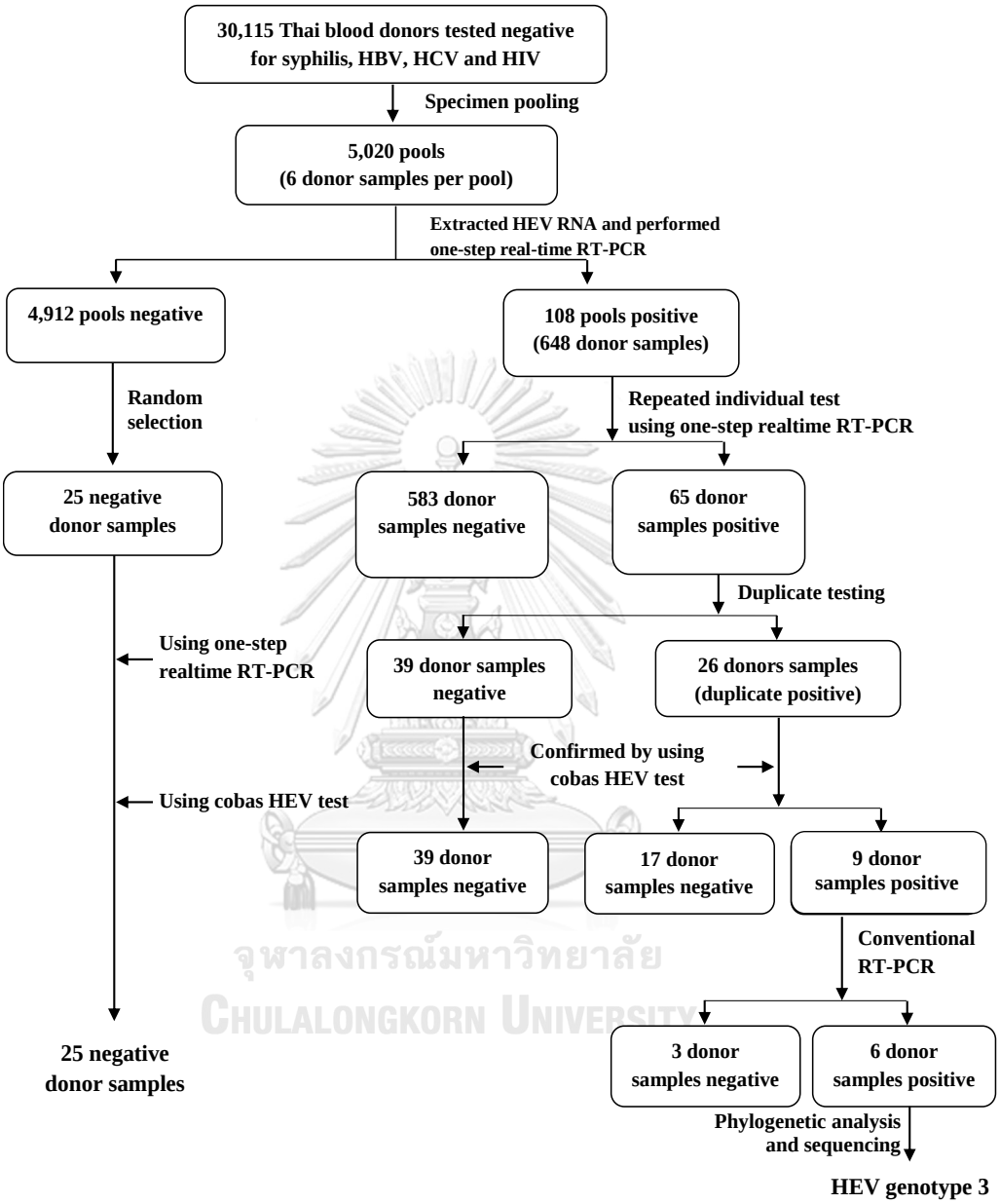


Figure 14 The schematic flow chart of HEV study of Thai blood donors.

Table 12 Characteristics of 26 individual donor samples tested three- reactive for HEV RNA using the in-house one-step real-time RT-PCR.

Donor No.	Pool No.	Donor		Ct value		cobas HEV test*	Nested PCR†		ELISA value (Euroimmun)		ELISA value (Wantai)	
		Sex	Age	P. of 6*	Individual triplicates (mean)	Ct value	ORF 1	ORF 2	IgM‡	IgG§	Ig M‡	IgG‡
1	NBC0009	M	31	33	36, 36, 36(36)	-	-	-	-	3.78	-	14.05
2	NBC0009	F	43	33	36, 36, 36(36)	-	-	-	1.27	1.25	-	8.657
3	NBC0011	M	50	34	34, 34, 34(34)	-	-	-	-	-	-	-
4	NBC0036	M	34	34	37, 34, 37(36)	-	-	-	-	-	-	-
5	NBC0036	M	33	34	38, 38, 34(36.7)	-	-	-	-	1.35	-	7.505
6	NBC0036	M	24	34	37, 34, 37(36)	-	-	-	-	-	-	-
7	NBC0293	F	18	34	36, 38, 36(36.7)	-	-	-	-	0.82	-	3.648
8	NBC0376	M	47	36	38, 38, 36(37.3)	-	-	-	-	5.13	-	>20
9	NBC0500	M	56	36	36, 36, 32(34.7)	-	-	-	-	-	-	1.095
10	NBC0507	M	50	36	34, 37, 37(36)	-	-	-	-	-	-	-
11	NBC0627	M	44	34	36, 34, 36(35.3)	-	-	-	-	3.49	-	7.033
12	NBC0960	M	45	34	36, 33, 36(35)	40.4	-	-	-	-	-	-
13	NBC1594	M	28	34	36, 38, 38(37.3)	-	-	-	-	-	-	-
14	NBC1612	M	37	34	34, 34, 36(34.7)	35.0	-	-	-	-	-	-
15	NBC2075	F	52	36	36, 36, 36(36)	-	-	-	-	-	-	-
16	NBC2204	F	33	36	36, 34, 36(35.3)	29.3	+	-	-	-	-	-
17	NBC2373	M	41	34	34, 34, 35(34.3)	-	-	-	-	-	-	-
18	NBC2581	M	42	37	36, 36, 36(36)	-	-	-	1.18	1.35	-	7.290
19	NBC2999	F	27	34	36, 36, 36(36)	-	-	-	-	-	-	-
20	NBC3081	M	25	36	34, 34, 34(34)	-	-	-	-	-	-	4.967
21	NBC3402	M	34	32	34, 34, 34(34)	26.7	+	+	-	-	-	-
22	NBC3561	M	33	34	34, 34, 34(34)	27.6	+	+	-	-	-	-
23	NBC3724	M	18	35	34, 34, 34(34)	34.2	-	-	-	-	-	-
24	NBC4446	F	21	36	33, 34, 32(33)	28.1	+	+	-	-	-	-
25	NBC4783	F	19	34	34, 34, 34(34)	30.2	+	+	-	-	-	-
26	NBC4890	F	30	32	32, 34, 34(33.3)	24.7	+	+	-	-	-	-

*P.of 6 = pools of 6
Dash line denotes negative result.
‡ OD ratio.
§ IU/ml.

Serological testing for anti-HEV IgM and IgG

For the serological test, 26 samples were performed the ELISA used for anti-HEV IgM and IgG antibodies detection (Table 12). Only 2 samples tested reactive for anti-HEV IgM ELISA with Euroimmun ELISA kit (7.7%, 2/26) but no reactive by using Wantai ELISA kit. Moreover, these samples were also tested for anti-HEV IgG ELISA. Nine samples were reactive, of which one sample was considered borderline for Wantai ELISA kit (30.8%, 8/26). While seven of 9 Wantai reactive samples were also reactive for Euroimmun ELISA kit, of which one sample was considered borderline for Euroimmun ELISA kit, so only 2 samples were discordant results.

Sequence analysis of ORF1 and ORF2 in blood donors

To identified HEV genotype in the samples, we performed manual RNA extraction and conventional RT-PCR on all 26 confirmed HEV RNA- reactive samples. Of these, HEV ORF1 and/or ORF2 regions were successfully amplified from 6 samples. Sequence data from both ORF1 and ORF2 were available from 5 samples, while only ORF1 nucleotide sequence was available from 1 sample. Phylogenetic analysis of both ORF1 (Figure 15) and ORF2 (Figure 16) showed that the HEV strains from this study consistently clustered with various reference HEV sequences from human, swine, and wild boar belonging to genotype 3. The majority of the HEV strains were genotype 3f. One HEV strain belonged to genotype 3c. Taken together, HEV viremia as measured by the presence of detectable viral nucleic acid resulted from genotype 3 infection.

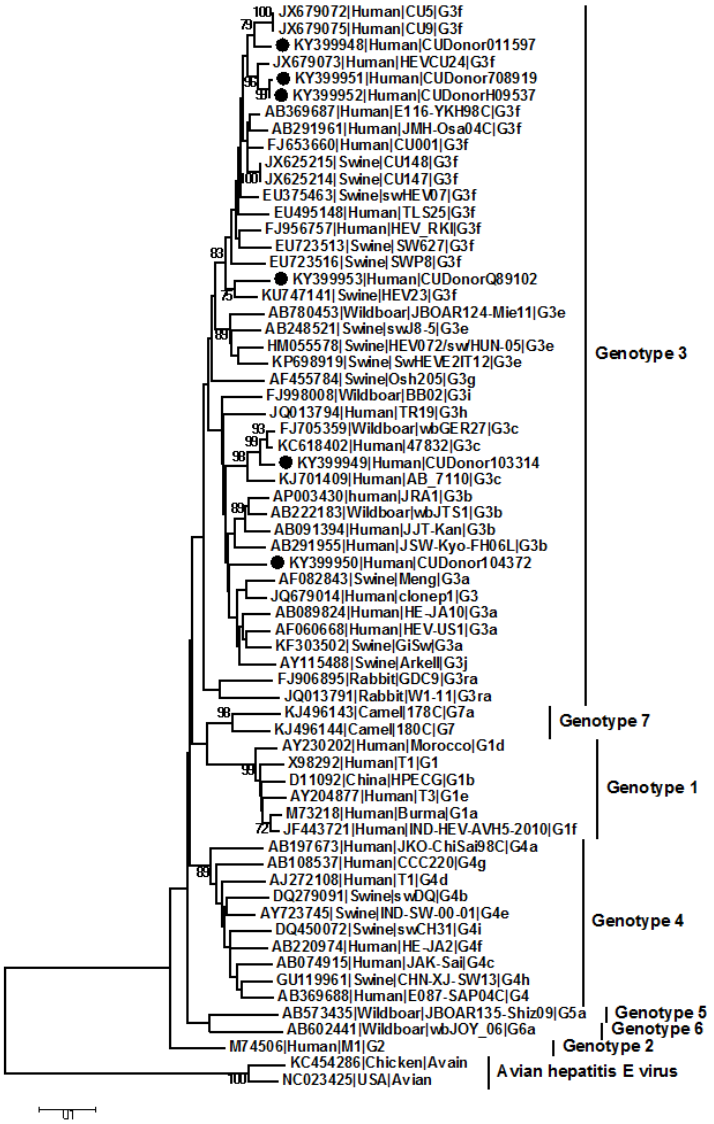


Figure 15 The phylogenetic analysis of the partial HEV ORF1 region.

Phylogenetic tree was constructed using the neighbor-joining method with bootstrap consensus inferred from 1,000 replicates. Only bootstrap values >70% are shown. Six HEV strains isolated from blood donor samples are shown on the tree denoted by black dots. Avian HEV sequence was included to represent an outgroup.

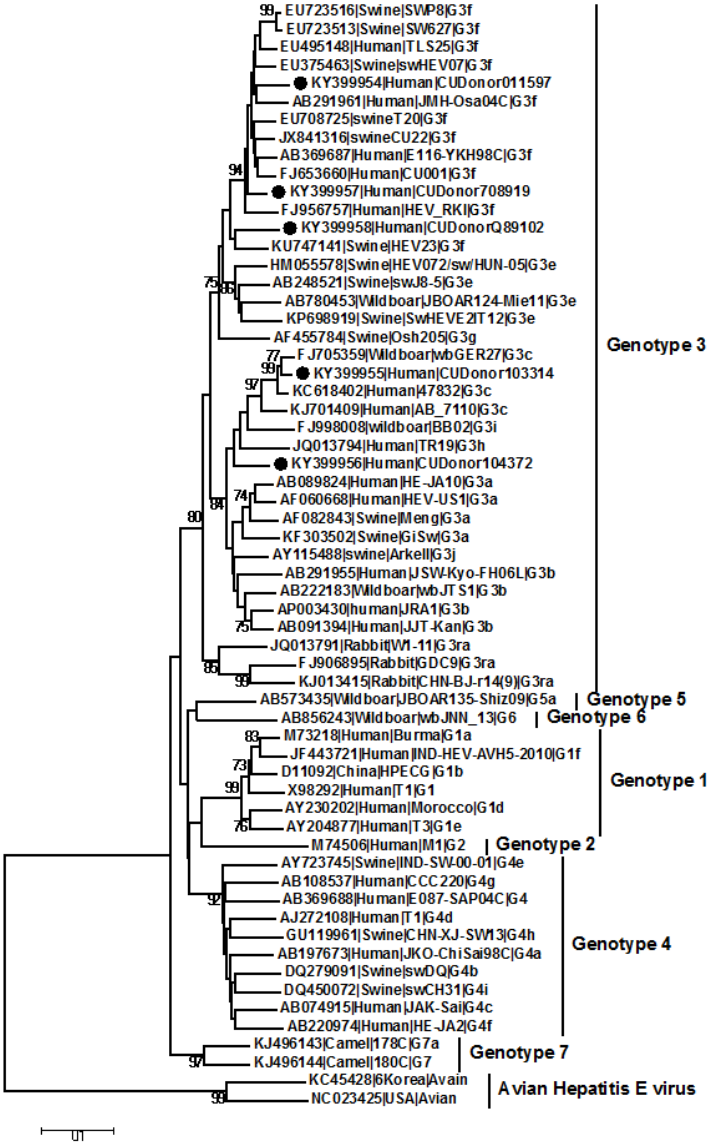


Figure 16 The phylogenetic analysis of the partial HEV ORF2 region.

Phylogenetic tree was constructed using the neighbor-joining method with bootstrap consensus inferred from 1,000 replicates. Only bootstrap values >70% are shown. Five HEV strains isolated from blood donor samples shown on the tree are denoted with black dots. Avian HEV sequence was included to represent an outgroup.

Part III: Genome analysis of HEV in swine and human circulating in Thailand.

(Manuscript in preparation)

Summary

Hepatitis, or the inflammation of the liver, is a major public health problem in Thailand and is most associated with viral infection. Hepatitis E virus (HEV) is an increasingly recognized serious health risk to pregnant mothers, immunocompromised individuals, and the elderly. Understanding HEV epidemiology and route of transmission is important to minimize or prevent the spread of HEV. Consumption of undercooked pork products is suspected to be the major cause of HEV infection in industrialized countries by HEV genotype 3. In addition, blood transfusion is another route that may be associated with hepatitis E transmission. I previously examined HEV RNA in pigs and blood donors, which showed that HEV prevalence in Thailand was similar to that in developed countries. Unlike in other developing Southeast Asian countries, only genotype 3 was found in Thailand. In my current study, aim to sequence the whole genome of HEV strains isolated in Thailand and analyze their sequences. The result showed the genotype 3f was predominated in Thai population and swine. Surprisingly, the other sub-genotype was found in Thailand as HEV genotype 3a and 3c which prevalent in Japan and Europe. Moreover, the evolutionary analysis was estimated the mean time of the ancestor for HEV genotype 1 to 4 with the sequence from Thailand was 1,624 years ago (95% HPD: 1222.40-2110.48) and the substitution rate was $5.63x10^{-4}$ base substitution per site per year which was lower than the previous study. Moreover, HEV in Thailand might be originated with swine infecting ancestor then intermix between human and swine. For the natural selection analysis, there are the positive selection in HEV genome sequence including ORF2 and ORF3 especially ORF2-ORF3 overlapping region whereas ORF1 found only negative selection. Thus, HEV was the highly conserve in the functional domains. Furthermore, the mutation associated with the clinical manifestation was identified in complete genome sequences. The mutation was found F1439Y, V1479I, and G1634R in ORF1 and N562D in ORF2 which were similar to the previous studies. Thus, the screening for the mutation should be carried out in the further study because it concerns with the

clinical presentation and the treatment of HEV in the patients especially the transplantation patient.

Background and Rationale

To date, the genetically of HEV have been reported and identified from various animal species led to broadened the host range and genetic diversity of virus (176). A novel strain was isolated to approve the possibility of HEV transmission whereas there are other animal strains are not studies yet. Nevertheless, the cross species can be occurred among the expanding host range that it should be suspected the genetic relationship between human and other animals. In addition, the host immune system and anti-viral drug are the causes of selective pressure lead to the mutation of HEV genome (95). The mutation rate of HEV was conducted in 2004 by Takahashi et al. that showed the substitution rate per of HEV is similar to hepatitis C virus, 1.5 base site per year (83). However, more studies are required to clarify the mutation in HEV structure which is crucial for HEV replication.

HEV structure consist of three open reading frames (ORF1, ORF2, and ORF3), 5′- and 3′-untranslated regions (UTRs), and a poly A tail at the 3′-end of genome (46). ORF1is different among genotype and encodes the nonstructural enzymatic activities which needed for replication. The sequence of amino acid in ORF1 region usually found a few mutations in the MeT and Y domain, while the domains with more mutation are PCP, macro domain, Hel, and RdRP domain. These mutations are lead to the difference length of ORF1 among genotype which genotype 3 and 4 are longer than genotype 1 and 2. Besides, hypervariable region (HVR) usually found amino acid substitutions which HVR may play a crucial role to regulate transcription and translation in viral replication (88). The other finding showed the PCP domain mutation associated with protease activity for HEV replication by suppression (96). Hel domain was reported the important mutation that V239A substitution which it can be concern with increased virulence in severe hepatitis patient (177). Furthermore, the amino acid change in ORF1 was identified in fulminant hepatitis failure and acute viral hepatitis patients (178, 179).

ORF2 encodes a structural protein like capsid protein that is immunogenic for neutralizing antibodies (95). Therefore, the mutation in ORF2 influence the virulence,

severity, and disease pathogenesis. Moreover, six mutations in ORF2 region including C5927T, C5933T, T6014C, C6032T, G6098A, C6104T, and amino acid mutation P259S may concern with fulminant liver failure (180). And ORF2 is widely used for vaccine development so that the mutation may concern with the effective of HEV vaccine challenges too (105). Due to ORF2 overlap with ORF3, the mutation in ORF3 also corresponds to ORF2 lead to affect the assembly of virus (113). Moreover, the interaction of ORF3 with host factors may be the cause that can be explanation the self-limiting in HEV infection by stimulation and enhance the immune response to clear viral rapidly. However, largely documents are unknown so more studies are required to clarify the functional of ORF3 region (95).

Presently, genotype 3 and 4 are reported in Asia and Europe that swine is stand out as the main reservoir associate with HEV infection in human (181). Additionally, HEV is poorly understood including biology, life cycle, natural history, and genetic variation which were important to treatment and prevention for human life (176). In Thailand, HEV genotype 3f is predominated which can be isolated from human and swine so the evidence of cross species can be occurred. Furthermore, the whole genome sequencing of Thai strain is insufficient for genome analysis. Thus, the aim of this study is to conduct the whole genome sequencing and analyze the HEV genome of Thailand. This significance of this study is useful for evaluation the future situation of hepatitis E infection by evolution study and understanding the drivers of virus replacement and genetic variation for HEV isolated from Thailand. In addition, this information is necessary for selection of effective vaccine and to devise appropriate preventive strategies.

Research Questions

1. Are there differences in the sequences of HEV found in Thailand and other countries?
2. What is the evolution pattern of HEV?
3. Are there significance in the mutation in HEV genome?

Objectives

To determine the whole genome sequence and analyze the HEV strains present in Thailand.

Expected benefits and applications

This finding will support the information of viral epidemiology, genetic variability, and the evolutionary dynamic in Thailand. Moreover, this information is useful for control and prevent the viral infection especially the way to choose the suitable vaccine and antiviral therapy for minimize the virus infection with less mutation.

Population study

This study used the positive samples from part I and II, and collect from patient who diagnosed as suspected cases of HEV infection from King Chulalongkorn Memorial Hospital and hospitals located in Bangkok.

Materials and Methods

RNA extraction and cDNA synthesis

RNA was extracted by using a commercially available Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, Taipei, Taiwan). Samples 200 μl was used for RNA extraction with Ribospin vRD II (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Complementary DNAs was synthesized using ImProm-II reverse transcriptase (Promega, Madison, WI). Briefly, the RNA template and random hexamers was incubated at 70ºC for 5 minutes and chilled on ice at 4ºC for 5 minutes. Reverse transcription mix was added and the reaction will be incubated at 25ºC for 5 minutes. The extension was performed at 42ºC for 2 hours and the reaction was inactivated at 70ºC for 15 minutes.

Primer in this study

The whole genome sequence of HEV uesed the overlapping primer in Table 13.

Table 13 The primer used for the whole genome sequence.

SET	Primer name	Sequence (5-3)	Position	Size
A	ECUF1(HE5-1)	TCGATGCCATGGAGGCCCA	1-20	543
	ECUR1(HE5-3)	TCRAARCAGTARGTGCGGTC	524-543	
B	ECUF2(F1)	CAGCGCTGGTATTCTGCC	355-372	504
	ECUR2(R122)	AACGAGGGTAGGGGACATAAG	839-859	
C	ECUF3(F11)	CTAGTGCAGGCTATAACCATGA	683-704	629
	ECUR4(R1)	GGCACTGTGCATAAAACTGG	1293-1312	
D	ECUF4(F2)	GGCTTATGCTTTTTGGTGCCA	974-994	912
	ECUF5(F3)	TCGCCAACGAGGGCTGGAA	1079-1097	
	ECUR5(R2)	CGCTGGGTGAACCTATTGTAC	1971-1991	
E	ECUF6(F4)	GAGTGCCGTACTGTGCTTGG	1693-1712	943
	ECUF7(F5)	TCTAATGGCTTGGATTGTACCG	1876-1897	
	ECUR6(R4)	CTTATTAGCCTCGAACCAGG	2801-2819	
F	ECUF8(F7)	GACTACAGGGTTGAGCAGAA	2611-2631	720
	ECUR7(R6-1)	GTATGAGCTCACAAACATCGG	3311-3331	
G	ECUF9(F8-2)	CCTCATCGGTCCATCTCCT	3179-3197	454
	ECUR8(R6)	ATCAGAAATGCCGACCTCACG	3613-3633	
H	ECUF10(F8-3)	CCAGAAGCTGGTCTTCACG	3405-3423	821
	ECUR9(R7)	GCGATAGTCTCACCTGTTGT	4207-4226	
I	ECUF11(F9)	CATTCTGATGTTCGCGAGTC	4012-4032	624
	ECUR10(R8)	CCATGTTCCAGACGGTGTT	4618-4636	
J	ECUF12(F10)	TCTCTCTTGGCCTTGAGTG	4454-4469	533
	ECUR11(R9)	CACGGGACACAACATCAACA	4968-4987	
K	ECUF13(F11)	TAGTGACTACCGTCAGCGCC	4713-4732	835
	ECUR12(R10)	AGCACCACGAGAATCAACATC	5528-5548	
L	ECUF14(F13)	TCCTCTTGGCGTGACCAGTC	5411-5430	707
	ECUR13(R14-1)	AACACGGGTGTTAGTGTTCC	6099-6118	
M	ECUF15(F10-2)	CAGGACGGCACCAATACTCA	5657-5676	771
	ECUR14(R12)	CCTTGTCCTGCTGTGCATTC	6409-6428	
N	ECUF17(F15)	CCGACAGAATTGATTTCGTCG	6308-6328	564
	ECUR15(R17)	CAATCAAAATCTGGTCACTAGCA	6849-6872	
O	ECUF18(F16)	ACCATCCAGCAGTACTCTAAGAC	6740-6763	475
	ECUR17(R18)	TTTTTCCAGGGAGCGCGA	7196-7215	

PCR of whole genome sequencing

The whole genome sequencing used the overlapping primers for sequencing the full length of HEV genome and amplified by using nested and semi-nested PCR (Table 6). Briefly, this study used the RNA positive samples from part I and II then cDNA synthesis is performed as followed; RNA template and random hexamers was incubated at 70ºC for 5 minutes and chilled on ice at 4ºC for 5 minutes. Reverse transcription mix was added and the reaction was incubated at 25ºC for 5 minutes. The extension was performed at 42ºC for 2 hours and the reaction was inactivated at 70ºC for 15 minutes.

After that cDNA was amplified by using condition as followed: cycling conditions started with denaturation at 94°C for 3 minute and then 40 cycles of PCR in each cycle of denaturation for 30 sec at 94°C, then annealing for 30 sec at 55°C and elongation for 1 min at 72°C, and ended with the final extension cycle at 72°C for 10 min. PCR products was resolved by 2% agarose gel electrophoresis and then visualized using staining with fluorescent ethidium bromide dye under ultraviolet light. The PCR products was purified using Agarose Gel Extract mini kit (5 Prime GmbH Hamburg, Germany) and sequenced by First BASE Laboratories (Seri Kembangan, Selangor, Malaysia). Sequences was subjected to BLAST analysis tool in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequence data analysis

The whole genome sequence was assembled and edited sequences into consensus sequences comparing with the reference in GenBank accession by using Seqman software (DNASTAR lasergene 6). Then consensus sequences were exported and used for the phylogenetic analyses.

Reference data set

A comprehensive HEV genome dataset was generated from the GenBank database and followed Smith et al. 2016 to classify the HEV genotype and subtype. All HEV sequences were known the host, collection time and geographic origin. We used 42 sequences of HEV genotype 1 to 4 which comprised 6 sequence of genotype 1, 1 sequence of genotype 2, 26 sequence of genotype 3, and 11 sequence of genotype 4 for

Phylogenetic and Evolution analysis which the Hypervariable region was removed from complete genome due to very poor alignment quality and avoided the recombination point (70). The reference strain for HEV genotype 2b and 3d were excluded because only partial sequence is available. In addition, HEV genotype 1e, 3h and 3ra were excluded as well because the sequences were short when compared with other reference sequences. For natural selection, we used the sequences of HEV genotype 3 which we divide to 3 open reading frames for comparing among regions comprising ORF1 30 sequences (exclude HVR)(182), ORF2 30 sequences, and ORF3 26 sequences.

Phylogenetic analysis

All complete genome data were aligned using BioEdit program (v7.2.5), and compared to known HEV sequences available in GenBank. Phylogenetic trees were constructed from Clustal W alignments of complete genome sequences by using the neighbor-joining method implemented in MEGA (v5.2). Bootstrapping was applied with 1,000 replicates to support tree topology. The phylogenetic analysis is used to investigate the phylogenetic relationship among reference sequence of HEV genotype and subtype with our complete genome sequences.

Evolutionary analysis

All completed genome sequences (exclude hypervariable region) were include with reported complete genome sequences and reference sequence were used in Evolutionary analysis. The nucleotide substitution rates and estimates for time to most recent common ancestor (tMRCA) were used Markov Chain Monte Carlo (MCMC) method with the General Time Reverisible (GTR) substitution model and a gamma distribution (G) rate. The substitution model GTR + Invarience site + gamma distribution (GTR+I+G) was used to be the Model test for estimation the rate of evolution. Then, all data were implemented in Bayesian Evolutionary Analysis by Sampling Trees (BEAST v1.8.0). Visual inspection of Bayesian sampled parameter estimates was conducted using Tracer v1.6 ensuring effective sampling size (ESS) of all parameters was showed as ESS (effective samples size) ≥200. Finally, the strict molecular clock was used for this study. Tree Annotator v1.8.0 was used to select the

maximum product of posterior probabilities and phylogeny visualized using FigTree v1.4.2.

Selective pressure

The selective pressures were elucidated by calculated the ratio between nonsynonymous (dN) and synonymous (dS) substitutions, (dN/dS). The ORFs (ORF1, 2, and 3) of HEV genotype 3 were selected for the selective analyses by implemented in Hyphy (183) on Datamonkey website (184, 185). For data analysis, Single-Likelihood Ancestor Counting (SLAC) and fixed effects likelihood method (FEL) (186, 187) were used followed the previous study. SLAC estimated the number of non-synonymous and synonymous substitution at each site based on the maximum likelihood reconstruction of ancestral codons. The selective pressure was defined as followed: dN/dS = 1 indicates neutral selection, dN/dS > 1 indicates positive selection and dN/dS < 1 indicates negative selection. A site with dN/dS > 1 and p-value less than 0.1 was considered to be positive selection site (188). For FEL methods, the analysis was iterated through every codon in the alignment and the cut off *p*-value was determined by using asymptotic chi-squared test. The positive and negative position were reported the estimate of ratio dN/dS > 1 and *p*-value less than 0.1 as reported from SLAC (189).

Mutational analysis associated clinical manifestation

For mutation analysis, all complete genome sequences from this study aligned with the NCBI reference sequence NP_056779 of nonstructural protein (pORF1), NP_056788 of capsid protein (pORF2) and YP_003864075 of hypothetical protein (pORF3) for the numbering of amino acid sequence. Then, mutation sites that concerned with the clinical manifestation followed the reported in previous study (Table 4) were observed across the genome. Only the mutation in HEV genotype 3 was performed in this study.

RESULTS

Whole genome sequence analysis

This study used the positive samples with high viral load from pork and variety meats in part I, blood donor samples from part II and patients who diagnosed as suspected cases of HEV infection from King Chulalongkorn Memorial Hospital and hospitals located in Bangkok. Unfortunately, the positive samples from blood donors were low viral load and insufficient for complete genome sequencing. So, samples from pork and variety meats, and patients samples were used for whole genome analysis in this study.

Twelve complete genome sequences were succeeded for whole genome sequencing in this study and all sequences were submitted to Genbank database and assigned the accession numbers MH450020-MH450022 for samples isolated from HEV patients and MH450023-31 for samples isolated from feces and bile of swine. The phylogenetic tree was constructed for comparing 12 complete genome sequences from this study with the reference sequence from Genbank database which were excluded the HVR region due to very poor alignment quality and recombination was reported in this region (70). Besides, the reference sequence of HEV genotype 2b and 3d were removed because these sequences were not the complete genome. Phylogenetic analysis in this study was observed both Neighbor-joining tree that was constructed by using *p*-distances, and Maximum likelihood analysis of entire genome sequence then the clusters were similar. The phylogenetic suggested that 12 complete genome sequences were closely related to HEV genotype 3 that was commonly found in Thailand (Figure 17 and Figure 18).

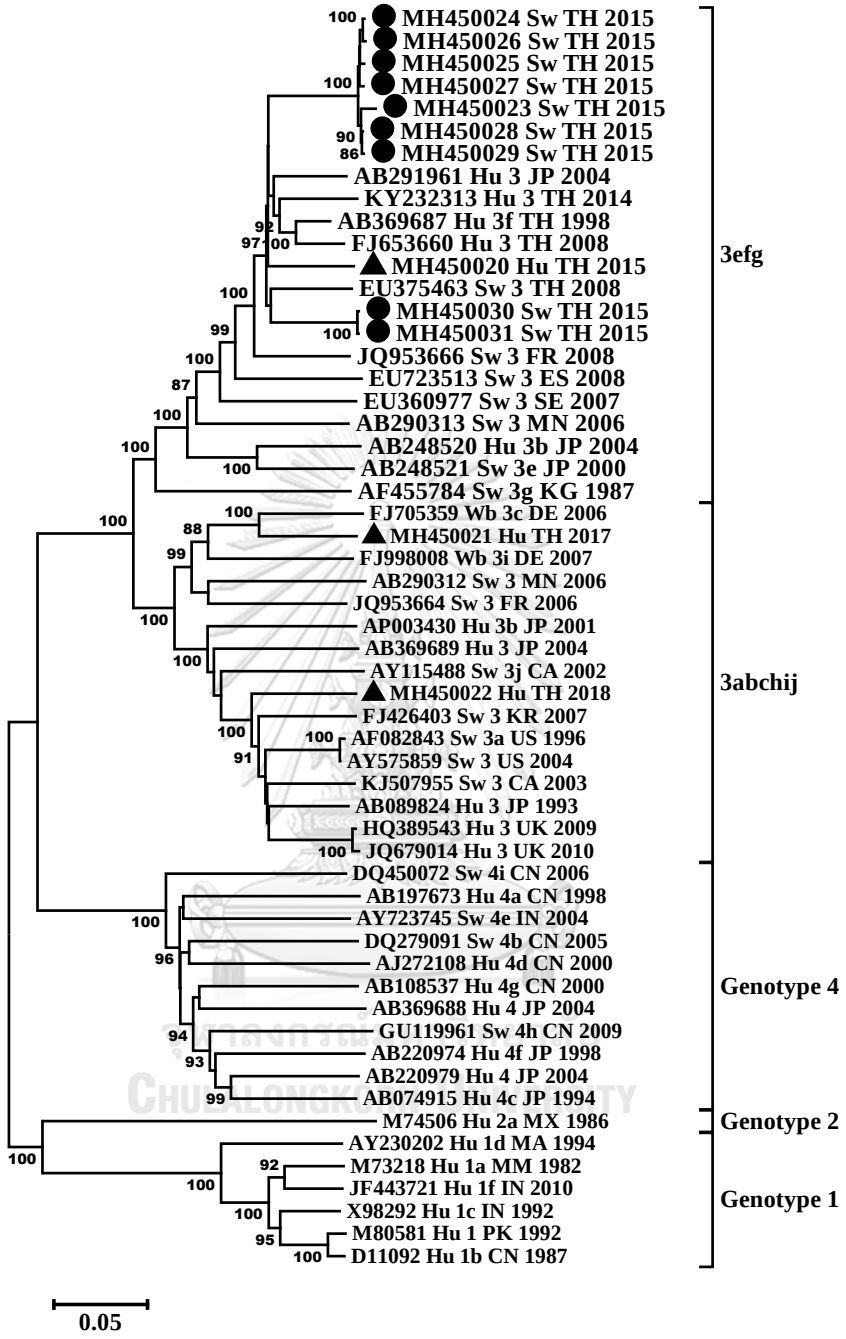


Figure 17 The phylogenetic analysis of the partial HEV ORF1 region.

Phylogenetic tree was constructed using the neighbor-joining method with bootstrap consensus inferred from 1,000 replicates. Only bootstrap values >70% are shown. Twelve HEV complete genome sequences isolated from patients (▲) and swine (●)samples are shown on the tree.

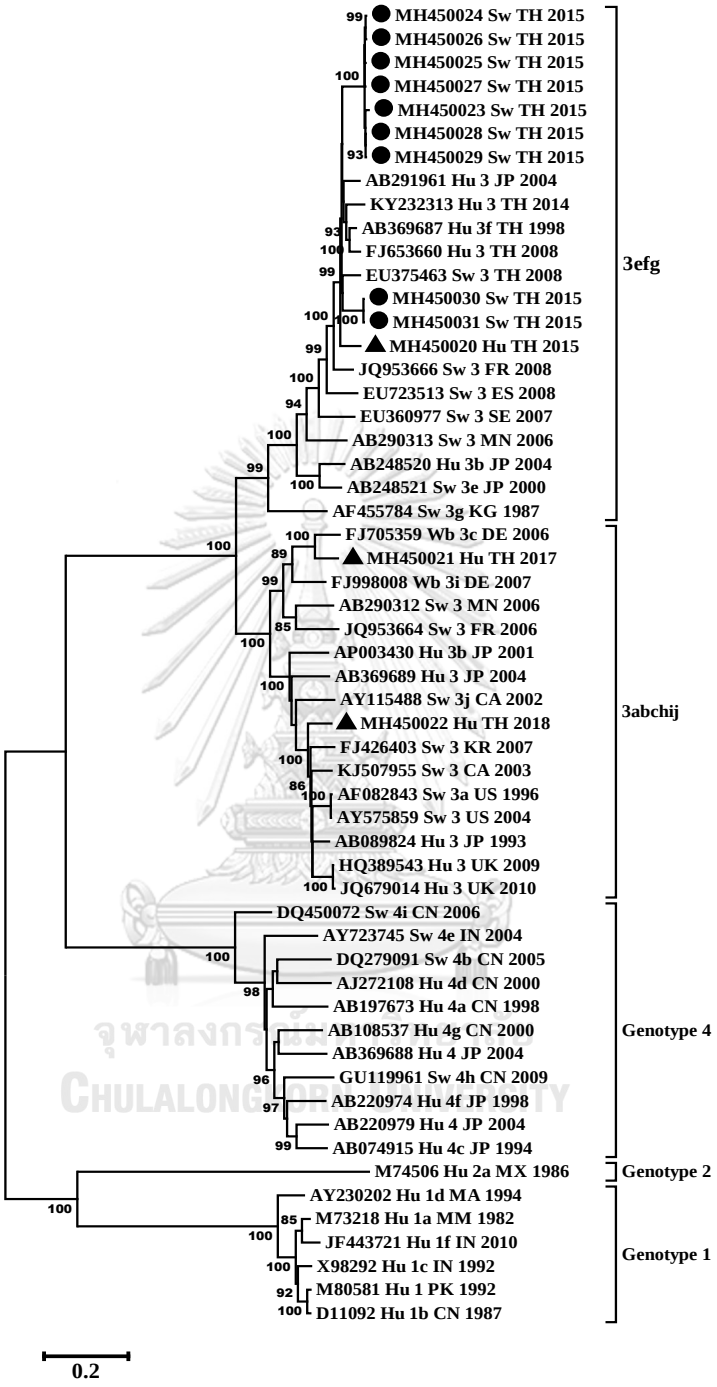


Figure 18 The phylogenetic analysis of the partial HEV ORF1 region.

Phylogenetic tree was constructed using the Maximum-likelihood distances method with bootstrap consensus inferred from 1,000 replicates. Only bootstrap values >70% are shown. Twelve HEV complete genome sequences isolated from patients (▲) and swine (●)samples are shown on the tree.

Characterization of the complete genome sequence for MH450020, MH4500021, and MH450022 isolated from human.

For three sequences that isolated from patients, these sequences were nucleotide blast with reference in NCBI database for search the similarity sequence. The result showed that there was the difference among 3 complete genome sequences which MH450020 appears to be closely to AB369387 and FJ653660 strain isolated from to HEV genotype 3f showed approximately 92-93% nucleotide identity. MH450021 showed 95% nucleotide identity with KX462160 that was clinical isolated from human in United Kingdom, 2014. Moreover, this sequence appeared 91% nucleotide identity with FJ705359 that was isolated from wild boar in Germany, 2016 and classified to be HEV genotype 3c. Another patient sample, MH450022 demonstrated 91% nucleotide identity with AB089824 which was isolated from human serum in Japan, 1993. In addition, MH450022 was closely resembling those found in HEV-US1 (AF060668) with the same percentage of nucleotide identity and classified to be HEV genotype 3a.

The pairwise comparison of our complete genome sequences were compared with reference complete sequences followed Smith at al. 2016 for HEV genotype and sub-genotype identification (156). The result showed that MH450020 had a genome-wide nucleotide similarity with reference sequence of HEV genotype 3f (AB369687) of 92.2%. And MH4500021 had a genome-wide nucleotide similarity of 89.5% with reference sequence of HEV genotype 3c (FJ705359) while MH450022 had a genome-wide nucleotide similarity of 89.9% with reference sequence of HEV genotype 3c (AF082843) shown in Table 14. For the pairwise comparison among ORF, ORF1 and ORF2 of three sequences had a genome-wide nucleotide similarity with the same HEV genotype with complete genome sequences but the similarity of ORF3 was different. MH4500021 had a genome-wide nucleotide similarity of 96.0% with reference sequence of HEV genotype 3i (FJ998008) and MH4500022 had similarity of 96.0% with reference sequence of HEV genotype 3b (AP003430) shown in Table 15.

Characterization of the complete genome sequence for MH450023 to MH4500031 isolated from swine.

For complete genome sequence isolated from swine samples, 9 complete genome sequences were clustered with genotype 3f and showed 91% nucleotide identity with the sequence isolated from an acute hepatitis E patient who returned from traveling in Thailand, 1998 (AB369687) (Figure17 and Figure 18). Additionally, the sequences were clustered with other reference sequence such as AB291961, FJ653660, and EU375463 which were reported from Thailand and Japan. The pairwise comparison of our swine complete genome sequences revealed that MH4500023 to MH4500031 had a genome-wide nucleotide similarity of 91.0-92.0% with reference sequence of HEV genotype 3f (AB369687) both complete genome sequence and separated to ORF 1, ORF2, and ORF3 shown in Table 14 and Table 15.

Table 14 Pairwise nucleotide similarity (%) of complete genome isolated from this study comparing with reference sequences of HEV genotype 3.
followed Smith at al. 2016 (156).

	REFERENCE SEQUENCES OF HEV GENOTYPE 3 (% SIMILARITY)									
VARIANTS	**3a AF 082843**	**3b AP 003430**	**3c FJ 705359**	**3e AB 248521**	**3f AB 369687**	**3g AF 455784**	**3h JQ 013794**	**3i FJ 998008**	**3j AY 115488**	**3ra FJ 906895**
MH450020	79.6	78.9	78.8	84.0	**92.2**	81.2	79.4	78.8	78.7	74.5
MH450021	83.2	82.0	**89.5**	77.8	78.6	79.8	83.5	84.8	82.2	74.2
MH450022	**89.9**	85.4	81.8	78.6	79.2	79.0	82.3	83.2	86.0	74.1
MH450023	79.6	78.9	77.9	82.9	**91.0**	80.5	78.4	78.6	78.5	73.6
MH450024	79.2	78.4	77.8	83.2	**91.7**	80.4	78.4	78.4	78.2	73.4
MH450025	79.2	78.4	77.6	83.4	**91.7**	80.4	78.2	78.3	77.9	73.4
MH450026	79.1	78.3	77.7	83.2	**91.7**	80.4	78.2	78.3	78.1	73.3
MH450027	79.2	78.4	77.6	83.3	**91.7**	80.4	78.3	78.3	78.1	73.3
MH450028	79.3	78.7	77.7	83.3	**91.5**	80.6	78.2	78.4	78.1	73.5
MH450029	79.3	78.7	77.7	83.3	**91.6**	80.6	78.1	78.5	78.1	73.6
MH450030	79.1	78.4	78.7	83.1	**92.0**	80.8	78.5	78.3	78.5	74.4
MH450031	79.1	78.4	79.0	83.1	**91.9**	80.9	79.0	78.5	78.3	74.4

Table 15 Pairwise nucleotide similarity (%) of ORF1, ORF2, and ORF3 isolated from this study comparing with reference sequences of HEV genotype 3.
followed Smith at al. 2016

Gene	Variants	Reference sequences of HEV genotype 3 (% similarity)									
		3a AF 082843	**3b AP 003430**	**3c FJ 705359**	**3e AB 248521**	**3f AB 369687**	**3g AF 455784**	**3h JQ 013794**	**3i FJ 998008**	**3j AY 115488**	**3ra FJ 906895**
ORF1	MH450020	78.0	77.2	76.9	83.0	**91.7**	79.6	77.6	77.1	76.6	71.6
	MH450021	82.2	80.9	**89.3**	75.4	76.7	77.9	82.7	83.8	80.7	72.0
	MH450022	**89.7**	84.9	80.5	76.5	76.8	76.9	81.0	82.0	85.0	71.7
	MH450023	77.7	77.1	75.9	81.2	**90.1**	78.6	76.5	76.9	76.3	70.3
	MH450024	77.1	76.4	75.7	81.8	**91.1**	78.3	76.4	76.6	75.8	70.0
	MH450025	77.1	76.5	75.5	81.9	**91.2**	78.4	76.2	76.5	75.6	69.9
	MH450026	77.1	76.4	75.6	81.9	**91.2**	78.5	76.4	76.5	75.8	69.8
	MH450027	77.1	76.4	75.5	81.8	**91.1**	78.4	76.2	76.5	75.7	69.9
	MH450028	77.2	76.8	75.7	81.7	**90.8**	78.7	76.2	76.6	75.6	70.1
	MH450029	77.2	76.8	75.7	81.8	**90.9**	78.6	76.1	76.7	75.7	70.1
	MH450030	77.4	76.6	77.4	81.8	**91.4**	79.4	76.9	77.1	76.6	71.7
	MH450031	77.4	76.7	77.5	81.8	**91.3**	79.5	77.0	77.1	76.6	71.8
ORF2	MH450020	83.3	82.6	83.2	86.3	**93.5**	85.0	83.4	82.8	83.3	81.1
	MH450021	85.4	84.3	**89.9**	83.5	82.9	82.5	85.4	87.0	85.6	79.3
	MH450022	**90.1**	86.4	84.8	83.6	84.8	83.6	85.3	86.0	88.4	79.7
	MH450023	84.0	83.1	82.3	86.7	**93.2**	85.1	82.7	82.5	83.7	81.2
	MH450024	84.3	83.0	82.5	86.4	**93.1**	85.1	82.8	82.5	84.0	81.1
	MH450025	84.1	82.9	82.3	86.6	**93.0**	85.1	82.9	82.5	83.4	81.2
	MH450026	83.8	82.9	82.2	86.3	**92.8**	84.9	82.2	82.3	83.5	80.9
	MH450027	84.1	83.1	82.4	86.7	**93.3**	85.0	82.9	82.4	83.8	81.0
	MH450028	84.2	83.1	82.3	86.6	**93.2**	85.1	82.7	82.6	83.9	81.2
	MH450029	84.0	83.1	82.3	86.7	**93.2**	85.1	82.7	82.5	83.7	81.2
	MH450030	83.0	82.8	81.7	86.0	**93.3**	84.0	82.2	81.0	83.1	80.3
	MH450031	83.0	82.8	81.7	82.4	**93.3**	84.0	82.2	81.0	83.1	80.3

Gene	Variants	Reference sequences of HEV genotype 3 (% similarity)									
		3a AF 082843	**3b AP 003430**	**3c FJ 705359**	**3e AB 248521**	**3f AB 369687**	**3g AF 455784**	**3h JQ 013794**	**3i FJ 998008**	**3j AY 115488**	**3ra FJ 906895**
ORF3	MH450020	93.7	94.6	94.2	96.1	**98.1**	94.0	94.6	94.6	92.4	87.4
	MH450021	92.7	94.2	95.4	93.3	94.2	93.0	93.6	**96.0**	92.0	85.2
	MH450022	94.2	**95.1**	90.7	92.4	93.3	93.4	94.2	93.3	93.6	85.3
	MH450023	94.3	95.1	94.2	96.3	**98.0**	93.9	95.2	93.9	93.0	87.3
	MH450024	94.0	94.8	94.5	96.1	**97.7**	93.6	94.9	93.6	92.0	87.0
	MH450025	94.0	94.8	93.9	96.1	**97.7**	93.6	94.9	93.6	92.7	87.7
	MH450026	94.0	94.8	94.5	96.1	**97.7**	93.6	94.9	93.6	92.7	87.0
	MH450027	94.3	95.1	94.2	96.3	**98.0**	93.9	95.2	93.9	93.0	87.3
	MH450028	94.3	95.1	94.2	96.3	**98.0**	93.9	95.2	93.9	93.0	87.3
	MH450029	94.3	95.1	94.2	96.3	**98.0**	93.9	95.2	93.9	93.0	87.3
	MH450030	93.6	93.9	94.2	96.3	**97.5**	94.0	94.6	94.6	92.4	86.6
	MH450031	93.6	93.9	94.2	96.3	**97.5**	94.0	94.6	94.6	92.4	86.6

Normally, HEV genotype 3 was segregated the subtypes into three clades namely 3abchij, 3efg, and 3ra (155, 156). For this study, the molecular phylogenetic tree was analyzed by individual ORF which the complete genome sequences of swine and another one from human in this study were clustered in 3efg clade whereas the complete genome sequences from human were clustered in 3abchij clade. The cluster by subtypes of ORFs complete sequences including ORF1, ORF2, and ORF3, was different which ORF1 and ORF2 were clustered the same while ORF3 was not clear separation into subtypes (155). Likewise, two sequences from human in this study showed the different subtype grouping different with other ORFs followed the % similarity and phylogenetic tree (Table 15 and Figure 19).

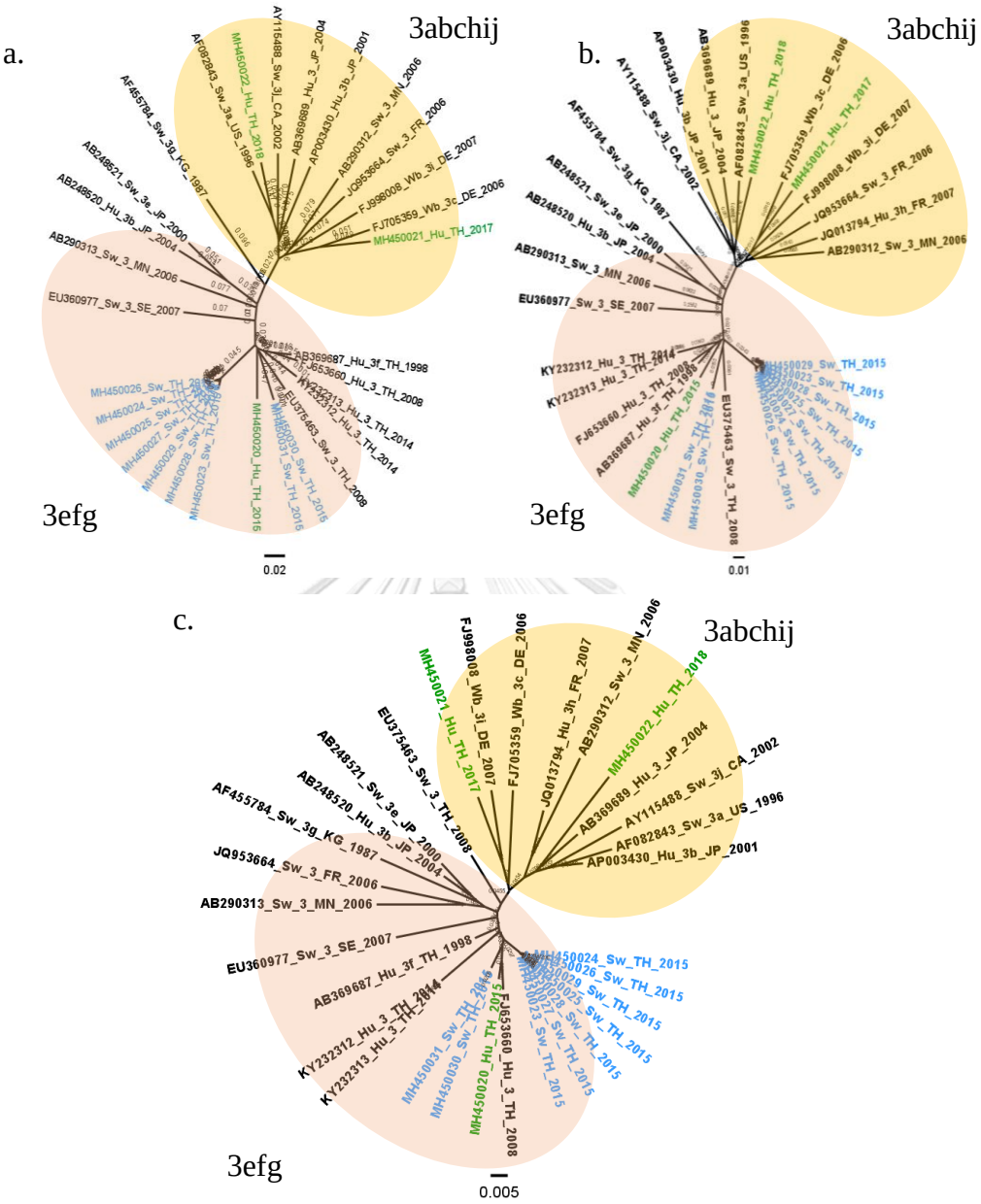


Figure 19 Molecular phylogenetic analysis of HEV genotype 3.

(a) Completed genome of ORF1 (exclude hypervariable region) (4908 bp) (b) Completed genome of ORF2 (1980 bp) (c) Completed genome of ORF3 (339 bp). Twelve of the complete genome sequences obtained in this study are included here (green color): complete genome sequences from patients, (blue color): complete genome sequences from swine.

Evolutionary analysis

To obtain an overview of the Evolutionary history and ancestral host of HEV isolated from Thailand comparing with reference complete genome sequences which this study was comprising our data, the reported complete genome sequences isolated from Thailand, and reference complete genome sequences for evolutionary study. All complete genome sequences of HEV genotype 1-4 must have the information concerning host species, place, and year of collection. The HVR of ORF1 was removed from all sequence owing to low quality and reliability of alignment. Then, the evolutionary history of these data was performed by using BEAST to calculate the Time to the Most Recent Common Ancestor (TMRCA) for HEV genotype 1-4. The mean of TMRCA and 95% highest posterior probability density with a coalescent constant size tree prior and finally the strict clock models are used which the effective sample size greater than 200. The data from this study estimated the mean time of the ancestor for HEV genotype 1-4 with the sequence from Thailand was 1,624 years ago (95% HPD: 1222.40-2110.48) and the substitution rate was 5.63×10^{-4} (4.06×10^{-4}-7.05×10^{-4}) or 0.00056 (0.00040-0.00070) base substitution per site per year (Figure 20).

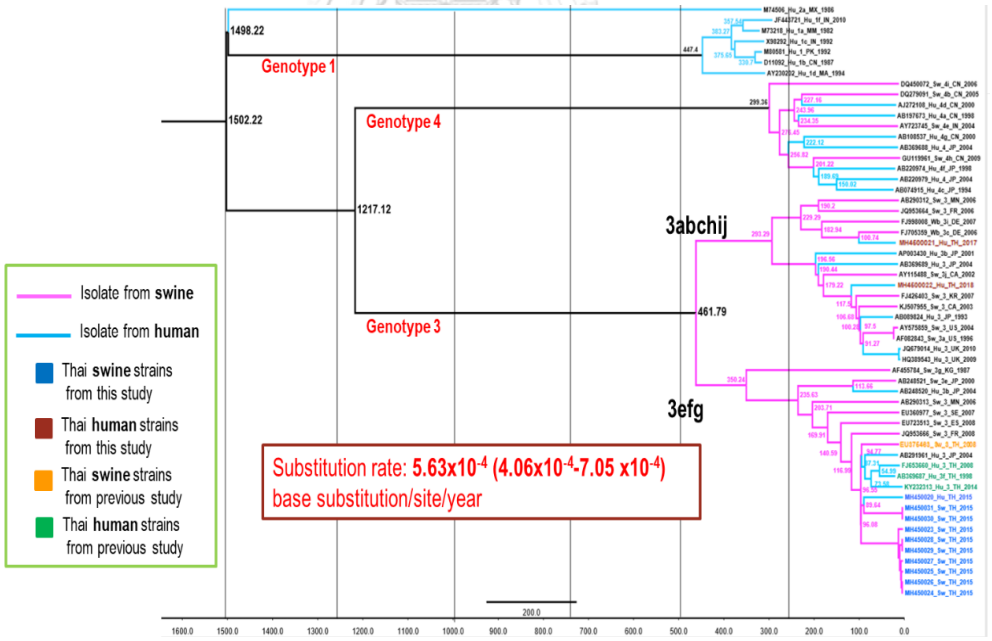


Figure 20 Molecular phylogenetic analysis of HEV genotype 1 to 4.

Analysis of phylogenetic tree showed that the earliest splits of HEV to separate the genotype exclusively for human and enzootic genotype was estimated the TMRCA around 1502 years ago. Then, the four main genotypes were segregated to HEV

genotype 1 and 2 at 1498 years ago whereas genotype 3 and 4 was split at 1217 years ago. The genotype 3 was seem to be the earliest genotype to diverge the genotype to subtypes which it could be divide to 3 clades comprising 3ra (data not shown), 3abchij, and 3efg. For the complete genome sequences in our study, the phylogenetic tree for genotype 1-4 showed that our sequences belong to HEV genotype 3 as the result in the previous study and clustered in two clades. Firstly, nine complete genome sequences from swine and one from human were identified to be HEV genotype 3f which contained in 3efg clade. The TMRCA of these sequences was most probably originated in Thailand in 1921 (97 years ago). Moreover, these sequences were grouped with the reported complete genome sequences isolated from human and swine in Thailand. As the result, the pattern of distribution of HEV genotype 3f in Thailand was likely to circulate in swine before human. Secondly, two complete genome sequences from human were identified to be HEV genotype 3a and 3c which was grouped in 3abchij clade. The TMRCA of MH4500021 was determined near the year 1918 (101 years ago) while MH450022 was older and have a TMRCA in 1900 (118 years ago). Moreover, these two complete genome sequences converged with the reported sequence isolated from wild boar and swine as well. Thus, the result of this study demonstrated the HEV genotype 3 originated in Thailand exist more than 100 years ago and likely from a swine infecting ancestor then spread among human and swine (Figure 20).

The skyline plot was created to demonstrate the population dynamic of HEV genotype 1 to 4. The strict clock model also used and stepwise constant skyline model for skyline plot. The skyline plot showed that the HEV genotype 1 to 4 population was stable for a long time and then started to increase around 1771 -1956. Thereafter, it started to plateau between 1956-1993 and then swift decline to original level (Figure 21).

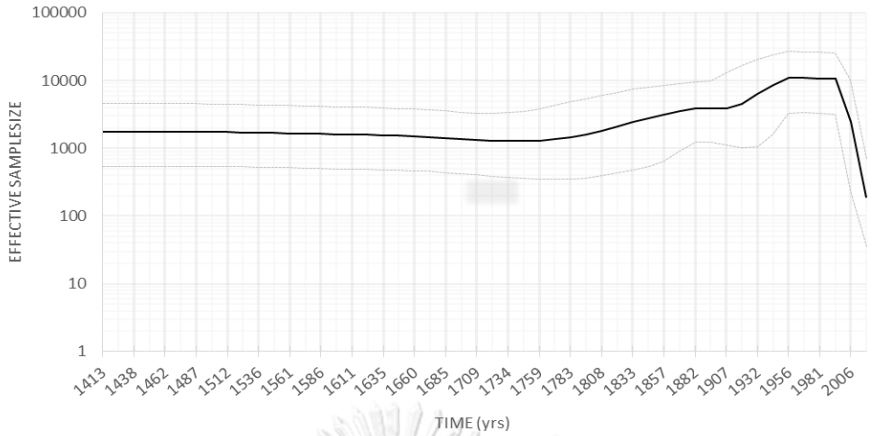


Figure 21 Skyline plot for HEV genotype 1 to 4.

HEV genotype 1 to 4 were used to construct this skyline plot using the strict clock model also used and stepwise constant skyline model. The solid black line is the mean value of highest posterior probability density (HPD). And the dashed grey line represents the limit of 95% HPD.

Selective pressure analysis

The selective pressure was performed by using two different method. First, the datasets of HEV genotype 3 sequences were analyzed by divided into 3 regions including ORF1 30 sequences, ORF2 30 sequences, and ORF3 26 sequences which the position of nucleotide was based on the HEV genotype 3 reference sequence AB248520 (182). Two algorithms were analyzed including SLAC and FEL. In this study, the site-specific positive and negative selection in HEV genotype 3 divide into ORF1, ORF2, and ORF3 were estimated by two different algorithms and reported positively selected site at least one method. Overall entire sequences of each gene, the mean of dN/dS ratio using SLAC analysis at significance level 0.1in the ORF3 was 0.228 which was higher than ORF1 and ORF2, 0.0271 and 0.2286, respectively. Most codons of ORF1 region was under the negative pressure with 82.76% whereas ORF2 and ORF3 were detected the negative codons around 68.33% and 5.60% respectively. For the significant positive selection site detected by SLAC analysis, no positively selected site located in ORF1 region while there were 3 positively selected sites (37, 70, and 95) at significance level 0.1 located in ORF2 region. For ORF3, the significant positive

selection sites were identified as well which there was only 1 site located in this region (Table 16 and Table17).

Table 16 Comparison of mean dN/dS and the number of positive selection site and negative selection site of HEV among ORFs.

Genes	Position	No.of codons	SLAC (Sig. Level =0.1)					FEL (Sig. Level =0.1)			
			Mean dN/dS	PS	%	NS	%	PS	%	NS	%
ORF1	26-7196	1636	0.0221	0	0	1354	82.76	0	0	1452	88.75
ORF2	5214-7196	660	0.0271	3	0.45	451	68.33	13	1.97	500	75.76
ORF3	5203-5544	339	0.2286	1	0.29	19	5.60	2	0.59	31	9.14

PS = Positive selection

NS = Negative selection

With regard to the selective pressure that identified by FEL analysis at significance level 0.1, the result accords with SLAC analysis which most of codons in ORF1 and ORF2 were under selective selection (88.75% and 75.76%, respectively). Meanwhile, the ORF3 region was detected less than other regions with 9.14%. For the positively selected site, the individual sites under positive selection were found in ORF2 and ORF3 especially in the ORF2-ORF3 overlapping region at N-terminus of ORF2 to C-terminus of ORF3 which concerned with viral replication. Thirteen positive selection sites were identified in ORF2 (10, 11, 13, 37, 38, 39, 64, 70,76, 95, 97, 98, and103) and two positive selection sites in ORF3 (66 and 103). Moreover, almost positive selection sites in ORF3 were located in functional domain 66 (P1 domain) and 103 (P2 domain) while all of positive selection sites in ORF2 only located at N-terminus (Table 17).

Table 17 The site-specific positive and negative selection of HEV genotype 3 using three different algorithm and Integrative selection analysis.

Gene	Domain	Site	SLAC (Sig. Level =0.1)		FEL (Sig. Level =0.1)	
			dN/dS	**p-value**	**dN/dS**	**p-value**
ORF2	N-terminal	10	0.774	0.158	0.337	0.031*
		11	0.919	0.117	0.404	0.019*
		13	0.64	0.201	0.300	0.061*
		37	1.124	0.059*	0.722	0.003*
		38	0.481	0.298	0.253	0.062*
		39	0.786	0.147	0.423	0.030*
		64	0.482	0.296	0.262	0.059*
		70	1.285	0.039*	0.873	0.001*
		76	0.482	0.296	0.270	0.080*
		95	1.36	0.032*	0.822	0.001*
		97	0.803	0.132	0.450	0.015*
		98	1.123	0.104	0.906	0.011*
		103	0.768	0.144	0.341	0.032*
ORF3	P1 domain	66	1.735	0.133	1.804	0.079*
	P2 domain	103	2.632	0.088*	4.675	0.041*

'*' is significantly difference

Mutation analysis and clinical manifestation

Twelve complete genome sequences isolated from patients and swine from this study were used for comparing with reference complete genome sequences at amino acid level for revealing the significant mutation. The NCBI reference sequence NP_056779 was used for numbering of HEV ORF1 (98). In the previous study, there were the reported of the mutation in HEV genotype 3 shown in Table 4 which distributed in entire genome. For this study, the mutation points were found in RdRp domain of ORF1 region including F1439Y, V1479I, and G1634R whereas ORF2 was found one sample (MH450022) with only one point in P domain as N562D. However, the mutation was not found in ORF3 when compared our complete genome sequences with the reported site of mutation. The observation of each complete genome sequences

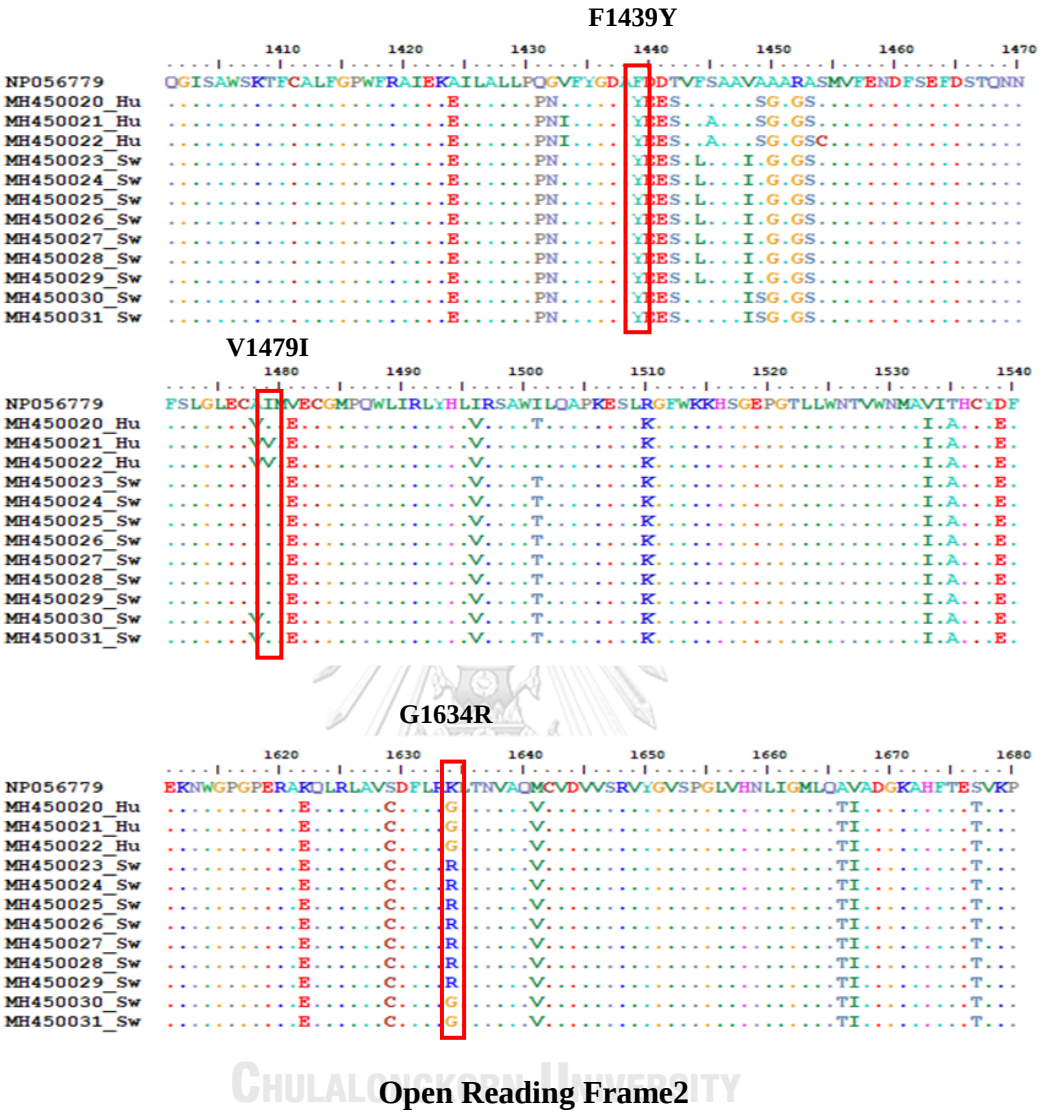
from this study, the mutation was found mainly in sequences isolated from swine but less in sequences isolated from human as shown in Table 18 and Figure 22.

Table 18 The observed HEV mutations were detected in complete genome sequences and their clinical manifestation.

Gene	Domain	Nucleotide substitution	Amino acid change (Reference)	Functional significance	Associated clinical manifestation	Observed the amino acid change in CG* sequences
ORF1	RdRp	T344A	F1439Y	Unknown	Ribavirin treatment failure	All complete sequences
	RdRp	NA	V1479I	Unknown	Ribavirin treatment failure	MH450020 MH450023-31
	RdRp	NA	G1634R/K	Increased efficiency of viral replication and infectivity	Ribavirin treatment failure	MH450023-29
ORF2	P domain	NA	N562Q/D/P/Y	Affect the dimerization of ORF2 protiein and HEV infectivity	-	MH4500022 (N562D)

*CG=complete genome

Open Reading Frame1



Open Reading Frame2

Figure 22 Deduced amino acid alignment of ORF1 and ORF2.

Mutation of RdRp domain of ORF1 and N-terminus of ORF2 in HEV genotype 3 complete sequences comparing with the completed genome sequences from this study.

CHAPTER IV
DISCUSSIONS AND CONCLUSION

All of the results in this study have been published and submitted in 2 different journals. And another is on process to prepare the manuscript. The publication as following;

Publication 1: Hepatitis E virus in pork and variety meats sold in fresh markets in Thailand. Food and Environmental Virology. 2017 Mar; 9(1):45-53.

Publication 2: Hepatitis E virus in Thai blood donors in Thailand
(2nd revised of Transfusion journal).

Publication 3: Genome analysis of HEV in swine and human circulating in Thailand (Manuscript in preparation).

The epidemiology and variation of HEV strain in Thailand

Previously, hepatitis E virus is known as an infectious disease that circulating only in developing countries and common cause of enterically transmitted viral hepatitis especially HEV genotype 1 and 2. Since HEV genotype 3 and 4 was found and confirmed as it could be isolated from animal species (190). HEV became to be the significant public health problem across the world. Several studies reported the HEV infection isolated from various animal reservoir including swine, wild boar, rabbit, camel, etc. and HEV could be identified in many countries varied by regions (74). For the genotype distribution of HEV, genotypes 1 and 2 are usually found in developing countries and are found only in human. Several developing countries have reported HEV outbreak from natural disasters such as flood and earthquake (191). A previous study reported no spike in HEV infection during the major flood of 2010 in Thailand (192). Genotypes 3 and 4 are found in developed countries whereby infections are sporadic and often found as complicated infection in immunocompromised patients or individuals under immunosuppression. Since swine remains the first probable animal source of HEV, it is important to determine whether swine can transmit this virus to human.

Evidence-based data suggest that HEV infection suspected to be a cause of acute hepatitis patients in Thailand. Potential zoonotic transmission of HEV especially from

autochthonous infection in industrialized countries is generally recognized from studies in experimental pig model and foodstuff containing pork products. Limited data on possible contamination of HEV in pork and variety meats in Thailand led us to analyze HEV in swine samples due to the abundance of fresh markets common in Southeast Asia. This study found that the prevalence rate of HEV of all the total specimens was 1.58 %. The prevalence is a lower rate compared to studies in other countries. The prevalence rate also was different by tissues examined. In addition, we found HEV RNA in market 0.23% and slaughterhouse 3.93% of the samples, which varied by specimen types. The prevalence rate of HEV RNA in pig liver (0.28%) and pork meat (0.36%) was significantly lower than those found in the bile (2.92%) and feces (5.24%), possibly due to viral shedding in animal excrement (193, 194)

As pigs comprise an economically important food animal for many countries, it is necessary to quantify the HEV risk to evaluate the zoonotic potential. Several surveys of swine farms in Thailand found between 10-65% of the pigs tested positive for anti-HEV IgG and 1.3-2.9% of pig feces contained HEV RNA depending on geographical regions (128, 129, 146). Since HEV is transmissible via fecal-oral route in pigs (195), a significant proportion of the swine raised even in industrialized countries such as France and The Netherlands are HEV-seropositive and their livers tested positive for HEV RNA at the time of slaughter regardless of the farming system used (conventional, free-range, or organic) (196, 197). Pork products remain the major probable animal source in acquiring HEV infection, consistent with the observation that the avoidance of pork consumption or close contact with pigs was associated with lower HEV sero-prevalence (158). As HEV replication is presumed to occur mostly in the porcine liver, HEV RNA has been detected in 1.9% of the liver sold as food in Japan (125), 0.83% in India (142), 1.5% in Madagascar (122), and 4.9% in Canada (198). Consequently, HEV contamination in pig liver sausages popularly consumed in Europe is not surprising (123, 124, 199, 200). Fewer studies have examined HEV contamination in the intestine, however, possibly because it is less popularly consumed. One study in an English slaughterhouse found HEV RNA contamination in 15% of the cecal content sample (140). It may be that intestinal fluid contains more HEV than smooth muscles and epithelial cells from the intestine because HEV was observed to replicate in bile epithelial cells and secreted into bile duct lumen (201). Our data

revealed lower prevalence (2.92%) than in pig bile in Italy (29.9%) (134). In a Spanish study, 37.7% of the pigs 1 week to 4 months examined were HEV-positive and that HEV was most commonly detected in the bile than any other sample types (195). China reported contamination had been found as high as 22.73%, possibly because sources of pig had been diverse and most of which belonged to genotype 4 (145). Overall, the prevalence of contamination in the liver, pork and intestine of the pig in Thailand was relatively low compared to other countries. Furthermore, even non-foodborne transmission of HEV linked to blood transfusion (159), pig handlers (202), and abattoir workers (203) has been reported. Therefore, HEV infection is not limited to only consumers, but also to healthcare and occupational safety.

The distribution of the four recognized HEV genotypes varied considerably worldwide. Although it has long been assumed that genotype 1 is prevalent in most Asian countries, a number of studies have confirmed the presence of mainly HEV genotype 3 in Thailand, all from symptomatic HEV infection (78, 82). This observation is consistent with the detection of genotype 3 in swine (146). Although evidence of specific HEV genotypes in Thailand has long been lacking in published literatures, the prevalence of genotype 3 and not 1 in Thailand may help explain a case report of an acquired HEV infection by an American who travelled to Thailand (204). Furthermore, the relative absence of genotype 1 and 2 is consistent with the absence of a spike in HEV infection during a devastating flood inundating Thailand in 2010 as would be expected as a result of natural disasters (191, 192).

As with other positive-strand RNA virus, potential genome recombination was observed in 3 samples, which exhibited ORF1 from genotype 3i and ORF2 from genotype 3f. Identification of genotype 3f in swine and genotype 3e in captive wild boar has been reported in Thailand (128, 131, 186). Emergence of recombinants was not unexpected since HEV has been reported to undergo intergenotypic and intra-genotypic recombination (205).

This study has several limitations. Although attempts were made to gather many types of samples, future studies will benefit from examining other tissues including the lymph nodes and blood. It is uncertain whether surveying markets outside of the metropolitan Bangkok will yield significantly different HEV prevalence or whether sensitivities in HEV testing may improve detection if all samples were

obtained directly from the slaughterhouse. Although the relatively low prevalence of HEV found in this study suggests that virus contamination in fresh meat is likely not widespread, the presence of HEV may depend on the age of the pig at slaughter, which typically is between 6 to 8 months. Additional studies to assess HEV burden in Thailand and elsewhere in Southeast Asia will enable the implementation of sound public health measures towards preventing HEV-related illness.

For the conclusion of this study, the result supported the assumption that swine is the possibly animal reservoir and seemingly food related of HEV infection in Thailand especially genotype 3. Thus, the food chain seems to be the next cause of HEV infection in human. Moreover, the other transmission routes are reported as well including blood borne transmission and organ transplantation. Presently, the blood transfusion has been of paramount importance for blood component therapy. Blood safety service should be implemented in the blood policy in every country. Many pathogens including HBV, HCV, and HIV must be screened before giving to patients. For HEV, many study showed the possibility of HEV infection in the patient through blood transfusion. So, the nationwide surveillance of HEV should be conducted in every country especially rural area.

In Thailand, the lack of scientific data on the potential risk of HEV transmission from donated blood led us to examine the presence of HEV RNA among healthy Thai blood donors (206). This study is the first investigation of the HEV infection among Thai blood donors with the large scale. By initially screening pooled plasma samples, and upon detecting HEV- reactive pools, samples from individual donors were then tested, we were able to screen >30,000 donor samples in a relatively short time. In this study, narrow sampling window of 3 months resulted in the exclusion of repeat donors, which was different from other similar studies whereby donations collected over several months or years were analyzed for HEV prevalence. Results from this study were also carefully validated using additional methods of nucleic acid test (cobas HEV test) and nucleotide sequence analyses and complemented with serological assays (anti-HEV IgM and IgG ELISA). From our study criteria of three-positives by the in-house real-time RT-PCR, the frequency of HEV RNA detected among healthy Thais was 26 in 30,115 donors, or approximately 1 in 1,158 individuals.

Approximately three times as many donors tested positive for HEV RNA by the in-house assay compared to the cobas HEV test. Specificity of the in-house assay was re-validated in this study against a wide variety of virus, so the greater number of positives was not likely due to non-specific cross-reactivity. Rather, it may be attributed to the differences in the limit of detection by the two assays. Although testing all samples with the cobas HEV test would have been ideal due to its fully automated platform, it was too cost-prohibitive on a large scale. This study was the first in Thailand to use the cobas for HEV testing and validation of the limit of detection was not done due to cost, therefore it was possible that the sensitivity of the cobas HEV test in this setting may differed than the published value. Samples with lower Ct values (between 33-35) obtained from the in-house assay were more likely to test positive in the cobas HEV test, although there was no absolute correlation between the Ct values of the two assays. Moreover, the in-house assay detected HEV RNA in donors with serological evidence of past HEV infection more often than the cobas HEV test. Thus, the results from the in-house assay alone suggest that as many as 1 in 1,158 donors could be HEV-infected at the time of donation.

For the blood safety, the testing with high sensitivity and specificity were used for blood donor screening due to prevent transfusion transmissible infection in recipients. False reactive results seem to be a problem in the interpretation result and lead to blood donor deferral and discard blood unit but it did not affect the recipients. In addition, some false reactive donors turn to be negative result after a suitable course of infection period and can re-entry to be a donor in the future (207). While the false negative results may jeopardize for the blood recipient because of the complications in post-transfusion (208). However, the gold standard testing for HEV infection was needed for confirmatory test (209). From the result of this study, we recommended to use both number of reactive results from in-house real-time RT-PCR. Although, the sensitivity of cobas HEV test was better than in-house real-time RT-PCR, less confounding factor due to fully automatic system and generally accepted, the limitation of financial resources and reagent supplying was the cause that we used the in-house real-time RT-PCR for HEV screening instead. So, the detectable HEV RNA was followed the result from both cobas HEV test and in-house realtime RT-PCR.

There is an increased consideration for the potential transmission of HEV to recipients of blood components (210-212). Several studies have assessed the risk of acquiring HEV from donated blood using a variety of methods to detect viral RNA, most frequently either in-house RT-PCR or commercial tests. In addition to varying methodologies and survey populations, results are sometimes not directly comparable because prevalence rates are expressed as per donations and not as the detectable HEV RNA among donors. Nevertheless, studies of HEV RNA prevalence conducted in large cohorts of healthy blood donors in Europe have ranged between 1 in 762 (The Netherlands) to 1 in 8,000 (Sweden) (167), (164). Intermediate rates were reported in France (1 in 2,200) (213), Denmark (1 in 2,300) (162), England (1 in 2,800) (75), Spain (1 in 3,300) (161), Germany (1 in 4,500) (164), and Ireland (1 in 5,000) (165). Lowest HEV burden appears to be in the U.S.A. (1 in 9,400) (174), Canada (0 in 13,993) (214), and Australia (1 in 15,000 and 1 in 74,131) (215), (216). In the majority of these studies, genotype 3 was most often detected. This is hardly surprising given the zoonotic potential for HEV genotype 3 and 4 from the consumption of infected pork and pig organs (217).

All HEV strains identified in this study also belonged to genotype 3 and displayed high sequence identity to strains previously described in human and swine found in Thailand (128). As HEV genotype can be confounded by recombinants, we analyzed both ORF1 and ORF2 and used the accepted reference sequences for genotyping (156). Since the consumption of undercooked pork, pork products, and to a lesser extent contaminated shellfish, contributes to the widespread exposure to HEV in the general population, it is presumed that donors most likely acquired HEV from dietary consumption, although occupation-related infection (e.g. abattoir and swine farms) cannot be excluded (123, 218, 219).

In addition to detecting HEV RNA, another important measurement to assess HEV burden is the serological values of anti-HEV IgM and/or IgG. ELISA-based determination of anti-HEV IgM and IgG antibodies in 26 donor samples with three-reactive by real-time RT-PCR showed that only 2 samples tested reactive for anti-HEV IgM ELISA with Euroimmun ELISA kit but no reactive by using Wantai ELISA kit. While seven concordant samples were found for anti-HEV IgG detection between Wantai and Euroimmun ELISA kit and only 2 samples were discordant results. The

discordant result between these two ELISA kit may occur when the sensitivity of ELISA kit was different. Vollmer and colleagues showed the comparative sensitivity of anti-HEV IgM and IgG ELISA commercial assay and they found that the sensitivity of Wantai ELISA kit was better than Euroimmun ELISA kit (220). Same with Norder and colleagues demonstrated that the performance of anti-HEV ELISA kit was depend on the sensitivity for the HEV detection (221). Otherwise, the reactive results of anti-HEV IgM ELISA in reactive samples showed very low titers indicated that might be false reactive or nonspecific reactive as well. Therefore, this study used the result from Wantai ELISA kit to interpret the result for reliability of serological result.

As in other studies, we found that many HEV RNA- reactive individuals did not demonstrate detectable IgM sero-positivity, therefore, the combined absence of anti-HEV IgM and IgG antibodies in these donors may indicate early stages of primary acute (163, 222, 223). Similar to the comparison of HEV RNA detection among countries, published sero-prevalence rates in different studies are difficult to compare due to possible sample duplications when samples are obtained over several months and years. We recently performed sero-prevalence survey in Thailand and found that approximately 1 in 3 Thais residing in central Thailand possessed anti-HEV IgG antibodies, which was three times higher than those residing in southern Thailand where residents are predominantly Muslims, and Islam region proscribes pork consumption (158). Nationwide, approximately 14% of Thai men in their 20's possessed anti-HEV IgG antibodies (127). Thus, past exposure of HEV in Thailand as measured by HEV sero-prevalence does not differ drastically from the rates found in western Europe (126, 224, 225).

Data regarding the prevalence of HEV in healthy adults have important implications in ensuring the safety of donated blood. At the very minimum, all donated blood is screened for HBV, HCV, and HIV. Further screening for HEV is currently implemented in only selected countries. Although extremely rare, transfusion-associated HEV transmission leading to clinical hepatitis has been reported in France, Germany, Japan, and the U.K. (49, 50, 75, 226). It may, therefore, be necessary to ensure the exclusion of HEV from donated blood and blood products when they are destined for at-risk recipients, such as pregnant woman, cancer patients on chemotherapy, individuals infected with human immunodeficiency virus, and solid

organ transplant recipients (227). Due to the narrow HEV viremia window in infected individuals, effective screening could rely on a nucleic acid test. Additional, the serological test for anti-HEV IgM and IgG detection may consider to be the supplementary test for diagnosis the acute, chronic, and past HEV infection (221). However, the additional test for HEV infection testing depends on the purpose of using and budget. Current challenges in HEV screening in donated blood include reliability and cost-effectiveness for the blood bank. Therefore, results from this study are expected to assist in the decision-making process towards further ensuring the safety of the blood supply in Thailand.

Many limitations of this study must be noted. Firstly, we used the remaining samples from routine, so the samples were limited and hardly repeated several times. Additional, we did not have back-up samples system so the look-back study was impossible and the IRB was not cover the patient study as well. These reasons led to lack of valuable data for supporting the evidence of HEV infection in Thailand.

In summary, the epidemiology of HEV infection in Thailand showed low prevalence and closely with the prevalence reported from developed countries. Moreover, the genotype 3 was still predominated circulated in Thailand which it could be isolated from human and animal species led to the evidence of cross species can be occurred. Whole genome analysis is the method that is useful for evaluation the HEV genotype and subtype in Thailand. Moreover, it could be used for prediction the evolutionary history, the origin and genetic diversity of HEV isolated from Thailand.

The classification and genetic diversity of HEV isolated from Thailand

Genotype 3 is the zoonotic and can infect both human and animal species led to develop the ability to efficiently transmit to other hosts species. Moreover, the new finding of the new host species was reported with increasing the new strains of HEV genotype 3(228, 229). The complete genome sequence analysis of HEV genotype 3 has been identified the genetically distinct due to various animal reservoirs including pigs, wild boars, deer, rats, rabbits, and chicken (116-121). In addition, the expanding host range has involved with the reported of genetic diversity and recombination in HEV among human and animal species significantly (176). Likewise, the mutation of HEV could be also detected because HEV is RNA viruses that showed high genetic

variability and rapid evolutionary change (230). In Thailand, HEV genotype 3f was mainly reported in Thailand and another finding was reported HEV genotype 3e as well that isolated from wild boar (131). Moreover, the study of case report an American who returned from a traveling in Thailand and infected with HEV genotype 1 (204). However, the evidence of specific HEV genotypes in Thailand has long been lacking in published literatures lead to ambiguous data. To address this concern, this study analyzed the complete genome sequences of HEV which were found from this study and evaluated the evolution for understanding the drivers of virus replacement and genetic diversity for HEV isolated from Thailand.

In our previous study, the result showed HEV genotype 3f circulating in Thailand but the results were performed the sequence from partly of ORF1 and ORF2. Some samples exhibited ORF1 from genotype 3i and ORF2 from genotype 3f so that the whole genome sequencing ought to perform. For the whole genome sequencing, twelve samples succeed for sequencing and the result of phylogenetic analysis showed all samples from patient and pork and variety meats were classified belonged to HEV genotype 3. All sequences were cluster with the reference sequences that isolated from both human and swine. In addition, the phylogenetic tree showed the percentage of identity among our HEV genotype 3f sequences that it can assume that these two groups were collected from different date or more than one strains circulating in the collection place. Then, the sub-genotype classification was performed by using pairwise comparison of our complete genome sequences compared with reference complete sequences followed Smith at al. 2016 (156) The criterion has been proposed that the difference of complete nucleotide sequence for genotype 1 and 2 range from 6.2-11.0% whereas those for genotype 3 and 4 range from 12.1-18.0%. For subtype of HEV genotype 3, there are 3 clades comprising 3abchij as the major clade, 3efg, and 3ra which classified by using complete sequence range from 12.1-18.0% for genotype 3 and 4 (14) According to our results, mainly samples from swine and only one sample from human were HEV genotype subtype f which almost studies reported this subtype was circulated among human and swine in Thailand (128, 129) Other two samples from human were identified to be sub-genotype 3a (Asian strain) groups with AF082843 and 3c (European strain) groups with FJ705359. This demonstrated that not

only HEV genotype 3f was circulating in Thailand but genotype 3a and 3c could be also found in Thai isolation.

HEV genotype 3 was extremely diverse and can be subdivided to many subtypes. Previously, high level of genomic intermixing and recombination were reported in HEV genome therefore the classification of ORF1 and ORF2 were useful for confirmation the subdivided of HEV strains (13). In this study, the result of the pairwise comparison of ORF1, ORF2, and ORF3 was performed as well. The percentage of identity in our sequences comparing with the reference sequences revealed that almost sequences of each HEV genes were conclusively identified as HEV genotype 3f. Except two sequences of ORF1 and ORF2 from human, MH450021 was identified as HEV genotype 3c which mostly found in Europe including The Netherlands, United Kingdom, Bulgaria etc. (167, 231, 232). Another sequence (MH4500022) was identified as HEV genotype 3a that was suspected to originate from Japan but some studies also demonstrated this subtype was found in USA, Korea, and the Netherlands (132, 167, 233). For the analysis of ORF3, these two sequences were segregated to HEV genotype 3i and 3b respectively. It can assume that ORF3 region is more highly variable than ORF1 and ORF2 which was not clear separation into subtypes. However, the complete genome sequence would be required to classification of HEV (13). The HEV genotype 3a and 3c were rarely found in Thailand and both samples were isolated from patient who were elderly males, not the transplantation case. Thus, this informative data is more useful for identification of HEV strain in Thailand. Although the significance among HEV diversity was not revealed now, the isolation of HEV was diverse and has different geographic distribution as HCV variant, may reflect to the transmission patterns as well. However, the data of HEV still unclear.

The evolutionary history, selective pressure, and mutational analysis of HEV isolated from Thailand

The evolutionary analysis was used to investigate the origin and history of HEV for understanding the pattern of HEV distribution especially in Thailand. As the result showed the estimated of the mean time of the ancestor for HEV genotype 1-4 with the sequence from Thailand was 1,624 years ago which was different from the previous study. The previous study focused the all genotypes of *Orthohepevirus A* sequences

with filtered the HVR and the estimated the TMRCA to be 6795 year ago whereas another study demonstrated the TMRCA of modern HEV genotype by using ORF2 non-overlapping region ranged from 536-1344 years ago (70, 182). So, the result of the origin time in each study depends on the using of dataset and region of HEV. Moreover, our study also estimated the TMRCA of genotype 1, 3, and 4 which was 447, 462, and 536 years ago, respectively. For HEV genotype 3 that mainly circulating in Thailand, the estimated TMRCA of this study was similar to the finding by Mirazo and coworkers estimated 320 years ago (236-420 years ago)(234). While another study by Zehender and colleagues showed the mean TMRCA of the tree root was 199 years ago (116-289 years ago), later than our study (235). For the substitution rate, our finding was $5.63x10^{-4}$ base substitution per site per year, lower than other study. The previous study showed the various of evolution rate. Purdy and colleagues obtained a mean of evolutionary rate of $1.13x10^{-3}$ by using Bayesian approach for ORF2 non-overlapping region whereas Zehender and colleagues also estimated in ORF2 region obtained the evolution rate of $1.8x10^{-3}$ with the same method. Another study of Tanaka and coworkers who used the RdRp of ORF1 by using the two different approaches obtained the rate of $0.84x10^{-3}$. Nevertheless, there were a different appropriate method, the different of gene studied, and sampling bias of selected complete genome sequences therefore the reported of TMRCA was various among the finding. In addition, the hypervariable region of HEV was various among genotype and subtype led to difficult for data analysis.

In Thailand, HEV infection has occurred like developed countries with the reported of sporadic case that's why the data is limited. There were a few reported complete genome sequences from Thailand lead to the origin of HEV in Thailand was not clear. HEV genotype 3 was predominate in Thailand especially subtype 3f which usually found in Thai swine. Interestingly, we found the other subtype of HEV as well that usually found in swine and human in the European countries and Japan so that the whole genome sequencing was required. For this study, we succeed the whole genome sequencing and analyzed the evolutionary of HEV. The TMRCA of HEV genotype 3 was most probably originated in Thailand in 1900 to 1921 (97-118 years ago) which HEV genotype 3a and 3c were likely circulated in Thailand before HEV genotype 3f. Interestingly, HEV genotype 3a and 3c were not shared the common ancestor with HEV

genotype 3f, which genotype 3a and 3c contain in 3abchij clade but genotype 3f contains in 3efg clade. In addition, HEV genotype 3a was Asian subtype and genotype 3c was European subtype which have not reported in Thailand (235). The reason was unclear but we assume that it may harbor in the environment and the acute self-limited disease of HEV genotype 3 led to underestimation and misdiagnosis. For the host of HEV, the phylogenetic tree demonstrated that HEV genotype 3 in Thailand was probably transmitted from a swine infecting ancestor then spread among human and swine. Like the previous study, Brayne and colleague showed the host-specific patterns of HEV genotype 3 was high jumps and indicated the HEV frequent transmission form swine to humans (93) Thus, our result support that HEV genotype 3 widely dispersed worldwide and the origin of HEV in Thailand was occurred more than 100 years ago and swine seem to be the swine-infecting ancestor.

The skyline plot for HEV genotype 1 to 4 in this study indicated that *Orthohepevirus A* likely originated for a long time that existed around 1600 years ago. The effective population size of HEV has been stable until 1771 then the effective population slightly expanded and increased. In 1914, the world war I was occurred which corresponded to the increasing of effective population size of HEV. Followed the study by Tanaka and coworkers who demonstrated that the effect of the war resulted to the effective population of HEV because people began to migrate to other setting and lack of the good sanitation (70, 182). Then, the effective population was continuous to increase in the period of the world war II in 1940 to 1945 after that it decreased rapidly around 1990. Moreover, the Japanese government introduce to import pig from England in 1990 which may possibly relate to the spread of HEV among pig for food in Japan (236). The reason is unclear about why the effective population decreased after the year 1990. However HEV became to know as the hepatitis viral and a lot of study reported the route of transmission like hepatitis A virus lead to awareness of HEV infection among world population (182).

The selective pressure was analyzed for studying the adaptive evolution as well. The HEV genotype 3 was performed in this study because it was reported in Thailand. Two different method comprising SLAC and FEL were used for identification the positive and negative selection in each gene of HEV sequence (ORF1, ORF2, and ORF3). According to the result of both method, the selective pressure was different

among HEV gene which the selective pressure on the functional domain of ORF3 was stronger than ORF1 and ORF2. The ORF1 was found no evidence of positive selection conversely all codons were detected under negative selection indicating that the mutation was not fitness or deleterious for the replication of virus. In addition, ORF1 region has many functional domains therefore more conserve in these domain (24). Although, this region contains the hypervariable region, this domain was not found more frequently than would be expected (237). For ORF2, the positive selections were found in this region especially N-terminal of ORF2 which plays a crucial role in RNA-protein interaction during viral replication (42). In the same way, the previous study showed the codon in the N-terminus of ORF2 detected high frequency of positive selection as well (186).

ORF3 was strongest selective pressure and found positive selection at C-terminus of ORF3 (N-terminal ORF2) or overlapping of ORF2 and ORF3 region. Two positive selection codons were located at P1 and P2 domain which P1 domain related with phosphorylated by MAPK during HEV infection and P2 domain contains a proline-rich PxxP motif for binding the Src homology domains concerned with the signal transduction pathway in the release stage of the viral assemble process (37). Thus, the positive selection of ORF3 may be the important for interaction with intracellular proteins (43). Furthermore, the positive pressure on ORF3 may affiliate with the diversity of HEV as well (186). And the overlapping of ORF2 and ORF3 (N-terminal of ORF2 and C-terminal of ORF3 was important in a mechanism of viral packing in the limited genomic space and it must translate to two protein product lead to have more substitution in ORF2-ORF3 overlapping which have an effect on the rate of evolution rate as well (93). Consequently, the distribution of positive pressure on ORF1, ORF2, and ORF3 of HEV genotype 3 were not randomly but all positive pressure and negative pressure were associated with the fitness for survival of the virus (238).

After the mutation, the synonymous and non-synonymous substitutions can occur over the entire HEV genome due to the transcription process. The viral RdRp lack the poof-reading activity associate with the viral replication lead to rise the variations in the HEV genome (239, 240). The main target cell of HEV is liver cells so the HEV mutation may occur and associate with the clinical manifestation of Liver.

From the previous study, many studies showed the mutation that concerned with the clinical presentation in the hepatitis patients shown in Table 4. The mutation in the HEV genome of Thai strain was not reported yet and almost patients was infected with HEV genotype 3 which was asymptomatic and self-limited due to lack of mutation data in Thailand (206). However, the organ transplantation in Thailand has increased the number of the patient which HEV could be transmitted by this route as well. Thus, the detection of HEV has been important to follow these patients because there were the reported of HEV infection in organ transplant patients and developed to be the chronic HEV infection later (241). For this study, we performed the investigation of HEV mutation in our complete genome sequence by comparing with the reference sequence for numbering the position of mutation (98). The mutation that we found in this study has 4 mutation which 3 of 4 were located on RdRp of ORF1 and concerned with the treatment with Ribavirin lead to the failure. Ribavirin is the only drug for using HEV infection treatment now which inhibit the HEV replication but its increased the error rate of RdRp in viral replication as well (91). G1346R is the small population that was changed after treatment with Ribavirin which the patient failed to response for Ribavirin therapy and the amount of virus became to increase again especially in immunosuppressive patients (91) For the functional of HEV mutation, the mutation of HEV genome may increase the efficiency of viral replication and infectivity lead to Ribavirin treatment failure (95). According to our study, the mutation of G1346R was occur mainly in complete genome sequence that isolated from swine but in human, amino acid was not changed. Furthermore, the other substitutions were detected as V1479I that together with G1634R in the mutation of ORF1, F1439Y was identified to associated with fulminant hepatic failure (91, 178) Another mutation, N562D was located in ORF2 which was evolved with the glycosylation and dimerization of ORF2 protein. Furthermore, this mutation may affect the activity of neutralizing epitopes of capsid protein but the clinical relevant was not reported yet (106, 242).

Conclusion of this study

The epidemiology and the evolutionary of HEV were revealed from this study. Firstly, the epidemiology of HEV in pork and variety meat from supermarket, bile and feces from slaughterhouse were investigated the prevalence which found lower than other countries and genotype 3 was the main strain that circulating in Thai isolation as European countries. Secondly, the screening of HEV in blood donors was performed which the detectable HEV RNA among healthy Thais blood donors of 0.09%, or approximately 1 in 1,159. Moreover, the HEV genotype 3 was predominated in Thai blood donors as European countries. Finally, the whole genome analysis of the complete genome sequencing isolated from Thailand showed that HEV genotype 3f was mostly circulating in Thailand. Moreover, the other subtype was also found in Thailand as HEV genotype 3a and 3c. The evolutionary analysis was estimated the mean time of the ancestor for HEV genotype 1-4 with the sequence from Thailand was 1,624 years ago (95% HPD: 1222.40-2110.48) and the substitution rate was 5.63x10-4 base substitution per site per year which was lower than the previous study. Moreover, the TMRCA of HEV genotype 3 in Thailand was most probably originated in Thailand in 1900 to 1921 (97-118 years ago) and might be originated with swine infecting ancestor then intermix between human and swine. For the natural selection analysis, there are the positive selection in HEV genome sequence including ORF2 and ORF3 especially ORF2-ORF3 overlapping region whereas ORF1 found only negative selection. Thus, HEV was the highly conserve in the functional domains. Furthermore, the mutation associated with the clinical manifestation was identified in complete genome sequences. The mutation was found F1439Y, V1479I, and G1634R in ORF1 and N562D in ORF2 which were similar to the previous studies. This informative data will provide the prevalence of HEV in Thailand. Including the evidence indicates that epidemiology may play a crucial role to help prevention of HEV infections in Thailand and useful for the further study of HEV infection.

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APPENDIX

Table S1 List of reference complete genome sequences information.

Accession No.	Strain	Length	Host	Geno-type	Countries	Year
M73218	Burma	7202	Human	1a	Myanmar	1982
D11092	HPECG	7207	Human	1b	China	1987
X98292	L1	7202	Human	1c	Indian	1992
AY230202	Morocco	7212	Human	1d	Morocco	1994
AY204877	T3	7170	Human	1e	French	1983
JF443721	IND-HEV-AVH5-2010	7217	Human	1f	India	2010
M74506	M1	7180	Human	2a	Mexican	1986
AF082843	Meng	7207	Swine	3a	USA	1996
AP003430	JRA1	7230	Human	3b	Japan	2001
FJ705359	wbGER27	7237	Wild boar	3c	Germany	2006
AB248521	swJ8-5	7241	Swine	3e	Japan	2000
AB369687	E116-YKH98c	7217	Human	3f	Japan	1998
AF455784	Osh205	7239	Human	3g	Kyrgyzstan	1987
JQ013794	TR19	7182	Human	3h	France	2007
FJ998008	BB02	7206	Wild boar	3i	Germany	2007
AY115488	Arkell	7255	Swine	3j	Canada	2002
AB290312	swMN06-A1288	7237	Swine	3	Mongolia	2006
JQ953664	FR-SHEV3c-like	7238	Swine	3	France	2006
AB369689	E088-STM04c	7215	Human	3	Japan	2004
AB290313	swMN06-C1056	7239	Swine	3	Mongolia	2006
EU360977	swX07-E1	7258	Swine	3	Sweden	2007
KJ873911	FR_R	7157	Human	3f	Germany	2013
EU723513	SW627	7193	Swine	3	Spain	2008
FJ906895	GDC9	7318	Rabbit	3ra	China	2009
KJ013415	CHN-BJ-r14(9)	7296	Rabbit	3	China	2013
JQ013791	W1-11	7271	Rabbit	3	France	2007
AB197673	JKO-ChiSai98c	7257	Human	4a	China	1998
DQ279091	swDQ	7234	Swine	4b	China	2005
AB074915	JAK-Sai	7236	Human	4c	Japan	1994
AJ272108	T1	7232	Human	4d	China	2000
AY723745	IND-SW-00-01	7262	Swine	4e	India	2004
AB220974	HE-JA2	7268	Human	4f	Japan	1998
AB108537	CCC220	7193	Human	4g	China	2000
GU119961	CHN-XJ-SW13	7264	Swine	4h	China	2009

Accession No.	Strain	Length	Host	Geno-type	Countries	Year
DQ450072	swCH31	7248	Swine	4i	China	2006
AB369688	E087-SAP04C	7227	Human	4	Japan	2004
AB573435	JBOAR135-Shiz09	7267	Wild boar	5a	Japan	2009
AB602441	wbJOY_06	7261	Wild boar	6a	Japan	2006
AB856243	wbJNN_13	7263	Wild boar	6	Japan	2013
KJ496143	178C	7224	Camel	7a	United Arab Emirates	2013
KJ496144	180C	7223	Camel	7	United Arab Emirates	2013

Table S2 List of reference complete genome sequences isolated from Thailand.

Accession No.	Strain	Length	Host	Geno-type	Nationality of host	Year	Comment
AB369687	E116-YKH98C	7217	Human	3	Thailand	1998	Derived from Japanese patients who had traveled to Thailand.
FJ653660	CU001	7216	Human	3	Thailand	2008	
KY232312	TH-hu-S45-1	7235	Human	3	Thailand	2014	Derived from serum
KY232313	TH-hu-F45-1	7235	Human	3	Thailand	2014	Derived from feces
EU375463	Thai-swHEV07	7245	Swine	3	Thailand	2008	

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