การศึกษาความชุกทางเซรุ่มวิทยาของไวรัสตับอักเสบอีและการตรวจพบอาร์เอ็นเอของไวรัสตับอักเสบอี ในเลือดและอุจจาระ ของผู้ป่วยหลังปลูกถ่ายตับในระหว่างการติดตามศึกษาเป็นเวลา 1 ปี

นางสาววินิตา โอฬารลาภ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2559

HEV Seroprevalence, Serum and Feces HEV RNA positivity in Post-Liver Transplant Patients During 1-year Follow-up Period

Miss Vinita Oranrap

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medicine
Department of Medicine
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Chulalongkorn University
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Thesis Title	HEV Seroprevalence, Serum and Feces HEV RNA positivity in Post-Liver Transplant Patients During 1-year Follow-up Period
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วินิตา โอฬารลาภ : การศึกษาความชุกทางเซรุ่มวิทยาของไวรัสตับอักเสบอีและการตรวจพบอาร์เอ็นเอของไวรัสตับอักเสบอี ในเลือดและอุจจาระ ของผู้ป่วยหลังปลูกถ่ายตับในระหว่างการติดตามศึกษาเป็นเวลา 1 ปี (HEV Seroprevalence, Serum and Feces HEV RNA positivity in Post-Liver Transplant Patients During 1-year Follow-up Period) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ. ปิยะวัฒน์ โกมลมิศร์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. ยง ภู่วรวรรณ, 47 หน้า.

ความสำคัญและที่มาของงานวิจัย:ไวรัสตับอักเสบอี (HEV) เริ่มมีความสำคัญในฐานะไวรัสตับอักเสบชนิดหนึ่งซึ่งมีอุบัติการณ์การติดเชื้อในผู้ป่วยที่มีภูมิคุ้มกันต่ำ โดยเฉพาะในผู้ป่วยหลังปลูกถ่ายตับ การศึกษาก่อนหน้านี้ในยุโรปและอเมริกาพบว่าความชุกทางเซรุ่มวิทยาของไวรัสตับอักเสบอีในประชากรทั่วไปเท่ากับ 5-12% และ 19% ตามลำดับ ในขณะที่การตรวจพบไวรัสตับอักเสบอีในเลือดในยุโรปและญี่ปุ่นพบว่าน้อยมากคือ 1.4% และ 0.12% ตามลำดับ งานวิจัยนี้ศึกษาความชุกทางเซรุ่มวิทยาและการตรวจพบไวรัสตับอักเสบอีทั้งในเลือดและอุจจาระ โดยมีสมมติฐานว่าในผู้ป่วยหลังปลูกถ่ายตับอาจมีการติดเชื้อไวรัสตับอักเสบอีแบบแฝงไม่แสดงอาการ

วิธีการวิจัย:จากผู้ป่วยหลังปลูกถ่ายตับทั้งหมด 106 คน ได้ถูกรวบรวมเข้าการศึกษาและได้รับการตรวจการติดเชื้อไวรัสตับอักเสบอีทั้งทางเซรุ่มวิทยาและการตรวจหา RNA ในเลือดและอุจจาระ หลังจาก คัดผู้ป่วยที่มีการติดเชื้อไวรัสตับอักเสบอี (n=3) และผู้ป่วยที่ไม่สามารถเจาะเลือดหรือเก็บอุจจาระ ได้ (n=13) ออกไป ผู้ป่วยทั้งหมด 91 คนได้เข้าร่วมการศึกษาโดยการตรวจทางเซรุ่มวิทยาของ ไวรัส ตับอักเสบอีทั้ง anti-HEV IgG และ anti-HEV IgM รวมทั้งการตรวจหา RNA ในเลือด และ อุจจาระด้วยวิธี RT-PCR ข้อมูลพื้นฐานของผู้ป่วยจะถูกรวบรวมจากประวัติบันทึกทางคลินิค และ นำมาวิเคราะห์ทางสถิติวิจัยแบบพรรณนา

ผลการศึกษา:ความชุกทางเซรุ่มวิทยาในผู้ป่วยทั้งหมด 106 คน เท่ากับ 53.8% หลังจากการคัดผู้ป่วยที่ไม่สามารถติดตามในระยะเวลาของงานวิจัยได้ จากผู้ป่วยในการศึกษา 91 คน ตรวจพบว่ามีผู้ป่วยที่ตรวจพบ anti-HEV IgG เป็นผลบวกคิดเป็น 50.5% และ anti-HEV IgG เป็นผลลบคิดเป็น 49.5% เมื่อพิจารณาที่ข้อมูลพื้นฐานและทางคลีนิกของผู้ป่วยทั้งสองกลุ่มแล้วพบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ในกลุ่มที่มี anti-HEV IgG เป็นบวกที่จุดเริ่มต้นของการศึกษาตรวจพบไวรัสตับอักเสบอีในเลือดและอุจจาระคิดเป็น 5/46 คน (21%) และ 1/46 คน (2%) ตามลำดับ ในกลุ่มที่มี anti-HEV IgG เป็นลบ ตรวจพบไวรัสตับอักเสบอีในเลือดและอุจจาระคิดเป็น 2/45 คน (4.5%) และ 3/45 คน (6.7%) ตามลำดับ การศึกษานี้เมื่อเก็บผลวิจัยจนครบ 1 ปีพบว่า ในการตรวจครั้งที่ 4 มีการตรวจพบไวรัสตับอักเสบอีในเลือดหรืออุจจาระที่มากกว่าปกติ ผู้วิจัยจึงมีความตั้งใจวิเคราะห์ผลการศึกษาในช่วง 8 เดือนแทน เมื่อสิ้นสุดการศึกษาที่ 8 เดือนพบว่าในกลุ่มที่มี anti-HEV IgG เป็นบวกตรวจพบไวรัสตับอักเสบอีในเลือดและอุจจาระคิดเป็น 11/46 คน (24%) และ 3/46 (6.5%) ตามลำดับ ส่วนกลุ่มที่ anti-HEV IgG เป็นลบตรวจพบไวรัสตับอักเสบอีในเลือด และอุจจาระคิดเป็น 9/45 คน (20%) และ 4/45 (8.8%) ตามลำดับ ในช่วงระหว่างติดตามการ ศึกษา 8 เดือนพบว่าตรวจพบไวรัสตับอักเสบอีเคสใหม่ในเลือดหรืออุจจาระในกลุ่มที่มี anti-HEV IgG เป็นผลบวกและผลลบคิดเป็น 8/14 คนและ 8/13 คนตามลำดับ ผู้ป่วยทั้งหมด 27 คนที่ตรวจพบไวรัสตับอักเสบอีในเลือดหรืออุจจาระมีทั้งหมด 2 คนที่มีค่าตับผิดปกติ หนึ่งในนั้นตรวจพบว่ามีท่อน้ำดีตีบตันและนิ่วในท่อน้ำดีซึ่งหลังจากส่องกล้องทางเดินน้ำดีเพื่อเอานิ่วออก

บทสรุปงานวิจัย:ความชุกทางเซรุ่มวิทยาในผู้ป่วยไทยหลังปลูกถ่ายตับค่อนข้างสูงเมื่อเทียบกับข้อมูลการศึกษาจากประเทศอื่นๆ แสดงให้เห็นว่าไวรัสตับอักเสบอีอาจมีความสำคัญมากกว่าที่คาดคิดในผู้ป่วยกลุ่มเสี่ยง การตรวจพบไวรัสตับอักเสบอีในเลือดหรืออุจจาระแม้ในกลุ่มที่ตรวจพบ anti-HEV IgG เป็นลบ แสดงให้เห็นว่าการวินิจฉัยการติดเชื้อไวรัสตับอักเสบอีไม่สามารถใช้การตรวจทางเซรุ่มวิทยาเพียงอย่างเดียว ยังคงต้องอาศัยการศึกษาต่อเนื่องในอนาคตเพื่อติดตามความสำคัญของไวรัสตับอักเสบอีในผู้ป่วยกลุ่มนี้ต่อไป

ภาควิชา	อายุรศาสตร์	ลายมือชื่อนิสิต	.....
สาขาวิชา	อายุรศาสตร์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

5874068030 : MAJOR MEDICINE
KEYWORDS:

VINITA ORANRAP: HEV Seroprevalence, Serum and Feces HEV RNA positivity in Post-Liver Transplant Patients During 1-year Follow-up Period. ADVISOR: ASST. PROF. PIYAWAT KOMOLMIT, Ph.D., CO-ADVISOR: PROF. YONG POOVORAWAN, M.D., 47 pp.

Introduction: Hepatitis E virus (HEV) has emerged as an important infectious disease in immunocompromised patients, especially those who are post-liver transplanted (LT). Reported HEV seroprevalence rates in general populations of Europe and the United States are 5-12% and 19%, respectively. Reported HEV RNA detection rates are remarkably lower, however, being 1.4% in Europe and 0.12% in Japan. We evaluated the HEV seroprevalence and RNA detection in post-LT patients to evaluate the hypothesis that HEV may pose potential subclinical risk in this particular immunocompromised patient population.

Method: 106 post-LT patients were enrolled and provided blood and feces samples. All patients were tested for HEV seroprevalence. After exclusion of acute/chronic HEV cases (n=3) and other unavailable cases (n=13), 91 post-LT patients were investigated for HEV serology (IgG and IgM) and HEV RNA detection (serum and feces) every 4 months during 1-year follow-up period. All patient samples were kept in -70C storage. HEV RNA in serum and feces were detected by real-time (in-house) RT-PCR technique (lowest level of detection=10 IU/mL). Demographic and clinical data were retrieved from the medical records for descriptive statistical analysis.

Result: The 106 post-LT patients had an HEV seroprevalence of 53.8%. After exclusion of the unavailable cases, 91 post-LT patients were prospectively investigated. HEV seropositive group was 50.5%, while the seronegative group was 49.5%. Baseline characteristics between two groups were not different. The serum and feces HEV RNA detection at baseline in seropositive group were 5/46 (21%) and 1/46 (2%), respectively. In seronegative group, the serum and feces HEV RNA detection were 2/45 (4.5%) and 3/45 (6.7%), respectively. Due to unprecedently high in proportion of patients with positive serum HEV RNA in both groups at the 4th visit, we decided to report our prospective result of the 8-month follow-up period. In seropositive group, serum and feces HEV RNA were detected in 11/46 (24%), 3/46 (6.5%), respectively. In seronegative group, serum and feces HEV RNA were detected in 9/45 (20%), 4/45 (8.8%), respectively. During 8-month period, 8 out of 14 and 8 out of 13 more cases of positive HEV in serum or feces in patients with and without IgG (+) were newly discovered, respectively. 2 out of 27 patients with positive serum or feces HEV RNA had abnormal liver function tests and one case was proved to be from anastomosis stricture with intrahepatic stone which was relieved after underwent ERCP.

Conclusion: Thailand has high prevalence of HEV seroprevalence in post LT patients. Post-LT patients could have subclinical HEV infection without obvious clinical clues. Without HEV RNA assays, active HEV infection could be missed even in HEV IgG seronegative patients. Feces HEV RNA detection adds on benefit of the diagnostic yield. However, clinical significance of these silence detection remains to be elucidated

Department: Medicine	Student's Signature .....
Field of Study: Medicine	Advisor's Signature .....
Academic Year: 2016	Co-Advisor's Signature .....

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CHAPTER 1: INTRODUCTION

1.1 Background problem and significance of the study

Hepatitis E virus (HEV) infection has now been recognized as new emerging infectious disease in the worldwide. The discovery of acquired cases in developed countries has changed our understanding of HEV infections. In recent years, HEV genotype 3 infections have been reported in Europe, New Zealand, and North America as in Asia countries such as China, Taiwan and Japan. Acute HEV infection usually self-limiting illness that last 4-6 weeks in normal healthy individual. Unlike the solid organ transplant recipients, HEV causes chronic infection, defined by persisting HEV RNA in serum or feces for 3 months or more. The clinical significance of chronic HEV infection in organ recipients were rapid progression of liver fibrosis and progress to cirrhosis. Due to immunosuppressive agent usage in these patients which resulted in false negative in serological report, the diagnosis of HEV infection should be based on molecular diagnosis as RNA detection in neither serum or feces.

In Thailand, only one study on HEV seroprevalence in normal Thai population which showed slightly seroprevalence. However, no previous HEV study in post-liver transplant (LT) patients has been explored. The early detection in asymptomatic post-LT patients would raise awareness of HEV chronicity and flare in the future.

1.2 Research questions

1.2.1 Primary research question

- To evaluate the HEV seroprevalence and serum/feces HEV RNA positivity at baseline in post liver transplant patients

1.2.2 Secondary research question

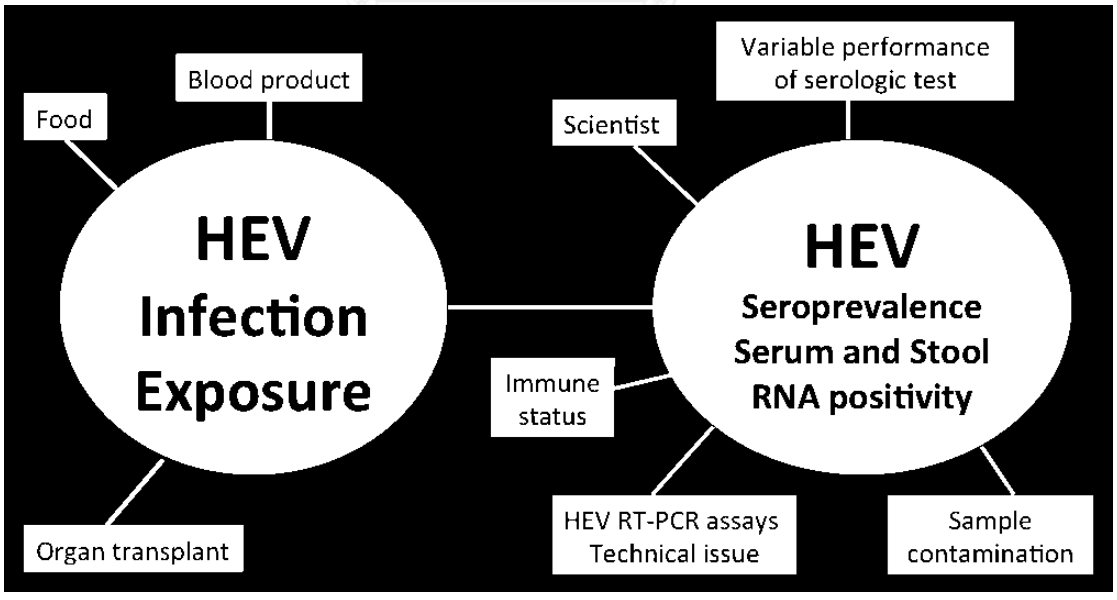
- To prospectively evaluate HEV serology and RNA positivity during 1-year follow period in post liver transplant patients.

1.3 Research hypothesis

- High HEV seroprevalence in post-LT patients in Thailand

- Post-LT patients who exposed to hepatitis E virus might have chronic infection during post transplant period.

1.4 Conceptual framework



1.5 Expected or anticipated benefit gain

- Evaluation of HEV seroprevalence in post-LT patients in Thailand

- Prospectively explored the changing of HEV serology during 1-year period

- Prospectively explored the HEV RNA detection in both serum and feces during 1-year period, and also the variability of RNA detection in each episode.

- May build-on the importance of HEV screening in pre-transplant period.

1.6 Ethical consideration

1.6.1 Respect for person

- The volunteers will receive total information about the study literally before signing the study inform consent.

1.6.2 Beneficience/Non-maleficence

- This study only collected the serum and stool sample from the volunteers which would not cause the life threatening or serious adverse event, also the detection of HEV virus either in serum or stool may benefit in post LT patients for the early detection, the appropriate treatment or the future follow-up.

1.6.3 Justice

- All the study volunteers will have to collect both serum and stool sample.

1.7 Limitation or challenges of the study

- The stool collection in some volunteers may considered as worrisome duty.

- The 1-year period of follow-up (every 4 months blood and stool collection) may be too long period for some volunteers.

- The period of 4 months in the study may not be accurate in all volunteers because the follow-up period in the study will depend on the date appointment and most compatible for the volunteers.

CHAPTER 2: LITERATURE REVIEW

Hepatitis E virus (HEV) is a small non-enveloped, single-stranded RNA virus that belongs to the *Herpesviridae* family. The HEV virus consists of three open reading frames (ORFs 1-3)[1-4]. The 5 known HEV genotypes arose due to a lack of proof-reading activity of the virus' RNA-dependent polymerase. Genotypes 1-4 cause disease in humans, while genotype 5 has only been identified in birds to date[4-5]. Genotypes 3 and 4 are zoonotic pathogens, with pigs being the reservoir species and human transmission occurring through consumption of contaminated foods[6-7]. Although, all human-infecting HEVs can be transmitted through blood products and transplanted organs[8-9].

The clinical symptoms of HEV in immunocompetent patients are similar to those of other viral prodromes and include fever, myalgia, weakness and loss of appetite. Transaminitis and jaundice also occur in a small proportion of these cases[10-11]. Only 2-5% of immunocompetent individuals present with symptoms of the HEV infection[12, 17-18] and the symptoms usually resolve spontaneously in 4-6 weeks. Cases of ongoing symptomology include individuals who are pregnant, have chronic liver disease or are immunocompromised, in who the condition can progress to fulminant hepatitis. The mortality rate in these groups range from 0.5% to 3%[10-11,13-16]. The HEV infection profile in the immunocompromised patients is distinctive, however. These patients, especially those who are organ transplanted or with human immunodeficiency virus infection, can experience prolonged viremia[17-19]. In those cases, 50-60% developed chronic hepatitis and 10% showed rapid progression to cirrhosis, graft failure and need for re-transplantation[20-21].

Diagnosis of HEV infection in immunocompetent patients requires detection of anti-HEV IgM antibodies[22], and delays in testing and diagnosis can be detrimental[23].

Unfortunately, the commercial serological assays have inconsistent test performance and limited sensitivities and specificities[23-25]. Diagnosis of HEV infection in immunocompromised patients, in contrast, is based on PCR detection of HEV RNA in serum or stool. Again, though, the HEV PCR test performance is inconsistent, with suboptimal sensitivity and specificity, and most of the published studies reporting on HEV RNA detection in this population have used PCR assays developed in-house[26-27].

National seroprevalence rates of HEV IgG has been reported in normal populations worldwide. The United States has a remarkably higher seroprevalence than the United Kingdom (25%[28] and up to 16.2%, respectively[29]). Germany and France have similarly high rates (34% and 22%, respectively[30-31]). The rates in Asian countries, however, vary widely, from 23.46% in China and 3.6% in Japan[32-33]. In our nation of Thailand, the reports of prevalence have ranged on the higher end of the worldwide spectrum, namely from 21.9% to 33% from military and general blood donor screenings[34-35].

The issue of HEV seroprevalence and RNA detection in the subgroup of transplant patients among the immunocompromised has become a topic of research interest. The first study, which involved a French population, found 10% seroprevalence among these patients, with persistent infection in 3.6%[36-37]. Later retrospective studies of German liver transplant patients found 4.4% seroprevalence, with persistent infection in 0.8%[38], and 1.4% HEV RNA detection[39]. A study of HIV-infected liver transplant patients in the United States found 18.9% seroprevalence and no patients showing HEV RNA positivity[40]. A nationwide survey of liver transplant patients in Japan found 2.9% seroprevalence and only 0.12% chronic HEV infection[41]. However, no HEV study of Thai post-liver transplant patients has been reported.

CHAPTER 3: MATERIALS AND METHODS

3.1 Research methodology

- Descriptive observational prospective study

- This study was approved by Chulalongkorn university institutional review board. Written (signature or thumbprint) informed consent was obtained from all the patients. Patients whose test were positive for antibodies or RNA testing were informed of their status and counseled using the Declaration of Helsinki and ethical guidelines for the clinical research

3.2 Population, samplings and location of research

- From October 2015 through February 2017, the post liver transplant patients in the King Chulalongkorn Memorial Hospital were enrolled for the study. 106-post liver transplant patients were recruited. The study protocol was showed in figure 1.

- The exclusion criteria of this study were the post-LT patients who could not continue the 1-year follow-up period (may be from active/chronic HEV infection, death or unable to follow-up)

3.3 Sample size calculation

- From the previous data in King Chulalongkorn Memorial Hospital, 78 post-LT patients were evaluated for HEV serology and showed anti-HEV IgG positive in 28 patients (35.59%) and anti-HEV IgG negative in 50 patients (64.1%). So the HEV seroprevalence that used in this study will be 36%

Define the confidence interval for sample analysis = 95%

$Z\alpha_{/2} = Z_{0.05/2} = 1.96$ (Two-tailed)

Formula $n = Z^2\alpha_{/2}\, PQ/d^2$

P = Prevalence = 0.36

Q = 1-0.36 = 0.64

d = acceptable error = 0.10

$n = (1.96)^2 (0.36) (0.64) / (0.10)^2$

= 88.5 ~ 86 (Estimated sample size)

3.4 Data collection

- All the patients underwent the 10 ml blood collection and also one pocket of fresh feces sample every 4 months during 1-year follow-up period.

- All the patients' sample were kept in -70c refrigerator storage until testing.

- The serum was tested for the anti-HEV IgG, IgM antibodies and HEV RNA.

- The feces was tested for HEV RNA detection.

- All the patients' clinical data including demographic data, medical history, current medication especially immunosuppressive agents and laboratory result were retrieved from the medical records.

3.5 Data analysis

- Descriptive statistical analysis by IBM SPSS Statistics 23
- Baseline characteristics:
    - Catagorical data: analyze in proportion
    - Ordinal data: analyze in median, interquatile (IQR)
    - Continuous data: analyze in median, SD

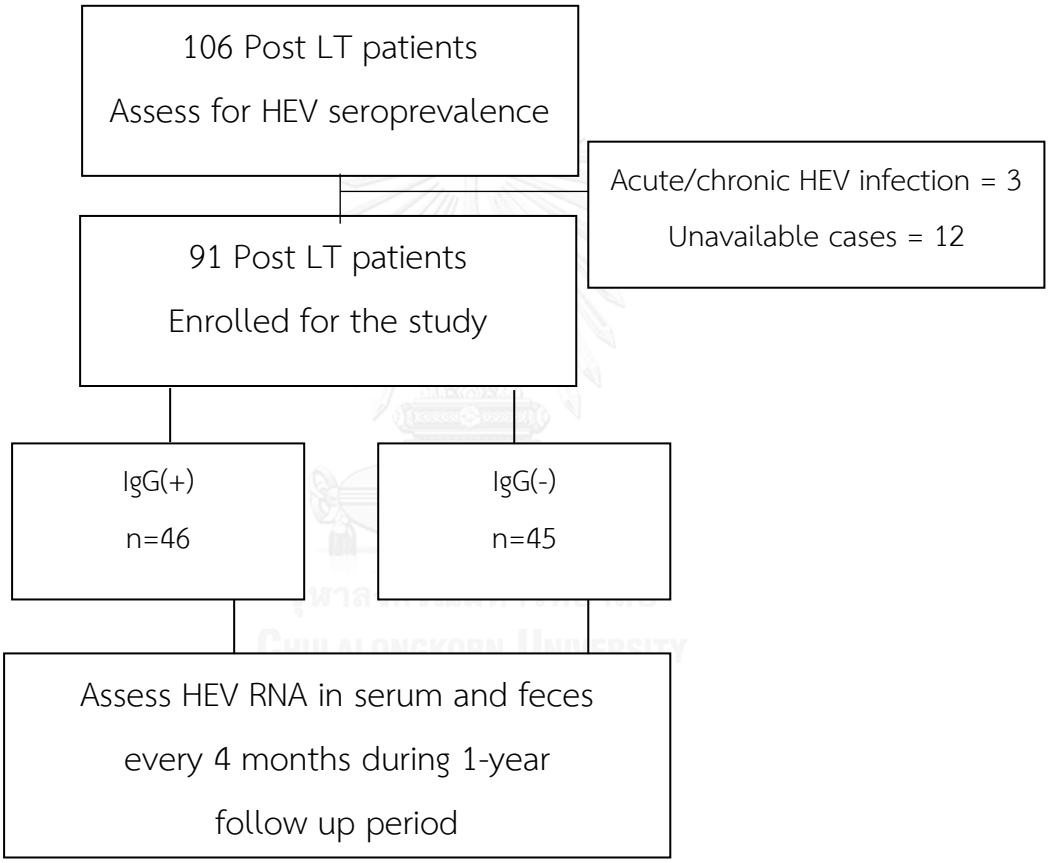


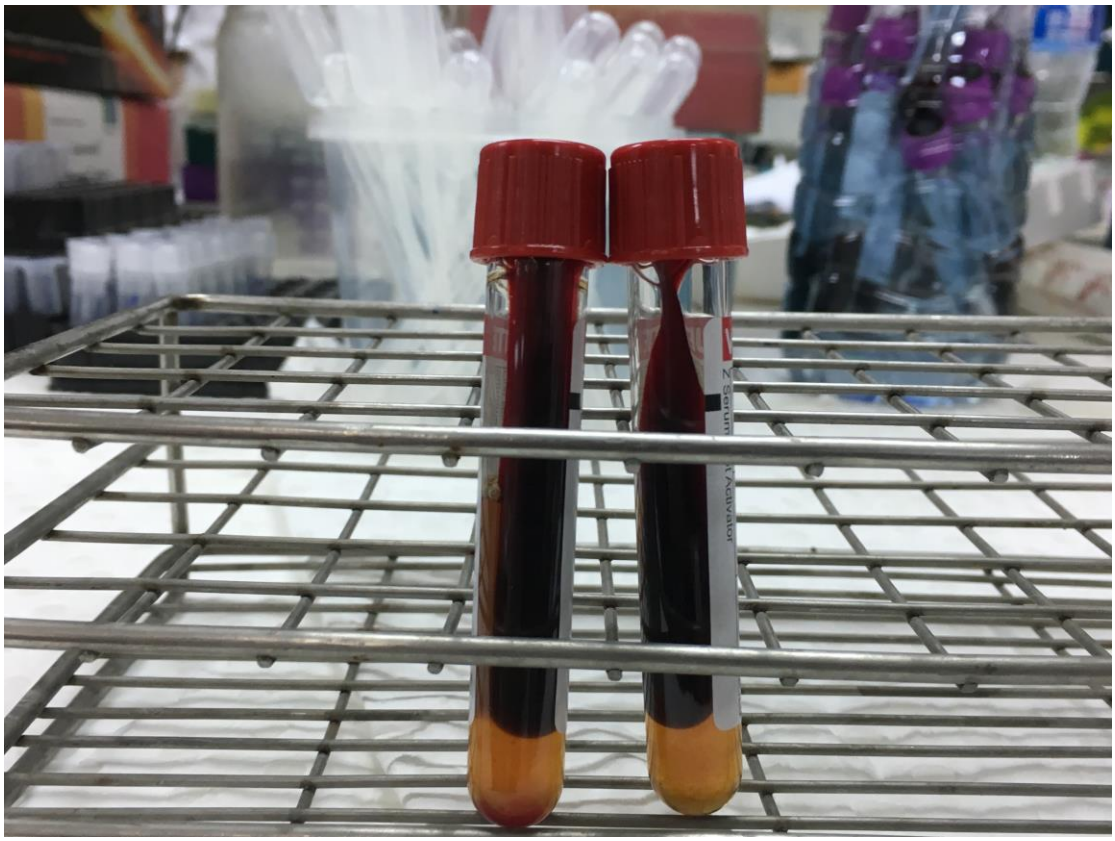
Figure 1: Flowchart of the study protocol. One-hundred-and-six post-liver transplant (LT) patients were enrolled and provided samples for testing of HEV seroprevalence. Acute/chronic HEV infection was detected in 3 cases and 12 cases were deemed unavailable (i.e. XXX), leaving 91 post-LT patients for evaluation of HEV seroprevalence and HEV RNA detection.

Due to unprecedently high in proportion of patients with HEV RNA detection at the 4th visit, for clinical accuracy, we decided to use 8-month follow-up period for prospective clinical evaluation, instead of the 12-month follow up period.

3.6 Laboratory methods

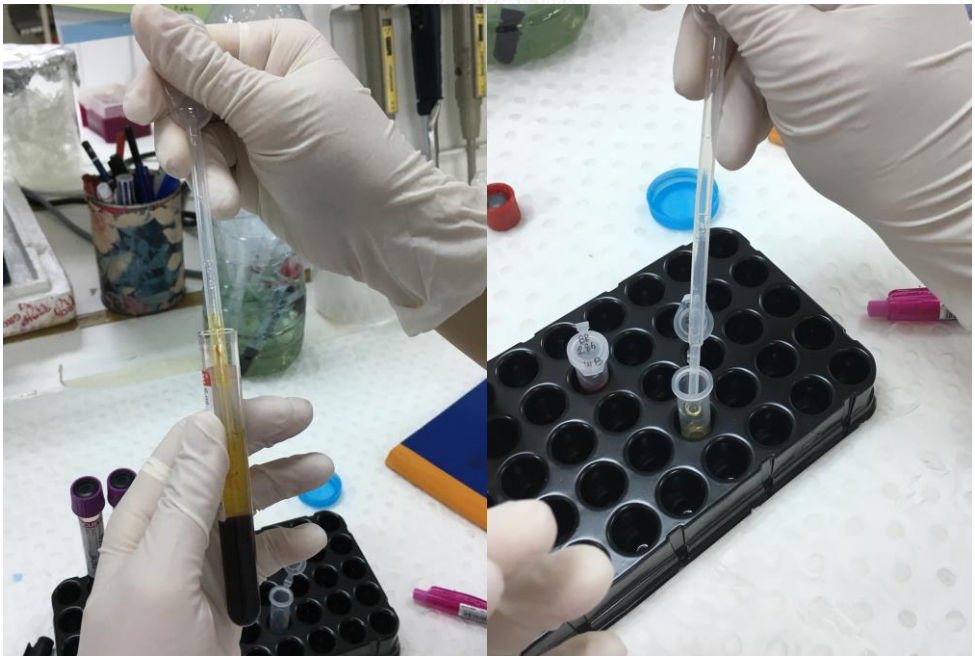
3.6.1 Serum and stool samples preparation

1. The blood samples were collected in clot blood tube. The fresh feces samples were collected in small pocket. (PICTURE 1.1)

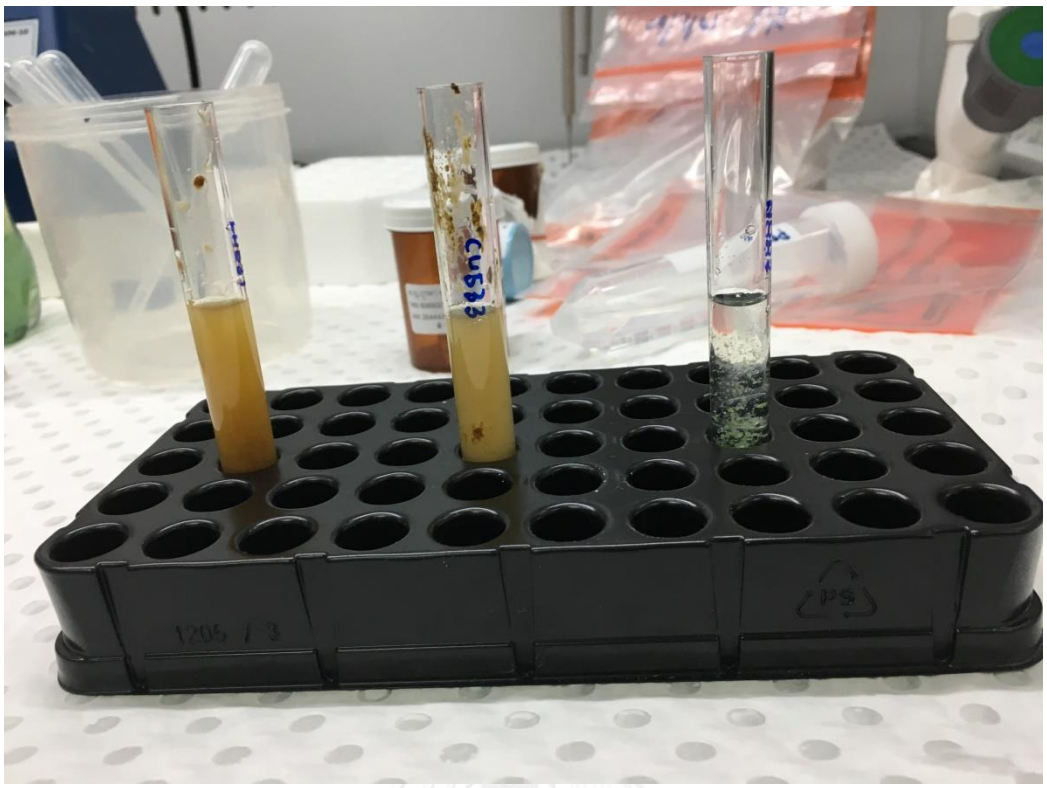


2. Applied the blood tubes in centrifuge at 3000 rpm in 3 minutes (PICTURE 1.2)

3. Transferred the serum part after centrifugation to the 1.5 ml centrifuge tubes. (PICTURE 1.3)



4. For the stool samples, used the phosphate-buffered saline (PBS) mixed with stool samples in ratio 9:1 and underwent the 3000 rpm centrifuge for 3 minutes. Collected the upper-clear part in 1.5 ml centrifuge tube (same as serum samples) (PICTURE 1.4, 1.5, 1.6 and 1.7)

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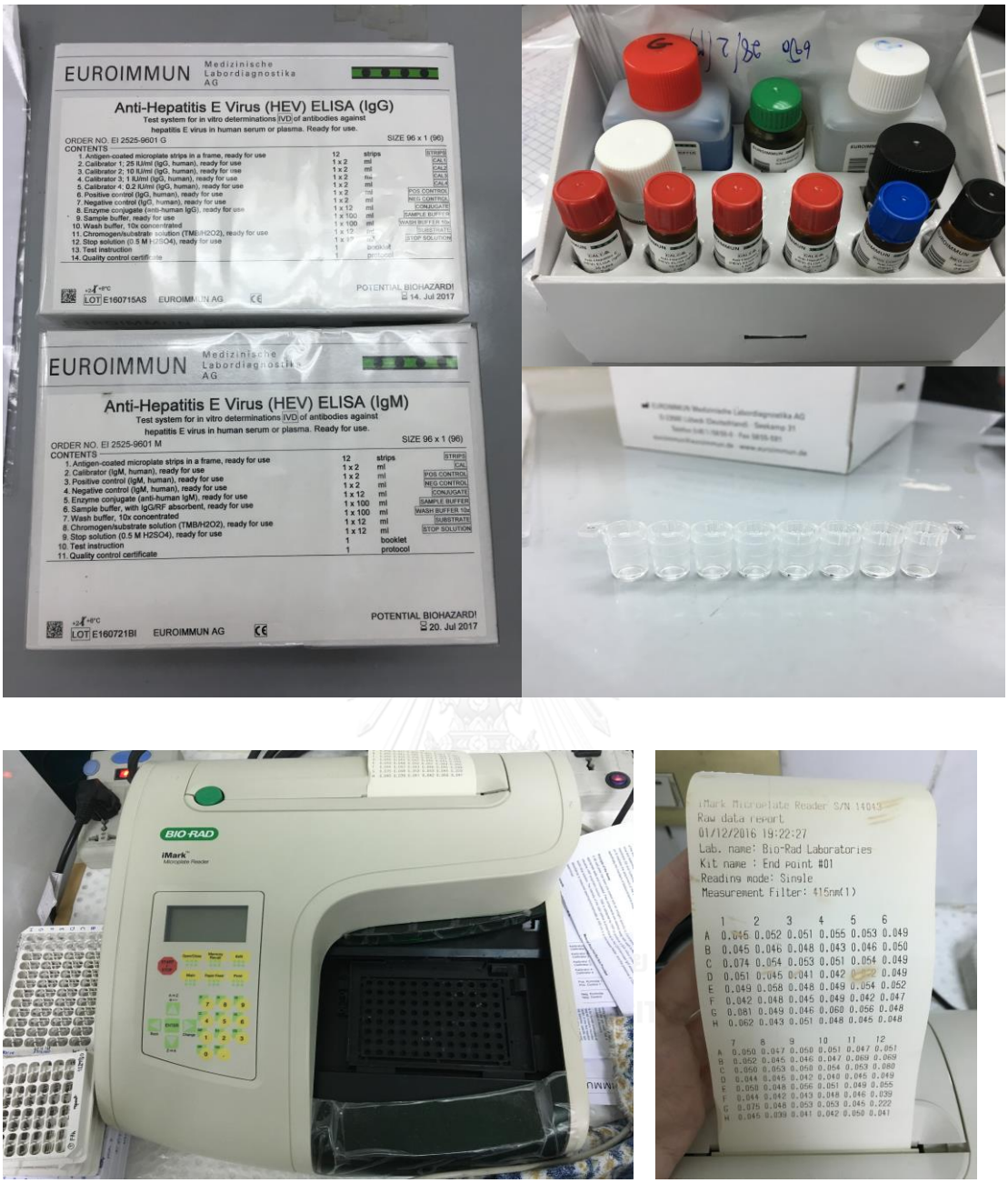
3.6.2 Serology assays

All serum and feces sample testing was carried out at the Center of Excellence in Clinical Virology, Chulalongkorn University. Anti-HEV 96-well plate enzyme-linked immunosorbent assay (ELISA) was used to test for the IgG and IgM HEV antibodies (D-23560; EUROIMMUN Medizinische Labordiagnostika AG, Lubeck, Germany), according to manufacturer's instructions. The limit of detection for anti-HEV IgG was 0.1 IU/mL and for anti-HEV IgM was ratio 0.0. The antigen coating was composed of a mixture of recombinant partial ORF2 genotype 1 and 3 antigens and detection-labelled rabbit anti-human IgG or IgM antibodies.



EUROIMMUN serology kit assay (above) for anti-HEV IgG and IgM (PICTURE 2.1)

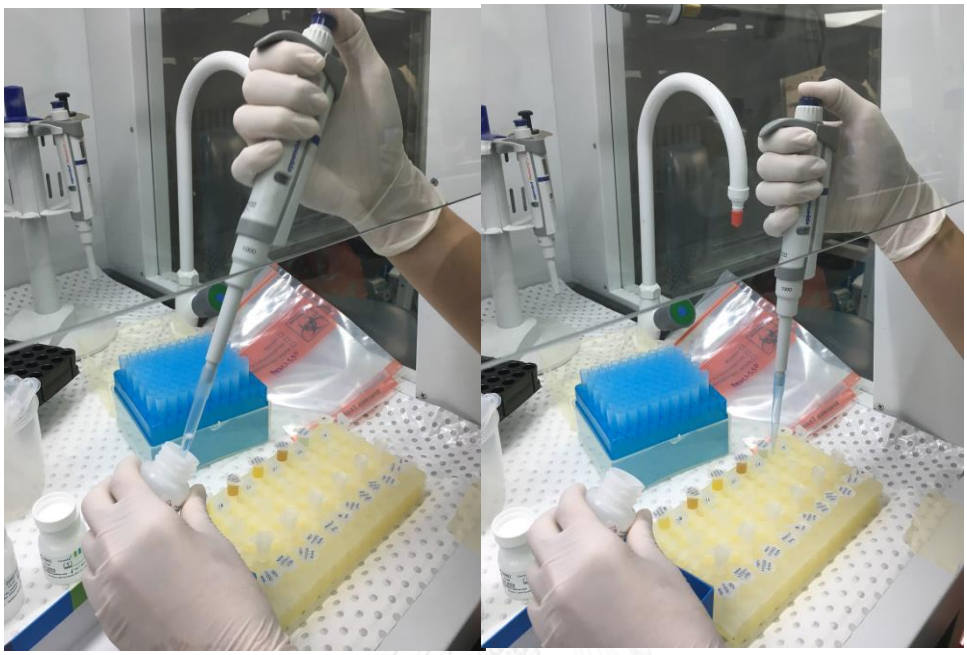
iMark^{TM} microplate reader (below) (PICTURE 2.2)

3.6.3 HEV RNA detection by real-time RT-PCR

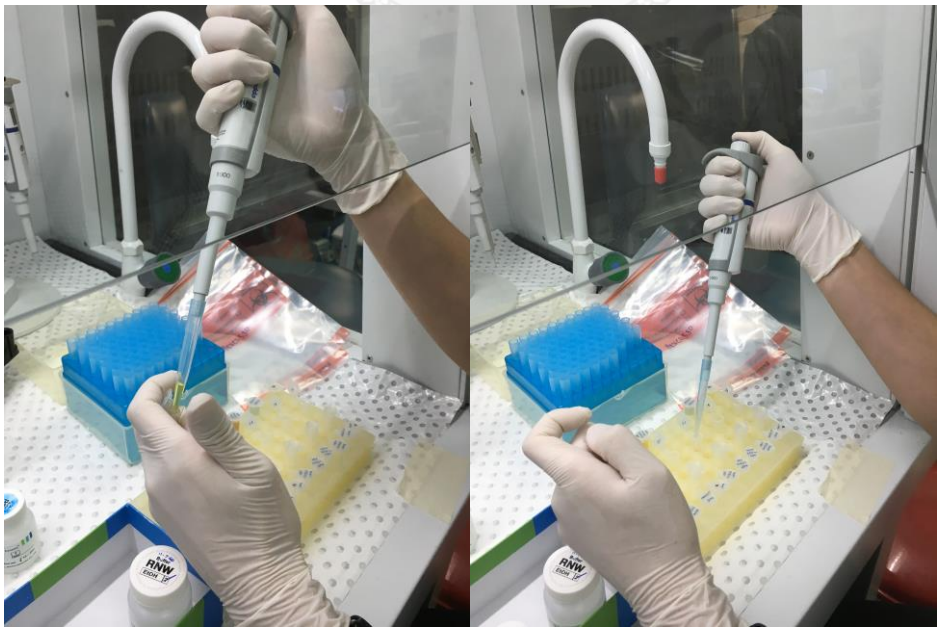
Serum samples (200 μL) and stool samples (250 μL feces suspended in phosphate-buffered saline, pH 7.4) were processed for isolation of viral nucleic acid using the Ribospin vRD II kit (GeneAll Biotechnology Co. Ltd., Seoul, Korea), according to the manufacturer's instructions. The HEV RNA was detected by one-step real-time PCR for HEV ORF2/3 using the published primer and probes[42]. Briefly, the *Taq*Man® RT-PCR reaction mixture (20 μL) was made by mixing 10 μL of 2× SensiFAST Probe No-ROX One-Step Mix (Bioline USA Inc, Taunton, MA, United States), 0.2 μL of reverse transcriptase, 0.4 μL of RNase inhibitor, 3 μL of RNA template, and primers and probe at concentrations of 400 nM and 100 nM, respectively. The ViiA™ 7 Real-Time PCR thermal cycler (Life Technologies Corporation, Carlsbad, CA, United States) was used for the real-time RT-PCR cycling reactions. Reverse transcription was carried out at 45 °C for 10 min, followed by denaturation at 95 °C for 2 min. DNA was amplified immediately with 40 PCR cycles at 95 °C (5 s) and 54 °C (20 s). The real-time RT-PCR data were collected after the reaction and the threshold cycle (Ct) value calculated by the ViiA™ 7 software.

3.6.3.1 RNA extraction by Ribospin™ vRD II (PICTURE 3.1, 3.2)

1. Add 300 µL of buffer **NVL** and 7 µL of Carrier RNA solution into a 1.5 ml microcentrifuge tube (PICTURE 3.3)



2. Transfer up to 100 µL of sample into the 1.5 ml microcentrifuge tube. (PICTURE 3.4)



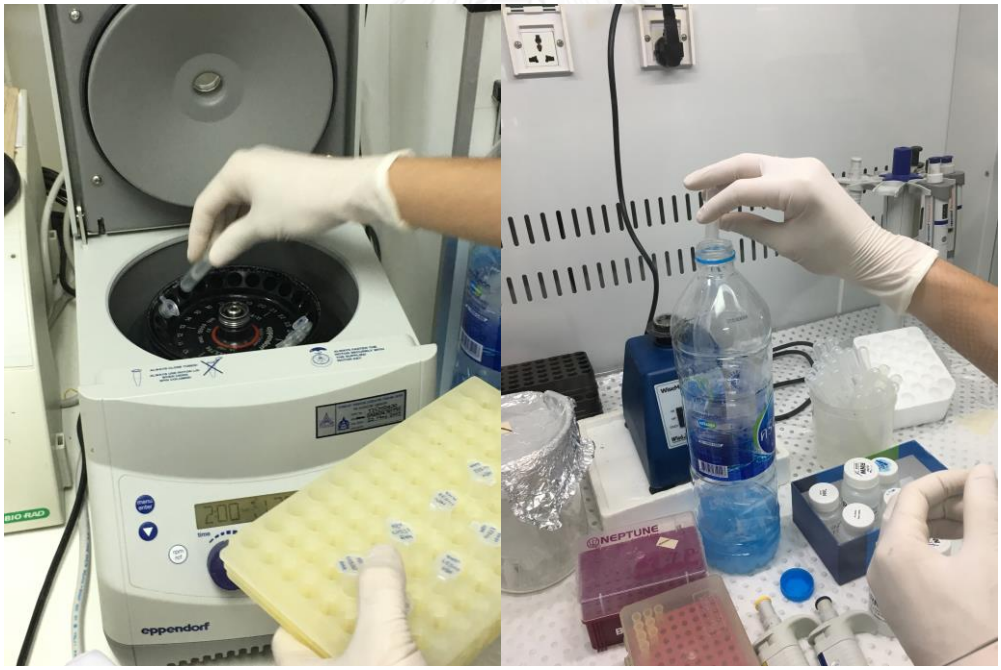
3. Mix thoroughly by vortexing for 10 seconds. For proper lysis, the complete mix of sample and buffer NVL is essential.

4. Incubate the mixture for 10 minutes at room temperature.

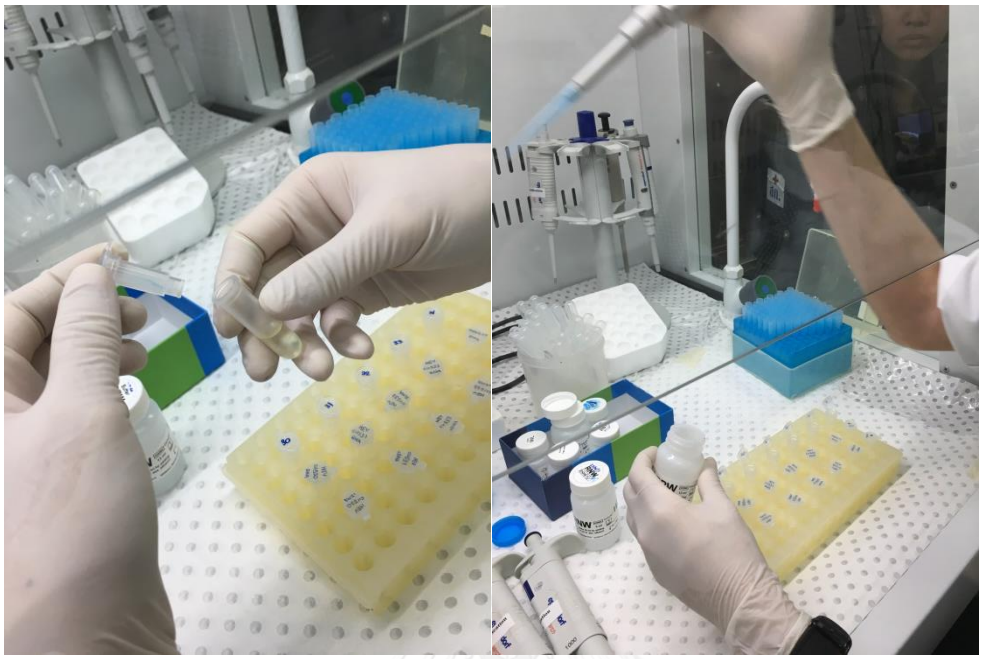
5. Add 350 µL of buffer **RBI** to the mixture and mix thoroughly by vortexing for 10 seconds.

6. Transfer up to 750 µL of the mixture to a spin column (Microcolumn type S, white).

7. Centrifuge at ≥10,000 x g for 30 seconds at room temperature. Discard the pass-through and reinsert the spin column back into the same tube. (PICTURE 3.5)



8. Add 500 μL of buffer **RBW** to the spin column. (PICTURE 3.6)



9. Centrifuge at ≥10,000 x g for 30 seconds at room temperature. Discard the pass-through and reinsert the spin column back into the same tube.

10. Add 500 μL of buffer **RNW** to the spin column.

11. Centrifuge at ≥10,000 x g for 30 seconds at room temperature. Discard the pass-through and reinsert the spin column back into the same tube.

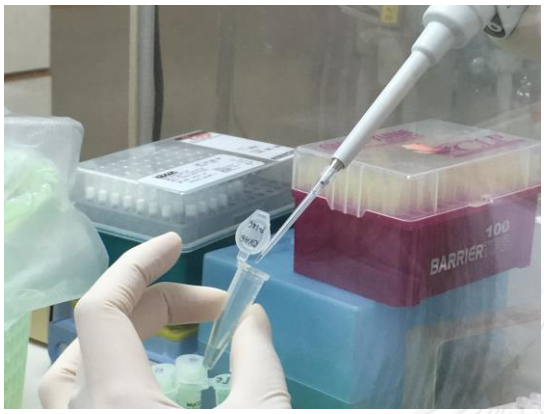
12. Centrifuge at full speed for an additional 1 minute at room temperature to remove residual wash buffer. Transfer the spin column to a new 1.5 ml microcentrifuge tube

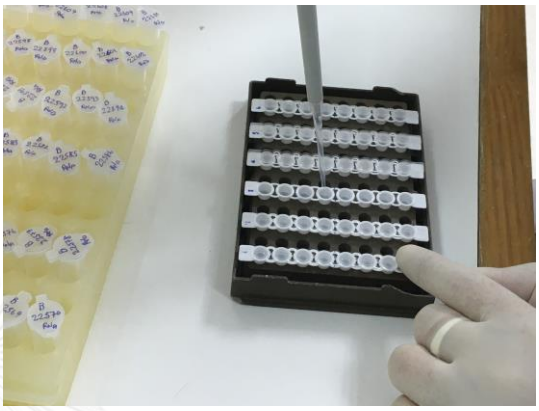
13. Add 20-50 μL of Nuclease-free water to the center of the membrane in the spin column. Let it stand for 1 minute.

14. Centrifuge at ≥10,000 x g for 30 seconds at room temperature.

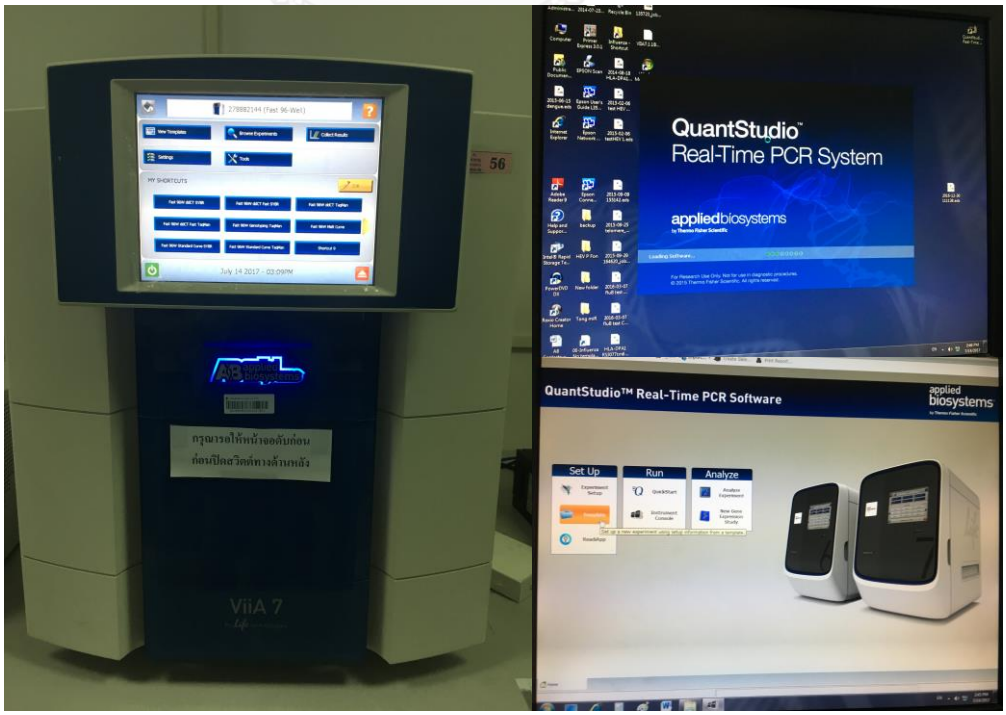
3.6.3.2 Real time RT-PCR by ViiA™ 7 software/QuantStudio™ application

1. Mixture of probe, primer and RNA template into the PCR plate. (PICTURE 4.1)

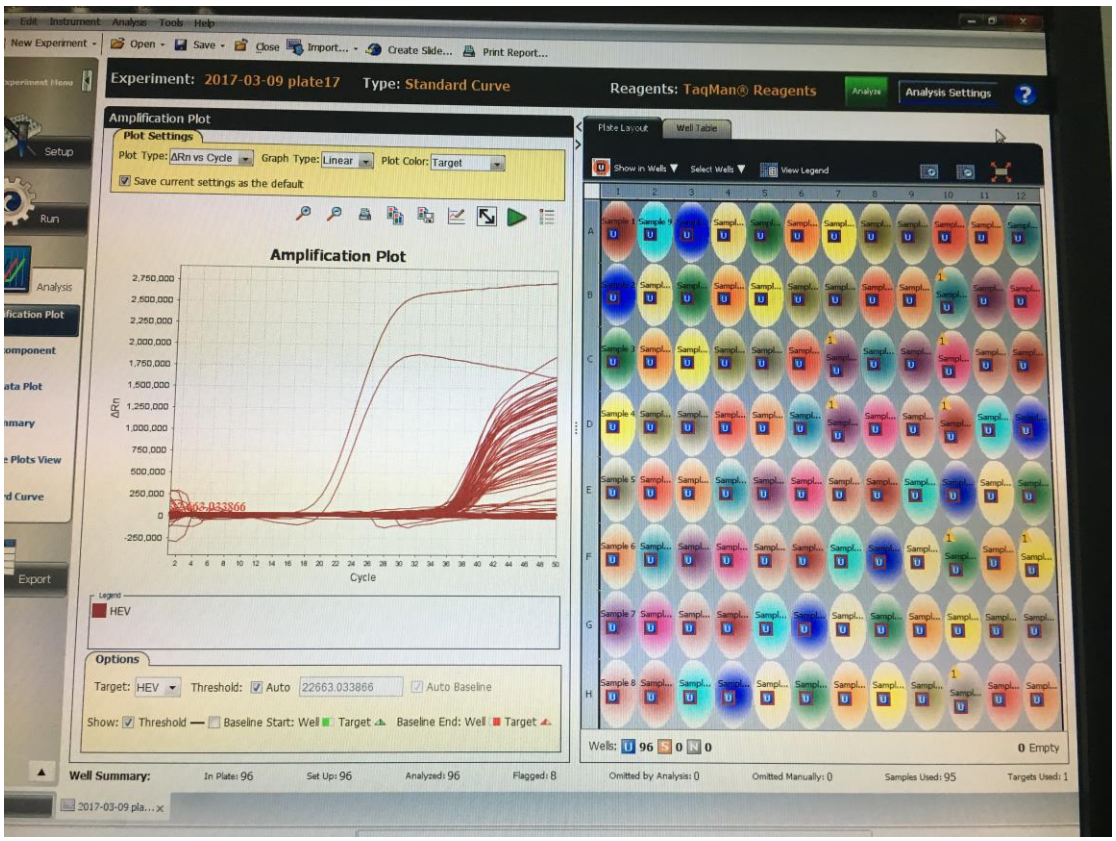


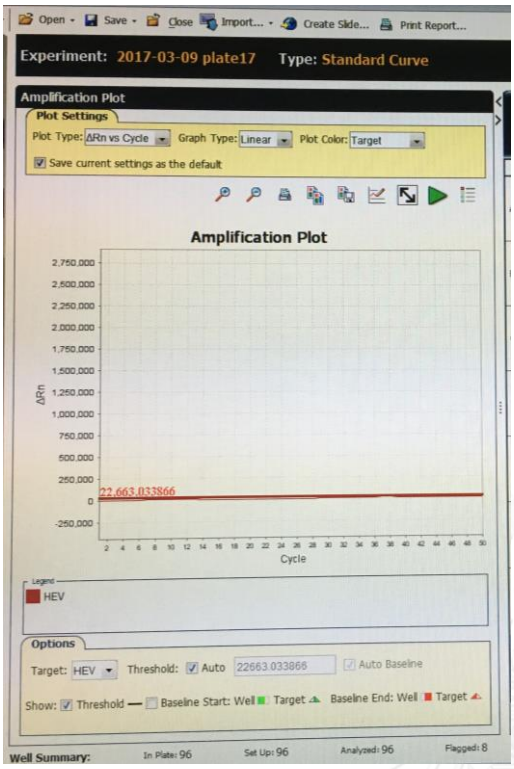


2. Using the ViiA™ 7 software/QuantStudio™ application (PICTURE 4.2)

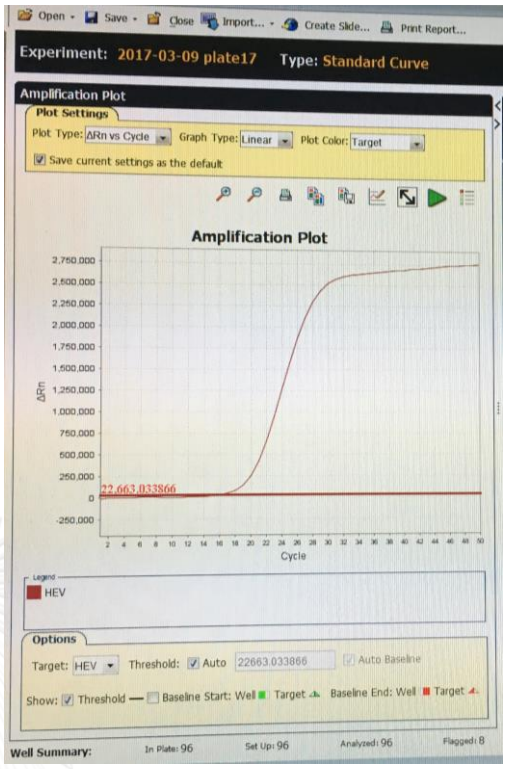


3. On the left, the PCR result was shown by amplification plot (in cycle threshold). On the right, the template was presented in multi-color oval-shaped pattern. (PICTURE 4.3)

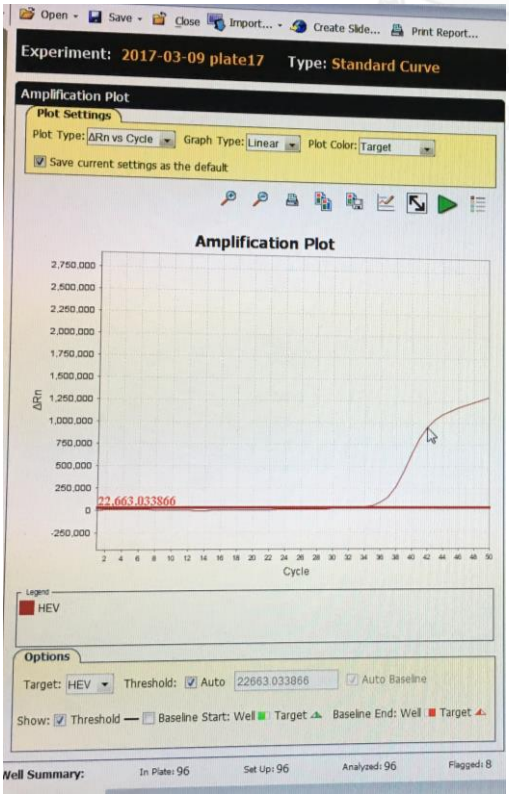


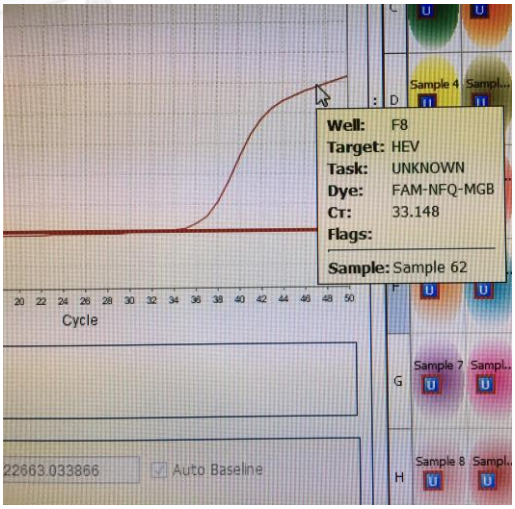


Negative control (PICTURE 4.4)



Positive control (PICTURE 4.5)





Positive result in patient's sample

(PICTURE 4.6, 4.7)

3.6.3.3 Converse RNA to cDNA for sequencing

1. Prepare RNA target and primer

    1.1 Use sterile, nuclease-free, thin-walled tubes, prechilled on ice

    1.2 For each 20 μL reverse transcription (RT) reaction, intubate at 70°C for 5 minutes and quick-chill at 4°C for 5 minutes and hold on ice

2. Prepare reverse transcription mix

    2.1 For each 20 μL reaction, vortex to mix and dispense 15 μL aliquots into the reaction tubes.

3. Add template and primers to the reaction mix

    3.1 For each individual reaction, add 5 μL of the appropriate template with primer mix to the 15 μL reverse transcription of RT-PCR mix. If necessary, overlay with nuclease-free mineral oil. The final volume for each individual reaction will be 20 μL. (PICTURE 5.1)

4. Reverse transcription

4.1 Anneal at 25°C for 5 minutes.

4.2 Extend the first strand for 60 minutes at 42°C. The extension temperature may be optimized between 37-55°C.

4.3 Heat-inactivate the IMPROM-II™ Reverse Transcriptase by incubating at 70°C for 15 minutes. (PICTURE 5.2)

4.4 Analyze cDNA, proceed with PCR or store frozen.

5. PCR amplification

5.1 The cDNA may be amplified by adding the products of the heat inactivated reverse transcription reaction directly to the PCR mix and proceeding with thermal cycling

5.2 Prepare PCR mix, minus the cDNA sample by combining the amplification reagents in a sterile 1.5 ml microcentrifuge tube on ice. Combine the components in the order listed, vortex gently to mix, and keep on ice.

5.3 Dispense the appropriate volume of PCR mix into each chilled reaction tube.

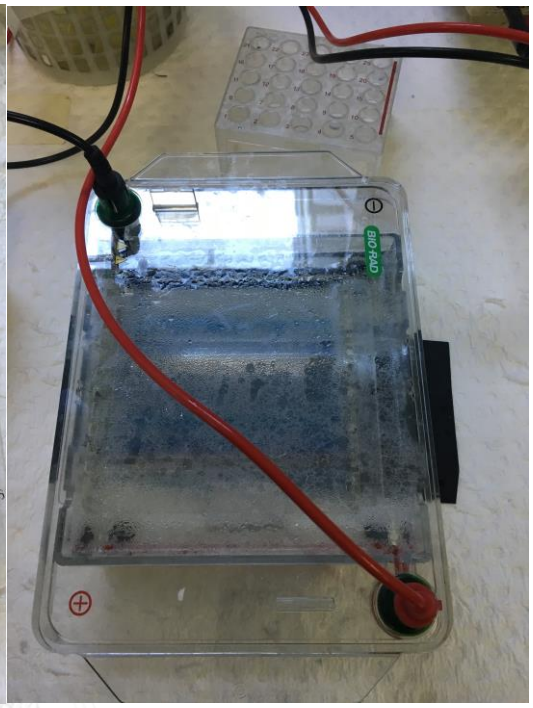
5.4 Add the appropriate aliquot of the reverse transcription reaction to the PCR mix.

5.5 If necessary, overlay the reaction with nuclease-free mineral oil and proceed with amplification program.

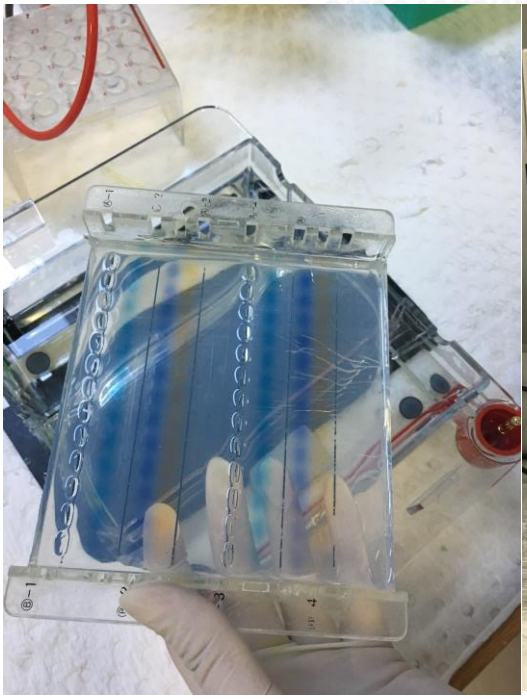
3.6.3.4 DNA sequencing

1. Prepare the agar plate for DNA sequence with Agarose and 1X TBE solution (4:1 ratio) (PICTURE 6.1)

2. Set the agar in the template for 30 minutes, insert the DNA template in the agar. (PICTURE 6.2)



3. The agar plate with color bar was read under the UV light interpretation. (PICTURE 6.3)



The DNA sequencing was interpreted under the UV light by the software.

(PICTURE 6.4)

CHAPTER 4 : STUDY RESULT

106-post liver transplant patients were evaluated for HEV seroprevalence. HEV IgG(+) was observed in 57 patients (53.8%), meanwhile HEV IgG(-) was observed in 49 patients (46.2%). After exclusion the acute/chronic HEV infection (n=3) and unavailable cases (n=12), 91-post liver transplant patients were evaluated for HEV RNA detection in both serum and feces. The acute/chronic HEV infection cases were showed in Table 1.

Case	Age	Sex	Cause	LT (M)	Clinical course	HEV		RNA		Treatment	Result
						IgG	IgM	S	F		
1	62	F	HBV, HCC	85	Chronic hepatitis	+	-	+	+	RBV 6 M	Response
2	57	M	HCV, HCC	19	Chronic hepatitis	+	-	+	-	RBV 6 M	Response
3	56	M	HCC	47	Chronic hepatitis	-	+	+	+	RBV 24 M	Relapse

Table 1: Baseline characteristics of acute/chronic HEV cases among the post-liver transplant (LT) patients. M: months, for 'LT' duration after liver transplant and for 'Treatment' duration; S: serum; F: feces; (+): positive; (-): negative; RBV: Ribavirin.

4.1 Baseline characteristics

Among 91-post liver transplant patients, the HEV seroprevalence was 50.5%, while the seronegative group was 49.5%. Between these groups, the patients'age, male population, the time of test after the liver transplant were not significantly different. The liver function tests were generally normal in both groups. With regarding to the immunosuppressive drug dosage, mycophenolate mofetil (MMF) were slightly higher but no statistical significance in the seropositive group (646.4±266 vs. 546.9±257 mg; p-value 0.157). In the seronegative group, dose of prednisolone and cyclosporine were higher but no statistical significance, compared with the seropositive group. According to dose prescribation, the immunosuppressive drug level (cyclosporine level) in the seronegative group were in higher level but no statistical significance (Table 2)

Table 2: Baseline characteristics in patients with anti-HEV IgG(+) [seropositive] and anti-HEV IgG(-) [seronegative]. *n/total (%); **mean±SD; †mean±SD (range).
ALT: alanine aminotransferase; AST: aspartate aminotransferase; LT: liver transplant.

Factor	Seropositive	Seronegative	p-value
Number of patients, n/total (%)	46/91(50.5)	45/91 (49.5)	-
Age (years)	56.3±13.1	59±11.3	0.313
Male sex, n (%)	33/46 (71.7)	30/45 (66.7)	0.605
Time of test after LT (months), range	70.4±39 (15-164)	74.7±47.7 (15-201)	0.647
Total bilirubin (mg/dL)	0.85±0.6	0.86±0.6	0.928

AST (IU/L)	37.2±30.5	35.2±45.3	0.812
ALT (IU/L)	42.7±46	38.7±58	0.725

Factor	Seropositive	Seronegative	p-value
Immunosuppressive dose			
- Azathioprine (mg) (2 vs 2 cases)	75.0	75.0	-
- Prednisolone (mg) (5 vs 6 cases)	4.5±3.3	12.5±11.2	0.159
- Mycophenolate mofetil (mg) (28 vs 29 cases)	646.4±266	546.9±257	0.157
- Tacrolimus (mg) (29 vs 25 cases)	2.4±1.6	1.8±1.1	0.087
- Sirolimus (mg) (9 vs 11 cases)	1.1±0.33	1.2±0.4	0.679
- Cyclosporine (mg) (8 vs 10 cases)	91.3±35	127.5±55.8	0.130
Immunosuppressive drug level			
- Tacrolimus (ng/mL)	4.5±3.3	4.0±4.4	0.691
- Sirolimus (ug/L)	5.0±1.7	5.9±2.2	0.396
- Cyclosporine (ug/L)	396±186.2	450±193.4	0.561

4.2 Serological changes during 12-month follow-up period

In HEV seropositive group, anti-HEV IgM(+) was detected in 2 out of 46 patients without any symptoms or abnormal liver function test . None of them had the HEV RNA detection in both serum and feces. The seroconversion to seronegative was found in 4 out of 46 patients (Table 3). In HEV seronegative group,

seroconversion to seropositive was found in 4 out of 45 patients. One of them had serum HEV RNA detection at the 2nd visit which was the seroconversion time.

4.3 HEV RNA detection at baseline and during 8-month follow-up period

HEV seropositive group

At baseline, the serum and feces HEV RNA detection were 5/46 (10.8%) and 1/46 (2%), respectively. During 8-months follow-up, serum and feces HEV RNA were further detected in 4/46 (8.7%) and 2/46 (4.3%), respectively. At the 8-month period, the overall serum and feces RNA detection were 11/46 (24%) and 3/46 (6.5%), respectively.

In this group, one patient had abnormal liver function tests from hepaticojejunostomy stricture with intrahepatic duct stone. The serum HEV RNA was detected in the 1st visit. After underwent ERCP, the liver function tests turned to normal level.

HEV seronegative group

At baseline, the serum and feces HEV RNA detection were 2/45 (4.5%) and 3/45 (6.7%), respectively. During 8-months follow-up, serum and feces HEV RNA were further detected in 7/45 (15.5%) and 1/45 (2.2%), respectively. At the 8-month period, the overall serum and feces HEV RNA were detected in 9/45 (20%) and 4/45 (8.8%), respectively.

In the seronegative group, one patient had mild transient hepatitis with positive serum and feces HEV RNA at baseline, but not detected later on.

Table 3: Number of cases with serum HEV serology and RNA detection in serum and feces; (+) represent positive test.

HEV status	Baseline n/total	2nd test n/total	3rd test n/total	Final report n/total (%) 8 months	4th test n/total	Final report n/total (%) 12 months
Serum HEV IgG(+), n=46 cases						
HEV IgG (+)	46/46	45/46	45/46	45/46 (97)	41/46	41/46 (89)
HEV IgM (+)	1/46	1/46	1/46	2/46 (4.3)	1/46	2/46 (4.3)
Serum RNA (+)	5/46	4/46	2/46	11/46 (24)	23/46	27/46 (58.7)
Feces RNA (+)	1/46	2/46	0/46	3/46 (6.5)	0/46	3/46 (6.5)
Serum HEV IgG(-), n=45 cases						
HEV IgG (+)	0/45	4/45	2/45	4/45 (8.8)	1/45	4/45 (8.8)
HEV IgM (+)	0/45	0/45	0/45	0/45 (0)	0/45	0/45 (0)
Serum RNA (+)	2/45	8/45	3/45	9/45 (20)	18/45	24/45 (53.3)
Feces RNA (+)	3/45	1/45	1/45	4/45 (8.8)	0/45	4/45 (8.8)

Note: The HEV RNA results of the 4th (at 12 months) were shown, but not included for the prospective evaluation due to contaminated RT-PCR result.

CHAPTER 5 : DISCUSSION OF THE STUDY

5.1 Discussion

HEV infection is generally agreed to have been underestimated in post-liver transplant patients until the seminal publication of Kamar et al[36], in which evidence of chronic HEV infection in solid organ transplant patients encouraged clinicians to know about HEV. Since then, several studies have reviewed case series of chronic HEV infection. In Europe and the United States, HEV seroprevalence is moderate (18.9-22%)[38-40], but much higher than in Japan (2.9%)[41]. Our study of post-liver transplant patients, the first of HEV seroprevalence in Thailand, showed a surprisingly high rate (53.8%), higher than that reported for healthy individuals[34-35].

The potential reasons for high HEV seroprevalence in post-liver transplant include blood transfusion, the organ transplant itself and the patient's lifestyle. Post-liver transplant patients usually receive multiple blood products during the transplant and post-operative periods. The blood donation screening program for HEV in Thailand is still not well established, and there is risk of infection from a donor with unrecognized occult hepatitis E. Indeed, previous studies have demonstrated HEV transmission through blood products and organ transplants[8-9]. In addition, liver transplant patients remain at risk of acquiring HEV through contaminated pork consumption[34].

In our study, the 1-year period serum HEV RNA detection was very high (62.6%) which predominantly in 4th visit serum collection. This unprecedently high HEV RNA detection rate may stem from laboratory error, likely to be contaminated RT-PCR process. We are on the process of in depth investigation to identify the cause of the error. This prohibit us to complete the 12-month cohort. However, the data

from 8-month follow up period were reasonably good enough to give us a clue of what it might be for the patients who had or did not have HEV IgG(+) at baseline.

Hence, we evaluated the 8-month study result. The HEV RNA detection in serum or feces were 29.6% which was high compared to the study in Europe and US (0-1.4%)[38-40] and Japan (0.12%)[41]. The HEV seropositive group has similar serum RNA detection rate with the seronegative group (24% vs. 20%). These finding may imply that the seropositive patients, who still have silence infection, are likely to have serum HEV RNA detection at some points, and the seronegativity does not mean that those patients have no active HEV infection. Regarding to the RNA detection test, we used broadly reactive TaqMan® RT-PCR assay for detection of HEV. This assay has been designed to target ORF2, allowing the detection of different genotypes of HEV without the use of degenerate primers or probes. The assay was determined to have a sensitivity of four GE copies, which is comparable the detection limit for a conventional nested PCR assay for HEV[44]. Also, real-time RT PCR has the benefit of shorter detection times, minimal potential for laboratory contamination[42].

On the other hands, the RNA detection in feces was higher in seronegative group (8.8% vs. 6.5%). There were three patients who solely detected RNA in feces without clinical hepatitis. According to natural history of HEV in immunocompromised patients, the HEV RNA can be detected either in serum or feces without specific pattern for viral shedding as well as the antibody detection which effected by the immunosuppressive agents. The feces RNA detection could be the add-on benefit for detection of HEV in immunocompromised host.

During follow-up period, some patients had HEV RNA detection in serum or feces more than one episode and some patients had RNA detection in both serum and feces in different episode which was consistently with the chronicity of HEV infection in immunocompromised patients. In addition, this study observed several patients who had HEV RNA detection in serum or feces with or without IgG (+).

Therefore, this could be implied that it is necessary for long-term closed monitoring for active HEV diseases in post-LT patients who had the silent infection.

Our study explored as many possible demographic and clinical factors from our medical records for their potential association with HEV infection in the post-liver transplant Thai population. These included age, sex, duration from liver transplantation, comorbid diseases, type and dosages of immunosuppressive drugs. Yet, no factor evaluated showed an association with HEV infection in these patients. Ribavirin had been used for the treatment of HEV infection in those who had clinical disease, and resulted in good responses. Duration of antiviral treatment for HEV has not been well established, but 6-12 months is a common recommendation[5, 14]. Patient 3 in Table 1 had relapsed disease after antiviral discontinuation following an initial 6-month therapy, and then received a second, prolonged course of ribavirin, up to 24 months; the patient has shown sustained response for over 1 year as of the writing of this report.

5.2 Limitation of the study

Our limitation in this study was the problem of laboratory error of the last episode's samples. Our team is planning to repeat the HEV RNA detection by the PCR technique in 4th serum samples as soon as possible to complete the 1-year study result. Other difficulty was patients' sample collection. As our initial plan was to collect the blood and feces sample on the same day, and also at the exact every 4-month period. However, these processes, ultimately, needed to be compromised and accommodated to the willingness of the patients, especially the feces collection. Even all these difficulties, all samples were collected from all patients as initial plan.

It is well recognized that the HEV antibody ELISA test has variable test performance for the different commercial assays and cutoff levels (Table 5)[43]. The EUROIMMUN antibody test that was used in this study has quite low-sensitivity, for both IgG and IgM (42% and 24%, respectively), but has very high specificity, at levels similar to other commercial assays (99% and 100 %, respectively)[43].

Table 4: Diagnostic performance of different commercial assays in HEV antibody testing (sensitivity and specificity). *Adapted from Norder H, et al. Diagnostic performance of five assays for anti-HEV IgG and IgM in a large cohort study. Journal of Clinical Microbiology*[43].

Antibody test	IgM Sensitivity	IgM Specificity	IgG Sensitivity	IgG Specificity
Euroimmun	24%	100%	42%	99%
Mikrogen	38%	99%	62%	99%
DSI	63%	99%	72%	99%
Axiom	29%	99%	95%	98%
Diapro	72%	100%	98%	96%

5.3 Conclusion

Thailand has high prevalence of HEV seroprevalence in post LT patients. Post-LT patients could have subclinical HEV infection without obvious clinical clues. Without HEV RNA assays, active HEV infection could be missed even in HEV IgG seronegative patients. Feces HEV RNA detection adds on benefit of the diagnostic yield. However, clinical significance of these silence detection remains to be elucidated by a study of the longer follow up period.

Nonetheless, from our result, clinicians should be aware of hepatitis E virus as one of the viral hepatitis that might act as an imitator in post liver transplant acute or chronic hepatitis.

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