The difference of plasma 16s ribosomal bacterial deoxyribonucleic acid level between cirrhotic patients with and without hepatic encephalopathy

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ความแตกต่างของระดับ 16 เอส ไรโบโซมอลดีเอ็นเอของแบคทีเรียในพลาสมาของผู้ป่วยตับแข็งที่มีและไม่มีอาการทางสมองเนื่องจากโรคตับ

น.ส.เกศรินทร์ ถานะภิรมย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Field of Study	Medicine
Thesis Advisor	Associate Professor PIYAWAT KOMOLMIT, Ph.D.

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เกศรินทร์ ถานะภิรมย์ : ความแตกต่างของระดับ 16 เอส ไรโบโซมอลดีเอ็นเอของแบคทีเรียในพลาสมาของผู้ป่วยตับแข็งที่มีและไม่มีอาการทางสมองเนื่องจากโรคตับ. (The difference of plasma 16s ribosomal bacterial deoxyribonucleic acid level between cirrhotic patients with and without hepatic encephalopathy) อ.ที่ปรึกษาหลัก : รศ. ดร. น.พ.ปิยะวัฒน์ โกมลมิศร์

ที่มาของงานวิจัย: การเคลื่อนย้ายของเชื้อหรือองค์ประกอบของแบคทีเรียออกนอกลำไส้และการอักเสบทั่วร่างกายมีบทบาทสำคัญในพยาธิกำเนิดภาวะแทรกซ้อนต่างๆ ในผู้ป่วยตับแข็ง การตรวจ 16 เอส ไรโบโซมอลดีเอ็นเอของเชื้อแบคทีเรียได้ถูกใช้อย่างแพร่หลายในการบ่งชี้ภาวะการเคลื่อนย้ายของเชื้อหรือองค์ประกอบของแบคทีเรียออกนอกลำไส้ ปัจจุบันยังไม่มีการศึกษาเกี่ยวกับความแตกต่างระหว่างระดับของ16 เอส ไรโบโซมอลดีเอ็นเอและการเกิดภาวะแทรกซ้อนทางสมองจากโรคตับ ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อจะ ศึกษาความแตกต่างระหว่าง 16 เอส ไรโบโซมอลดีเอ็นเอ และอาการแทรกซ้อนทางสมองในผู้ป่วยโรคตับแข็ง

ระเบียบวิธีการวิจัย: ผู้ป่วยตับแข็งที่ไม่มีการติดเชื้อแบคทีเรียที่ยินยอมเข้าร่วมการศึกษาที่โรงพยาบาลจุฬาลงกรณ์ระหว่างเดือนสิงหาคม พ.ศ. 2564 ถึงธันวาคม พ.ศ. 2565 ผู้ป่วยทุกรายได้รับการประเมินระดับของอาการทางสมองโดยใช้เกณฑ์เวสต์ฮาเวน แบบประเมินอาการทางสมองในผู้ป่วยโรคตับที่คะแนน ≤ -5 และตรวจค่าพลาสมา 16 เอส ไรโบโซมอลดีเอ็นเอ ซีรัมไลโปโพลีแซคคาไรด์บายดิ้งโปรตีน ทูเมอร์เนคโครซิสแฟกเตอร์ อินเตอร์ลิวคิน-6 โซลูเบิ้ลซีดี 14 และระดับของแอมโมเนียในหลอดเลือดดำ

ผลการศึกษา: ผู้ป่วยตับแข็งทั้งสิ้น 294 รายเข้าร่วมการศึกษา ในจำนวนนี้ร้อยละ 31.3 มีภาวะแทรกซ้อนทางสมองแอบแฝง และร้อยละ 19.7 มีภาวะแทรกซ้อนทางสมองแบบเด่นชัด ตรวจพบดีเอ็นเอของแบคทีเรียร้อยละ 31.3 ในผู้ป่วยที่ไม่มีอาการทางสมอง ร้อยละ35.9 ในผู้ป่วยที่มีอาการทางสมองแอบแฝง และร้อยละ 48.3 ในผู้ป่วยที่มีอาการทางสมองแบบเด่นชัดตามลำดับ ผู้ป่วยที่มีอาการทางสมองแบบเด่นชัดจะพบการเคลื่อนย้ายของดีเอ็นเอแบคทีเรีย และมีระดับซีรัมไลโปโพลีแซคคาไรด์บายดิ้งโปรตีน สัดส่วนของเม็ดเลือดขาวนิวโตรฟิลต่อลิมโฟไซท์ ทูเมอร์เนคโครซิสแฟกเตอร์ อินเตอร์ลิวคิน-6 และแอมโมเนียสูงกว่าผู้ที่ไม่มีอาการทางสมอง ระดับของดีเอ็นเอของแบคทีเรียมีความสัมพันธ์เชิงบวกเล็กน้อยแบบมีนัยสำคัญทางสถิติไปกับระดับทูเมอร์เนคโครซิสแฟกเตอร์ อินเตอร์ลิวคิน-6 และแอมโมเนีย ขณะที่ระดับของดีเอ็นเอของแบคทีเรียไม่มีความสอดคล้องไปกับระดับซีรัมไลโปโพลีแซคคาไรด์บายดิ้งโปรตีน ทูเมอร์เนคโครซิสแฟกเตอร์ โซลูเบิ้ลซีดี 14 อินเตอร์ลิวคิน-6 แอมโมเนียและคะแนนจากแบบประเมินทางสมอง

สรุป: นอกจากระดับแอมโมเนียที่สูง การเคลื่อนย้ายของเชื้อแบคทีเรียอาจจะเป็นอีกพยาธิกำเนิดที่สำคัญของการเกิดอาการทางสมองแบบเด่นชัดในผู้ป่วยโรคตับแข็ง

สาขาวิชา	อายุรศาสตร์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Bacterial translocation, Hepatic encephalopathy, Cirrhosis, Plasma 16S ribosomal bacterial DNA, Lipopolysaccharide binding protein, Interleukin-6, Tumor Necrosis Factor alpha, Ammonia, Soluble CD14

Kessarin Thanapirom : The difference of plasma 16s ribosomal bacterial deoxyribonucleic acid level between cirrhotic patients with and without hepatic encephalopathy. Advisor: Assoc. Prof. PIYAWAT KOMOLMIT, Ph.D.

Background: Bacterial translocation (BT) and systemic inflammation play a key role in the pathogenesis of cirrhotic complications. The 16S ribosomal bacterial DNA (bactDNA) has been widely used as a marker of BT. The data on the relationship between BT, systemic inflammation and hepatic encephalopathy (HE) are scarce. This study aimed to assess the difference between plasma 16s ribosomal bactDNA and HE in patients with cirrhosis.

Method: Cirrhotic patients without bacterial infection were enrolled at Chulalongkorn University, Bangkok, Thailand, from August 2021 to December 2022. Grading of HE was classified by the West Haven Criteria and Psychometric hepatic encephalopathy score (PHES) ≤ -5. BactDNA, lipopolysaccharide-binding protein (LBP), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), soluble CD14, and venous ammonia levels were all measured.

Results: Overall, 294 cirrhotic patients were enrolled, with 92 (31.3%) and 58 (19.7%) having covert and overt HE, respectively. BactDNA was found in 31.3%, 35.9%, and 48.3% of patients with no HE, covert HE and overt HE, respectively. Patients with overt HE had more bactDNA translocation, and higher levels of serum LBP, neutrophil-to-lymphocyte ratio, TNF-α, IL-6, and ammonia than those without HE. Patients with detectable bactDNA had higher white cell counts and serum LBP and IL-6 levels than those without. Levels of plasma bactDNA had a weak significantly positive correlation with venous ammonia, TNF-α and IL-6 ($r=0.13$-0.32, $p<0.05$). While there was no significant correlation between bactDNA, serum LBP, sCD14, TNF-α, IL-6, ammonia level and the PHES score ($p>0.05$).

Conclusion: Apart from hyperammonemia, bactDNA translocation-related systemic inflammation might be a potential pathophysiological mechanism of overt HE in cirrhotic patients.

Field of Study:	Medicine	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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Kessarin Thanapirom

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CHAPTER 1

INTRODUCTION

BACKGROUND AND RATIONALE

Hepatic encephalopathy (HE) is a term that describes a variety of neuropsychiatric symptoms associated with liver insufficiency and/or portal-systemic shunting (PSS), ranging from minor changes in brain function to marked disorientation and coma (1). HE is one of the most common complications in cirrhosis patients that result in hospitalization and re-admissions (2). As a result, the healthcare burden and costs associated with HE management are extensive and growing (3). In addition, the incidence and prevalence of HE are associated with the severity of the underlying hepatic diseases and PSS (4-6). HE has traditionally been categorized into overt HE (clinically indicate neurological or psychiatric abnormalities) and covert HE (abnormalities on neuropsychological or electrophysiological tests without obvious clinical manifestation). At the time of diagnosis, the prevalence of overt HE is 10–14% in cirrhosis (7, 8), 16–21% in decompensated cirrhosis (9, 10), and 10–50% in patients having transjugular intrahepatic portosystemic shunt (TIPS) insertion (11, 12). Overt HE is an event that determines the acute decompensation of the disease, such as variceal bleeding or ascites, in patients with cirrhosis (9). The prevalence of developing the first episode of overt HE is approximately 25% within five years following the first diagnosis of cirrhosis. The potential risk factors for the development of HE in cirrhotic patients are diabetes, hyponatremia, the presence of portal hypertension, alcoholic-related cirrhosis, and chronic hepatitis C infection (13-16). Several medications, including benzodiazepines, gamma-aminobutyric acid (GABA)ergies, opioids, and proton pump inhibitors, represent potentially independent modifiable risk factors for HE(16). HE is associated with in-hospital and 30-day mortality (17) and has a significant negative

impact on health-related quality of life (HRQOL) and sleep-wake pattern (18) in patients with cirrhosis, which has clinical and psychological consequences (19, 20). Furthermore, HE exerts a multidimensional burden on the caregivers of patients and the national healthcare system.

Although advances in this research area have elucidated complex pathogenesis, the exact mechanism that leads to HE is not fully understood. Hyperammonemia has been considered as the important cause responsible for brain dysfunctions in HE (10). Increased brain ammonia is related to the impaired blood-brain barrier (BBB), astrocyte swelling and dysfunction, and cerebral edema in cirrhotic patients with HE (10, 21). However, the direct correlation between the degree of hyperammonemia and its diagnostic role as well as determining the severity of HE has not been confirmed in several clinical studies. A prospective study by Shawcross D.L. *et al.* showed that HE grade and coma score did not correlate with blood ammonia in patients with cirrhosis admitted to a liver Intensive Care Unit (22). Furthermore, patients without evidence of HE (grade 0) frequently had ammonia levels more than the upper limit of normal (47 μmol/L), while some patients with overt HE (grade 2-4) had normal ammonia levels (23). This raises the point that other contributing factors may involve the development and progression of HE. Apart from ammonia, other systemic factors e.g., inflammation, oxidative stress, increased bile acids, and lactate, contribute to the precipitation and progression of HE (24).

Systemic inflammation precipitated by gut bacterial translocation, superimposed infection, or liver injury causes BBB dysfunction and neuroinflammation. *In vitro* studies have shown that the presence of IL-1b can compromise the BBB through intracellular cyclooxygenase (COX) and tumor necrosis factor-alpha (TNF-α) activity, which promotes brain inflammation and disrupts the permeability of cerebral microvascular endothelial cells (25, 26). Pro-inflammatory cytokines are linked to

poorer cognitive performance in cirrhotic patients with HE compared to those without HE (27). Serum interleukin-6 (IL-6) and TNF-α were correlated with the severity of HE in cirrhotic patients (28, 29), while their reduction improved HE (27). Bacterial infection and systemic inflammatory responses (SIRS) were associated with advanced HE (grade 3 or 4) in cirrhotic patients (22). Moreover, systemic inflammation modulates the effect of ammonia on brain function. Inflammatory mediators, e.g., nitric oxide and pro-inflammatory cytokines, exacerbate the neuropsychological effects of hyperammonemia in cirrhotic patients (30).

Bacterial translocation (BT) is defined as the migration of bacteria and/or bacterial products from the intestine to the mesenteric lymph nodes and extraintestinal sites and exerts immunological response (31). BT is found in 25% of patients with cirrhosis, and its prevalence is increasing in advanced liver diseases. The prevalence of BT in patients with Child-Pugh A, B, and C is 3.4%, 8.1%, and 30.8%, respectively (32). Furthermore, BT is more frequent in cirrhotic rats with ascites than in those without (33). Several mechanisms explain why patients with cirrhosis have increased BT, including small bowel bacterial overgrowth, high intestinal permeability, and impaired immune response (34). BT plays a crucial role in developing complications of cirrhosis by inducing systemic inflammatory states and aggravating hemodynamic imbalances. Several biomarkers of BT have been proposed, such as bacterial DNA (bactDNA), lipopolysaccharide (LPS), peptidoglycan, lipopolysaccharide-binding protein (LBP). BT triggers systemic inflammatory state and hemodynamic derangement in patients with cirrhosis. Toll-like receptors (TLR) are a type of membrane protein expressed in peripheral blood mononuclear cells that recognize various conserved molecular patterns of the pathogen. Activation of TLR leads to the release of pro-inflammatory cytokines that can exacerbate hemodynamic abnormalities, especially splanchnic arterial vasodilation and cardiovascular

dysfunction, in patients with cirrhosis (35). Translocation of bacterial products in non-infected patients with cirrhosis and ascites is associated with aggravation of peripheral vasodilation and a worsening of intrahepatic endothelial dysfunction; this was related to an increased systemic inflammatory state as shown by the presence of higher plasma TNF-α (36). Plasma bactDNA level is positively correlated with serum nitric oxide and inflammatory cytokines, including TNF-α, IL-12 and IFN-γ (37).

In terms of HE, it is well known that bacterial infection is the common precipitating cause of HE in patients with cirrhosis. However, evidence of the relationship between BT and HE is scarce. No previous studies have been conducted to investigate the association between bactDNA translocation and HE in cirrhotic patients. In recent decades, several studies have indicated how inflammation and infection have synergistic effects with ammonia on the pathogenesis of HE(30, 38). The BT-associated inflammatory response may play a role in the pathogenic mechanisms that lead to HE. Therefore, this study aimed to investigate the difference between plasma bacterial DNA levels and the presence of HE in cirrhotic patients.

RESEARCH QUESTIONS

Phase 1 Developmental phase

1.1 What is the optimized protocol for quantification of 16s ribosomal bactDNA from human blood samples by the real-time polymerase chain reaction technique?

1.2 Is the plasma 16s ribosomal bactDNA level able to comprehensively detect the presence of different bacterial pathogens in positive blood cultures?

1.3 What are the normative data of the PHES in healthy Thai population?

Phase 2 Clinical study

PRIMARY RESEARCH QUESTIONS

Is there any difference with a greater than moderate effect size (0.5) in plasma 16s ribosomal bactDNA levels between cirrhotic patients with and without hepatic encephalopathy?

SECONDARY RESEARCH QUESTIONS

1. Does plasma 16s bactDNA level have the correlation coefficient more than 0.2 with serum LBP, IL-6, TNF-α, and venous ammonia level in cirrhotic patients?

2. Does plasma 16s bactDNA level have the correlation coefficient more than 0.2 with PHES score in cirrhotic patients with covert HE?

3. Is there any difference with a greater than moderate effect size (0.5) of serum LBP, IL-6, TNF-α and venous ammonia levels between cirrhotic patients with and without hepatic encephalopathy?

OBJECTIVES

Phase 1 Developmental phase

1.1 To optimize the protocol for quantification of 16s ribosomal bactDNA from human blood samples by real-time polymerase chain reaction technique.

1.2 To assess the feasibility of using the 16s ribosomal bactDNA level for the detection of various different bacterial strains from positive blood culture samples.

1.3 To determine the normative data of the PHES in healthy Thai population

Phase 2 Clinical study

PRIMARY OBJECTIVE

To investigate the differences with a greater than moderate effect size (0.5) of plasma 16s ribosomal bactDNA levels between cirrhotic patients with and without hepatic encephalopathy.

SECONDARY OBJECTIVE

1. To investigate the correlation with the value of coefficient more than 0.2 between plasma 16s ribosomal bactDNA and serum LBP, IL-6, TNF-**α**, and venous ammonia level in cirrhotic patients.

2. To demonstrate the correlation with the value of coefficient more than 0.2 between plasma 16s ribosomal bactDNA level and PHES score in cirrhotic patients with covert HE.

3. To assess the differences with a greater than moderate effect size (0.5) of serum LBP, IL-6, TNF-**α** and venous ammonia level between cirrhotic patients with and without HE.

HYPOTHESIS

Phase 1: Developmental phase

1. Optimized plasma16s bactDNA level assay has high sensitivity and feasibility to detect broad-range different bacterial pathogens.

2. Based on the normative data of healthy volunteers, the cirrhotic patients were classified as having covert HE when their PHES was less than -5.

Phase 2 Clinical study

1. Cirrhotic patients with HE have a higher plasma 16s ribosomal bactDNA level than patients without HE with an effect size of more than 0.5.

2. Plasma 16s ribosomal bactDNA level is positively correlated with serum LBP, IL-6, TNF-α, and venous ammonia levels in cirrhotic patients who have HE with a correlation coefficient of more than 0.2.

3. Plasma 16s ribosomal bactDNA level is significantly correlated with the PHES score in cirrhotic patients with covert HE with a correlation coefficient of more than 0.2.

4. Cirrhotic patients with HE have a higher serum LBP, IL-6, TNF-α and venous ammonia level than patients who do not have HE with an effect size of more than 0.5.

RESEARCH DESIGN

Cross-sectional analytic study

CONCEPTUAL FRAMEWORK

The conceptual framework of the developmental phase and clinical study are shown in Figure 1 and 2.

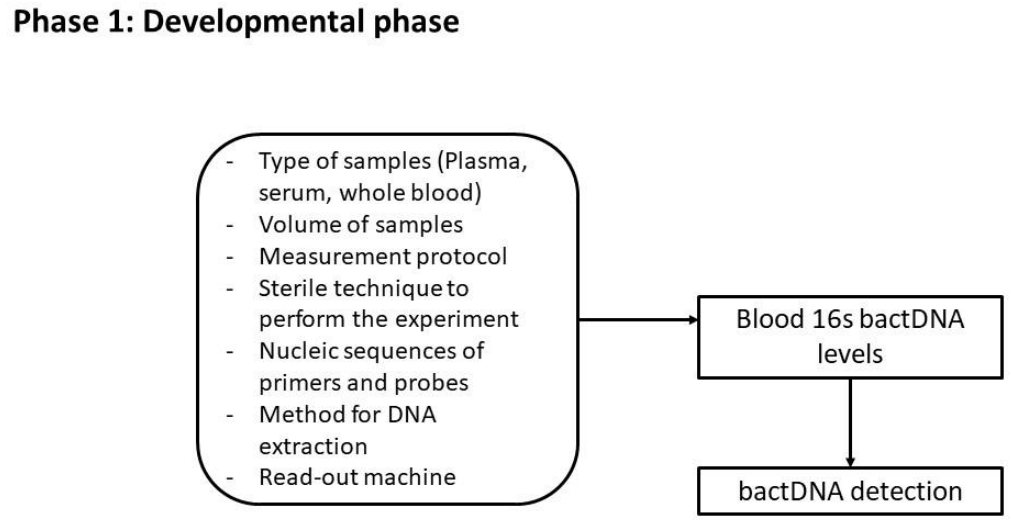

Figure 1 Conceptual framework of the developmental phase

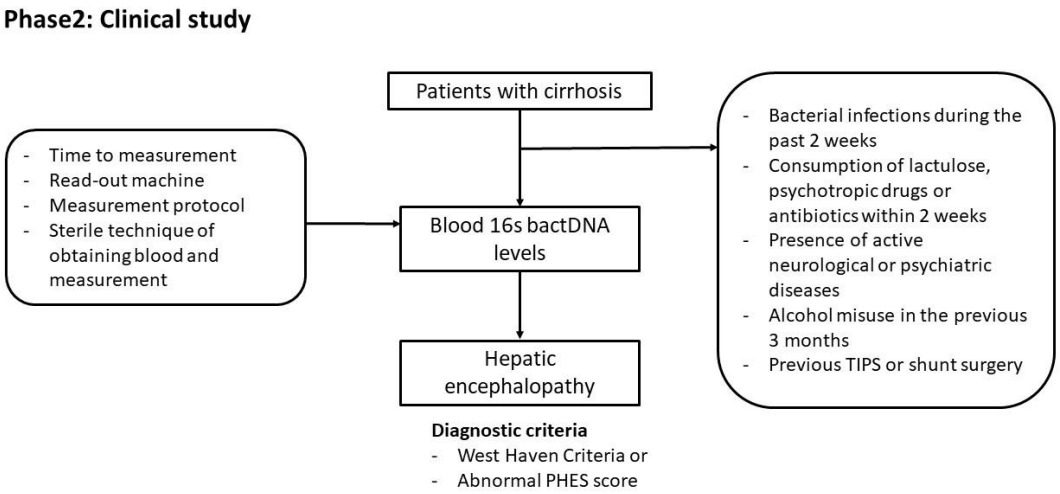

Figure 2 Conceptual framework of the clinical study

CHAPTER II

LITERATURE REVIEW

Hepatic encephalopathy: definition and epidemiology

Hepatic encephalopathy (HE) is defined as a brain dysfunction caused by liver impairment and/or porto-systemic shunting (PSS) after the exclusion of any other known brain disease (1). Patients with HE have a broad spectrum of neurological or psychiatric manifestations, from subclinical alterations to coma. HE is one of the most severe complications of cirrhosis (39). HE is associated with an increased mortality rate, risk of hospitalization, falls, motor-vehicle accidents, poor health-related quality of life, and psychosocial burden (40-42). HE is a manifestation of severe liver impairment; its prognosis is determined by the severity of the underlying liver disease. Compared to cirrhotic patients without HE, cirrhotic patients who present with overt HE have a median survival time of only a few months and a 2-fold increased risk of death over a year (7, 43).

The overall incidence of HE is 11.6 per 100 person-years (16). In cirrhosis, the 1-, 5-, and 10-year cumulative incidence of HE is 0-21%, 5-25%, and 7-42%, respectively. Regards to the severity of cirrhosis, the incidence of developing the first episode of overt HE during 1-year follow-up in Child A and B patients is 10% and 25%, respectively (44). Overt HE is present in 10–14% of cirrhotic patients at the time of diagnosis (7, 8), 16–21% in decompensated cirrhosis (9), and 10–50% in patients who underwent transjugular intrahepatic portosystemic shunt (TIPS) placement (11, 12). Despite receiving standard treatment, patients with a prior episode of overt HE had a 42% chance of recurrence after a year, and those with recurrent overt HE had a 46 percent chance of another episode within six months (45, 46). The prevalence of minimal HE is 37-80% in cirrhotic patients (47-49). The potential risk factors for HE are

alcohol-related cirrhosis, the presence of portal hypertension, minimal HE, grade 1 HE, diabetes, and hyponatremia (13-16).

Pathogenesis of hepatic encephalopathy

The pathophysiology of HE has yet to be determined. The general agreement is that a high ammonia level and inflammation cause astrocyte swelling and increased permeability of the blood-brain barrier (BBB).

Ammonia

Ammonia is mainly produced in the gastrointestinal tract and enters the bloodstream through the portal vein. Ammonia is generated by enterocytes from glutamine, and colonic bacterial break down nitrogenous substrates, e.g., ingested protein. Apart from the gut, ammonia is produced in the kidney and muscles. Ammonia is metabolized into urea through the urea cycle in a healthy liver and subsequently excreted through the kidney. In liver dysfunction, the ability to remove ammonia is reduced, resulting in increased blood ammonia. In the setting of hyperammonemia, astrocytes quickly metabolize glutamate and ammonia to glutamine by glutamine synthetase enzyme, leading to increased intracellular osmolarity, astrocyte swelling, and brain edema (50). Low-grade brain edema and a prominently neuro-inhibitory state are pathognomonic of HE in cirrhotic patients (51, 52). Although plasma ammonia level is higher in cirrhotic patients with HE than those without HE (27, 28). The ammonia level may be increased in patients without HE and normal in patients with overt HE (23). This reflects that ammonia is not the only cause of all the neurological changes in patients with HE (53). Figure 3 shows ammonia metabolism in normal and liver dysfunction.

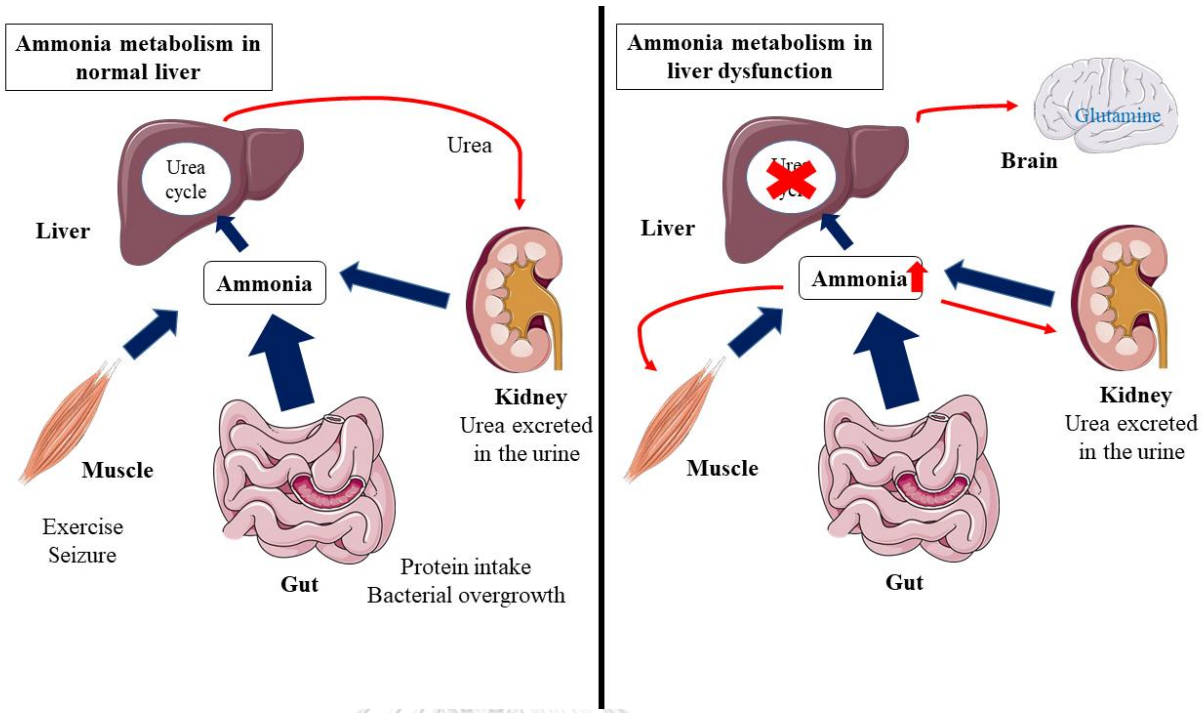

Figure 3 Ammonia metabolism

Inflammation

Systemic inflammation, aggravated by several factors such as the inflamed liver, bacterial translocation, and infection, induces BBB dysfunction and neuroinflammation. Increased evidence supports the synergistic role of inflammation and infection in modulating the cerebral effects of ammonia. Shawcross *et al.* investigated the impact of induced hyperammonia in cirrhotic patients who were hospitalized with SIRS from infection. The patients were induced hyperammonia by administering a special oral amino acid solution during SIRS and following the resolution of SIRS and evaluated for pro-inflammatory cytokines and neuropsychological test battery. Induced hyperammonia led to a substantial deterioration of neuropsychological scores in patients who showed signs of SIRS, but not after SIRS was resolved, indicating the inflammation plays a crucial role in the neuropsychological effects of hyperammonemia in patients with cirrhosis. (30). The presence of ammonia sensitizes

the brain to a systemic inflammatory stimulus, allowing it to elicit an inflammatory response involving both proinflammatory and neurotransmitter pathways.

The possible mechanisms by which systemic inflammation affects neurological abnormalities are not clearly elucidated. There are several possibilities. First, the cytokines might modulate ammonia diffusion and compromise the endothelial BBB within the central nervous system (54). Second, IL-1β and TNF-α enhance the expression of peripheral-type benzodiazepine binding receptors, which may affect cellular osmolarity in cultured astrocytes (55). Third, there are several receptors of proinflammatory cytokines in brain endothelial cells. These can transmit signals that result in nitric oxide and prostanoid production in the brain. Finally, these cytokines might affect at perivascular cells of the macrophage (56).

Recently, a new theory showing the important role of systemic inflammation in the development and progression of cirrhosis complications has been proposed (57). Patients with advanced cirrhosis demonstrate a state of systemic inflammation with increased circulating pro-inflammatory cytokines and chemokines. These contribute to circulatory dysfunction and multi-organ dysfunction, and failures in cirrhotic patients (58).

Oxidative stress

Increased production of reactive oxygen and nitrogen species (ROS and RNS) in cultured rat astrocytes that are treated with ammonia, pro-inflammatory cytokines and benzodiazepines (59, 60). In addition, systemic oxidative stress and ROS are associated with the development of cerebral edema in rats (61). Cirrhotic patients with HE exhibited higher systemic oxidative stress (serum 3-nitro-tyrosine) than those without HE (62). Moreover, in patients with chronic liver disease, the production of antioxidant

proteins, such as albumin and glutathione decreases while the synthesis of ROS increases, resulting in oxidative stress and cellular dysfunction (63).

Neurosteroids

Neurosteroids are produced mainly by myelinating glial cells, e.g., astrocytes. Translocator proteins on the mitochondrial membrane in astrocytes and control neurosteroid production (64). Increased density of translocator protein expression in the central nervous system in cirrhotic patients with minimal HE evaluated by PET scan with specific ligand binding (65). In addition, neurosteroids are positive modulators of $GABA_A$ receptors (66).

Bile acid

Bile acids are synthesized in the liver from cholesterol and metabolized in the gut by microbiota. The vast majority of bile acids are recycled by enterohepatic recirculation. In patients with end-stage liver disease, bile acids are increased, probably due to the release of bile acid content from destroyed hepatocytes and a decrease in bile acids reuptake from the bloodstream. Bile acids affect the brain via bile acid transporters on BBB, which is related to HE development (67). Bile acids have been found in the brain of rats that developed HE from bile-duct ligation, which causes neuroinflammation, increased BBB permeability, and cerebral edema (68).

Manganese

Manganese is one of the neurotoxins that deposits predominantly in the basal ganglia. Manganese deposition has been identified by magnetic resonance imaging in the basal ganglia of patients with cirrhosis and has been exhibited to improve after normalization of liver function (69, 70). Manganese-induced brain dysfunction by stimulating translocator protein on astrocytes further increases neurosteroid synthesis and GABAergic tone (71).

Lactate

Lactate is an organic compound derived from glucose and metabolized by lactate dehydrogenase in neutrocytes and astrocytes. Lactate is transferred to the extracellular space and used as a source of energy by neurons. The changes in lactate metabolism are associated with neuronal impairment and HE (72). Patients with HE have more lactate concentration in blood and brain than those without HE (72). In rats with bile-duct ligation, brain lactate and glutamine levels rise parallel with brain edema, while inhibiting lactate synthesis reduces brain lactate and edema (72). Figure 4 concludes the pathogenesis of HE.

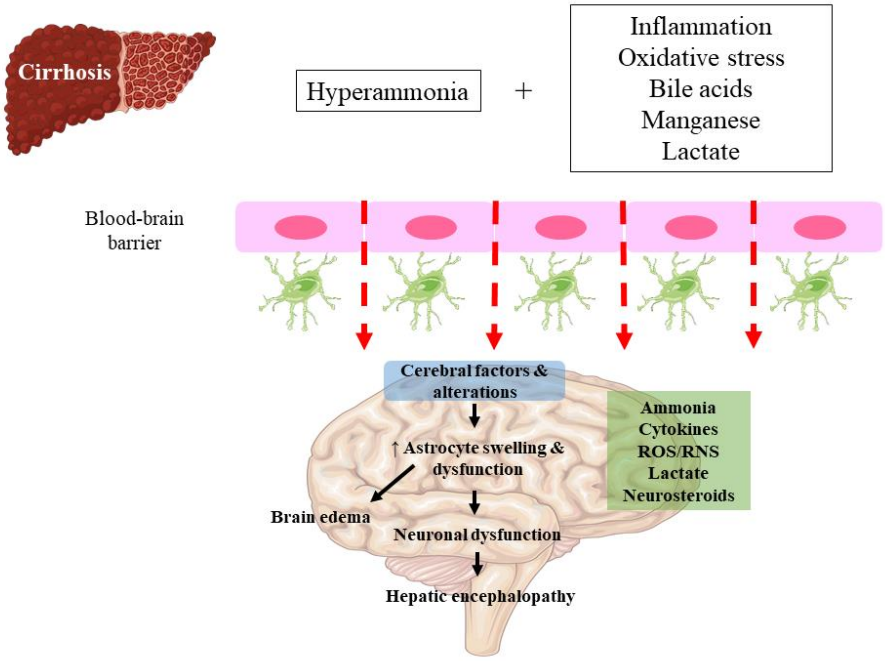

Figure 4 Pathogenesis of HE in cirrhosis

Hepatic encephalopathy: grading and classification

HE is classified based on four factors; the underlying disease, the severity of clinical manifestations, the time course, and the presence or absence of precipitating factors (73).

Based on the underlying disease, three subtypes of HE have been proposed.

- Type A: HE is occurring in the setting of acute liver failure. This type may be associated with increased intracerebral pressure and brain herniation (74).

- Type B: HE occurring in the setting of portal-systemic shunting with no intrinsic hepatocellular disease.

- Type C: HE is occurring in the setting of liver cirrhosis

Based on the severity of liver disease: The severity of HE is graded based on the clinical manifestations. The West Haven criteria (WHC) are the most widely used for grading HE (1). These criteria grade a patient's mental state through subjective behavior assessments, intellectual function, consciousness alteration, and neuromuscular function. The original version of WHC consisted of 4 grades, ranging from grade 1 (which includes trivial changes in behavior, mild confusion, slurred speech, disordered sleep) to grade 4 (which indicates an unresponsive patient with a coma) (75). However, the finding of studies using these criteria revealed significant interobserver variability in their assessments of low grade of HE. As a result, Amodio and colleagues proposed a modified WHC in 2004 that introduced objective detail for evaluating the individual parts of the criteria in patients with HE (76, 77). Table 1 shows the grading of HE based on modified WHC and clinical description.

Pronounced asterixis is seen in patients with grade II or III HE, while asterixis is usually absent in grade IV HE due to decorticate or decerebrate posturing (78). Patients with minimal and grade I HE are classified as having covert HE, while patients with grade II to IV HE are described as having overt HE, which has clinically apparent neurological deficits. Minimal HE is defined as HE without apparent neurological abnormalities, but the psychometric or neuropsychological tests reveal cognitive deficits.

According to the original descriptions, diagnosing grade I HE is difficult and challenging due to vague symptoms and signs. In addition, there is poor interrater variability in defining this stage. Therefore the American Association for the Study of Liver Disease (AASLD) and European Association for the Study of Liver Disease (EASL) proposed the operative criteria for diagnosis grade 1 HE ("despite being oriented in time and space, the patient appears to have some cognitive/behavioral decay concerning his/her standard on clinical examination or the caregivers") (75). However, the problem still persists in patients who do not have a caregiver. Therefore, in 2011, the ISHEN (International Society for Hepatic Encephalopathy and Nitrogen Metabolism consensus has recommended that minimal HE and grade I should be combined and categorized as "covert HE"(79) (Table 2). Patients with covert HE diminish their level of daily functioning, quality of life, and driving capacity (80, 81).

Table 1 West Haven criteria and description (77)

	Description
Grade 0	Normal
Minimal HE	Psychometric or neuropsychological alterations of tests
Grade 1	- Trivial lack of awareness - Euphoria or anxiety - Shortened attention span - Impairment of addition or subtraction - Altered sleep rhythm
Grade 2	- Lethargy or apathy - Disorientation to time - Obvious personality change - Inappropriate behavior - Dyspraxia

	- Asterixis
Grade 3	- Somnolence to semistupor - Response to stimuli - Confused - Gross disorientation - Bizarre behavior
Grade 4	Coma

Table 2 Proposed HE classification in cirrhosis according to International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) consensus (82)

	Unimpaired	**Covert HE**	**Overt HE**
Mental status	Not impaired	Not impaired	From disorientation to coma
Specialized tests (according to local expertise)	Not impaired	Impaired	Not specifically required but will be abnormal
Asterixis	None	None	Present (except in coma)

Based on time course

The time course of HE can be categorized into three types: episodic, recurrent (within six months or less), and persistent (behavioral changes that are always shown, interspersed with bouts of overt HE).

Based on the presence of precipitating factors

HE episodes are classified as either non-precipitated or precipitated. If precipitation occurs, the precipitating factors should be mentioned, for instance, medications, dehydration, hepatocellular carcinoma, portosystemic shunting, excess dietary intake of protein, gastrointestinal hemorrhage, infection, electrolyte imbalance, constipation.

Hepatic encephalopathy: diagnosis

Diagnosis and testing for overt HE

The diagnosis of overt HE is based on clinical signs and symptoms. The gold standard is the WHC (Table 1). Disorientation and asterixis have high inter-rater reliability and thus have been selected as marker symptoms of overt HE (76). There is good interrater reliability in determining disorientation to time in grade II HE (83). The Glasgow Coma Scale (Table 3) is widely used in patients with significantly altered consciousness and provides an operational description (84). However, overt HE remains a diagnosis of exclusion since this group of patients is usually susceptible to cognitive dysfunction from several factors, including psychoactive medications, alcohol withdrawal, drug use, electrolyte disturbances, hypoglycemia, and psychiatric disorder (e.g., dementia and depression) (Table 4). Currently, there are no gold standard laboratory tests that can be utilized to diagnose overt HE. Brain imaging should be evaluated in patients with an uncertain diagnosis or those with localizing neurological signs.

Table 3 Glasgow Coma Scale (84)

	Glasgow Coma Scale					
	1	**2**	**3**	**4**	**5**	**6**
Eyes	Does not open eyes	Opens eyes in response to painful stimuli	Opens eyes in response to voice	Opens eyes spontaneously	N/A	N/A
Verbal	Makes no sounds	Incomprehensible sounds	Utters inappropriate words	Confused, disoriented	Oriented, converses normally	N/A
Motor	Make no movements	Extension to painful stimuli (decerebrate response)	Abnormal flexion to painful stimuli (decorticate response)	Flexion/ withdrawal to painful stimuli	Oriented, converses normally	Obeys commands

N/A, not applicable

Table 4 Other causes of encephalopathy in cirrhotic patients

- Hypoxia	- Hypercapnia
- Acidosis	- Uremia
- Psychoactive medications	- Electrolyte imbalances
- Prior seizure or stroke	- Delirium tremens
- Wernicke-Korsakoff syndrome	- Intracerebral hemorrhage
- Central nervous system sepsis	- Cerebral edema +/- intracranial hypertension
- Hypoglycemia	- Drug intoxication

Blood ammonia level

For decades, the role of blood ammonia level in diagnosing HE has been widely debated. The EASL-AASLD guidelines do not recommend the routine measurement of ammonia level in HE (1). Several factors affect plasma ammonia levels. The level rises after a high protein intake, persistent fasting, gastrointestinal hemorrhage, strenuous exercise, cigarette smoking, decreased renal function, and transjugular intrahepatic portosystemic insertion (TIPS) (85). Volume expansion reduces plasma ammonia concentration by increasing urinary ammonia excretion and reducing ammonia genesis (86). In addition, measurement of blood ammonia level needs specific conditions on blood collection, including completely fill the ethylenediaminetetraacetic acid (EDTA) tube and immediately centrifugation, using tourniquet can falsely increase ammonia levels, need quickly transporting on ice at +4 °C and it was interfered with hemolysis, and marked jaundice. Venous and arterial ammonia had a moderate correlation in

hospitalized cirrhotic patients (23, 87). Thus, venous sampling is the most convenient and adequate method for ammonia measurement.

The performance of venous ammonia ≥ 55 μmol/L to diagnose HE was shown in Table 5 (88). There are false positive and false negative of using ammonia levels for this purpose. Gundling F study showed 21.7% of patients without HE had high plasma venous ammonia level, while 61.1%, 38.5% and 60% of patients with HE grade 1, 2, 3, respectively had normal ammonia level. However, ammonia levels have a high negative predictive value (81%); normal ammonia in cirrhotic patients with altered consciousness/coma should be referred to a differential diagnosis of other diseases rather than HE (89). Blood ammonia is not a reliable test for diagnosis HE in patients with cirrhosis. Ammonia level does not correlate with the severity of HE. Significant overlap in blood ammonia levels between patients with HE grade 1 and 2 compared to patients with grade 3 and 4 (87). However, higher arterial and venous ammonia levels are associated with more severe HE and worse clinical outcomes (23, 89, 90). Treatment with ammonia lowering agents is associated with good outcomes and improvement of HE (91, 92).

Table 5 The diagnostic performance of plasma venous ammonia for detecting hepatic encephalopathy in cirrhotic patients (88)

	%
Accuracy	59.3 %
Sensitivity	47.2 %
Specificity	78.3 %
Positive predictive value	77.3 %
Negative predictive value	80 %

Diagnosis and testing of minimal HE/covert HE

In minimal/covert HE, cognitive impairment is characterized by attention deficiency, working memory issues, visuo-spatial coordination, psychomotor speed/reactions, and executive function deficits, e.g., response inhibition (79, 93, 94). Long-term memory and language function are intact. Minimal HE/covert HE should be diagnosed by using neuropsychological tests that are nationally and culturally validated, as well as accessibility and local expertise. Neuropsychological tests, such as psychometric hepatic encephalopathy score, critical flicker frequency, continuous reaction time, Stroop test, and Animal Naming Test, have been validated and could be recommended to investigate minimal/covert HE among patients with cirrhosis. Even though these tests are sensitive, their specificity is in doubt since other metabolic causes and traumatic injuries to the brain can have similar impairments. Therefore, the test results should be interpreted regarding the patient's history, clinical signs, and socio-economic position. There are three categories of tests that have been proposed for the diagnosis minimal/covert HE, including psychometric tests, electrophysiologic tests, and computerized tests. Of these three categories, at least one test needs to be abnormal to diagnose minimal/covert HE for single-center studies in current international guidelines (1, 82).

1. Psychometric tests

1.1 **Psychometric Hepatic Encephalopathy Score**

A paper-and-pencil battery test called Psychometric Hepatic Encephalopathy Score (PHES) has been widely validated and accepted as the best clinical standard for diagnosis minimal/covert HE (73). PHES is the sum score obtained from the five sub-tests, including the Number Connection Tests (NCT) A and B, a Digital Symbol Test (DST), the Serial Dotting Test (SDT), and the Line Tracing Test (LTT) (Figure 5). This battery evaluates psychomotor speed and precision, visual perception, visuo-spatial coordination, visual construction, concentration, attention, and short-term memory. The tests are relatively easy to perform by health care personnel and have good external validity. Several potential factors could affect PHES, including age, gender, educational level, ethnicity, and occupation (79, 95). The sensitivity and specificity of PHES to determine minimal/covert HE was 96% and 100%, respectively (79). The test-retest reliability has been exhibited to more than 0.81(96). A previous study showed the absence of learning effects for the PHES when the test battery was repeated within six months (97). Apart from diagnostic tools, PHES has been studied for its role in evaluating prognosis. PHES was a predictor for the development of overt HE, survival, and risk of falls within one year in patients with cirrhosis (79, 98-100). Interestingly, PHES and the sub-tests results have a significant association the brain glucose metabolism in cirrhotic patients with grade 0-II HE. The cortical glucose utilization reduces with decreasing PHES total points especially in inferior frontal and dorsolateral frontal regions (101). Currently Hannover Medical School holds the copyright for using PHES and allows the test via request.

The original PHES battery was developed and validated by Professor Karin Weissenborn from the Department of Neurology, Medizinische Hochschule Hannover, Germany (79). To date, this test has been validated, translated, and normalized in

many other countries (95, 102-105). However, no previous normative data of PHES score in the Thai population has been published in the full-paper original article. A previous abstract was presented in the 34th Annual Meeting of the Royal College or Physician in April 2018 by Wongwandee M. *et al.* (106). The author replaced the alphabet in NCT-B with the Thai alphabet in the same order since some enrolled participants were not familiar with the English alphabet. The study enrolled 93 healthy Thai volunteers with a mean age of 41.3 ± 13.0 years and a mean education duration of 14.6 ± 4.2 years. Mean PHES was -0.58 (+1 to -6) with a standard deviation of 1.34. The study suggested that the cut-off score of -4 should be used for differentiating normal from minimal HE. PHES score was associated with education and gender.

To evaluate the NCT A and B; subjects are instructed to join the dots between numbers or numbers and alphabets in a timed manner, with the result being the time in seconds required; the DST; subjects are asked to match numbers with special symbols; the score is determined by the number of correct pairs obtained within a 90-second time frame; SDT; the subjects are required to dot the middle of a group of blank circles and the time to finish is the outcome; LTT; subjects are asked to draw a line between two parallel lines. The results from each subtest are compared to age-related normative data and transformed into scores ranging from +1 to - 3 based on how many standard deviations of patient's test value differs from the mean in the general population. PHES score can range from +5 to - 15 depending on how long the patient takes to complete the test, the number of correct pairs in DST, and how many errors the patient makes on the LTT. Table 6 shows the method of scoring when assessed by all five subtests. However, scoring LTT is different from other tests; it needs time and numbers of errors to complete the track. The error points are assigned every time the drawn line touches (1 point) or crosses the border line (2 points). There is no maximum score. Then combined scores are calculated with individual time (second)

and error points. Raw scores are converted to Z-scores by standardizing all scores to mean and SD of the control group similar to other tests. The cut-off of the PHES score of ≤ -4 is recommended to differentiate normal from minimal HE in German and Thai studies (79, 106). Twenty-five percent of patients with grade 0 HE achieved abnormal results of the PHES test and were categorized as having covert HE. If the single test findings were higher than the mean plus 2SD range, they were considered to have covert HE (107).

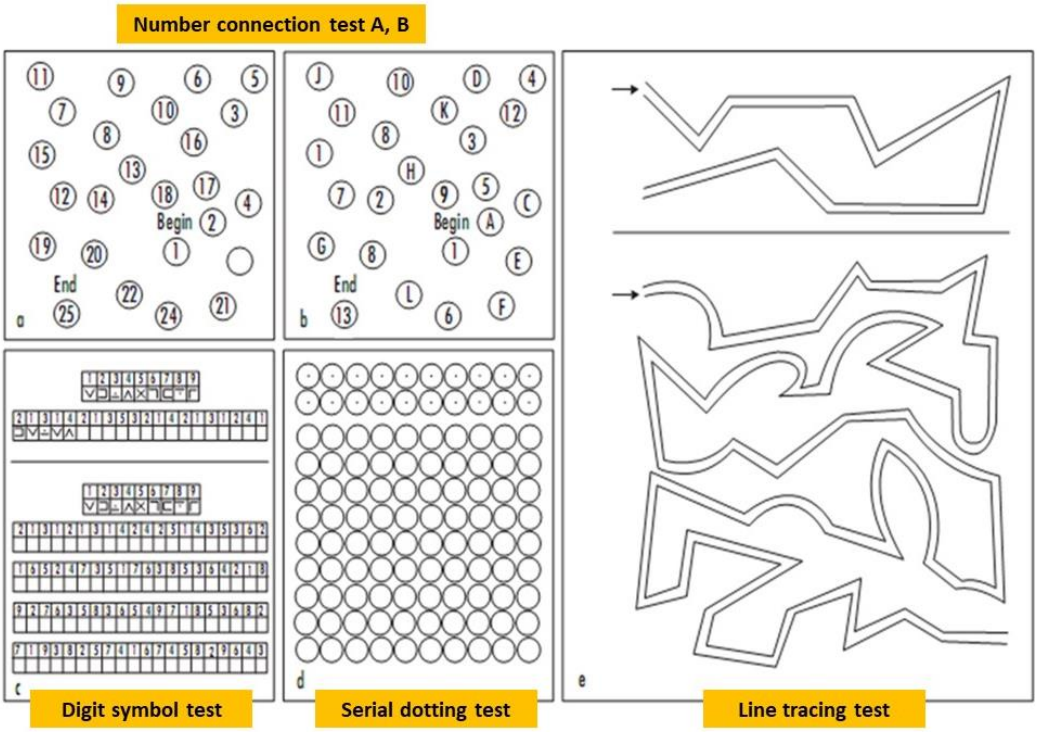

Figure 5 Psychometric Hepatic Encephalopathy Test (79, 108)

Table 6 Method of scoring when assessed by all five subtests of PHES battery.

Test results within	**Score**
Less than the mean -1SD	1
+/- 1 SD range	0
+1 to +2 SD beyond the mean	-1
+2 to + 3 SD beyond the mean	-2
Worse than the mean + 3SD	-3

1.2 Stroop app

A smartphone application called EncephalApp_Stroop or Stroop test was developed and validated by Bajaj et al. as the test to screen for covert HE. The Stroop effect is a psychomotor speed and cognitive flexibility test by measuring time to correctly recognize a series of symbols with different colors (Offtime) and printed words with different colors (Ontime). The test effectively detects cognitive dysfunction in patients with covert HE (109). The EncephalApp is free and available on iTunes and Google Play Store. It is straightforward to administer, score, and interpret. A cut-off of > 274.9 seconds (Ontime plus Offtime) has an accuracy of 89%, a sensitivity of 78%, and a specificity of 90% for diagnosing minimal HE compared to PHES (110). Test-retest reliability in healthy controls and patients without prior overt HE is good (110). However, there is no available test in the Thai version, which needs further studies to optimize and validate.

2. Electrophysiologic tests

2.1 Electroencephalography (EEG) is an electrophysiological technique that can diagnose cirrhosis-related neuropsychiatric dysfunctions (111). The sensitivity of EEG to diagnose HE is approximately 43%-100% (76). The sensitivity of the EEG was lower than NCTs A and B (108). However, EEG has several limitations, including high inter- and intra-observer variability, requiring neurological expertise to perform and interpret the test, and high cost. EEG tracings show posterior dominant alpha rhythm slowing in mild HE, followed by theta and high irregular delta waves as the HE proceeds into grade IV(111). However, EEG results are non-specific and could be altered by other metabolic causes. A normal EEG finding can occur in a patient with clinically overt HE, while an abnormal EEG result may be found in a patient without clinical manifestation of HE. The EEG abnormalities regard to mean dominant frequency and the percentage of theta and delta activity are found in 7% of patients with normal signs and neuropsychological tests, 15% in patients with normal clinical manifestation but abnormal findings in PHES battery, and only 50% of patients with overt HE(112). Thus, EEG is not recommended to diagnose minimal/covert HE.

2.2 Critical flicker frequency (CFF)

CFF can identify various neuropsychological disorders, from visual signal processing to cognitive activities. Patients are shown light pulses with a beginning frequency of 60 Hz, subsequently decreasing by 0.1 Hz/second. Patients are instructed to determine the time at which obviously fused light appears to flicker. CFF threshold is defined as the frequency at which a flickering light cannot be discriminated from a constant, non-flickering light. A CFF of < 39 Hz has 61% sensitivity, 65% specificity, and 84% accuracy in diagnosing CHE in cirrhosis patients (113, 114). This CFF threshold is correlated with the PHES score (115). The benefits of the test include its objectivity and unaffected by the patient's age, level of education, or literacy (114, 116). Several

factors influence CFF, including medications, age, and equipment (39). CFF is associated with the development of overt HE and mortality (117).

3. Computerized tests

3.1 The Inhibitory Control Test (ICT). ICT is a computerized attention span and response inhibition. The subject is asked to respond to the targets (e.g., X and Y) and not to lures (non-X and Y targets). Response rate and times of lures and targets are analyzed after the end of the test. CHE is detected if patients have longer lures and target reaction times, lower target responses, and a higher rate of lure responses. The lure threshold of >5 was determined as the diagnostic criteria of covert HE (118). The sensitivity and specificity of ICT to diagnose covert HE is 87% and 77%, respectively (118). The test shows good test-retest reliability (118). The ICT is simple to use, free and well-validated, but it needs highly functional patients. Several studies have demonstrated that ICT results were poorly correlated with the results of PHES for the diagnosis of covert HE (119, 120). In addition, the ICT result is influenced by patients' demographic factors and learning effects (120).

Bacterial translocation

Bacterial translocation (BT), also known as microbial translocation, is defined as the migration of viable microorganisms or their products, such as bacterial deoxyribonucleic acid (DNA), endotoxin, and peptidoglycan from the intestinal lumen into the mesenteric lymph nodes and other tissues and organs (31). The Enterobacteriaceae family (*E. coli*, *Klebsiella* spp.), *Enterococci*, and S*treptococci* spp. are the most common organisms found in BT in humans (121). In a mouse model study, gram-negative bacteria translocate in great numbers to the mesenteric lymph nodes, whereas Gram-positive and anaerobic bacteria translocate at much lower levels (122). Translocation of viable bacteria, endotoxin, or bacDNA exerts a systemic

inflammatory state and aggravates the hemodynamic derangement by stimulating pro-inflammatory cytokines and nitric oxide synthesis.

In patients with decompensated cirrhosis, bouts of transient bacteremia caused by the bacterial passage from the gut to the systemic circulation occur. Several of these episodes may be undetected, while others may cause infection and cirrhosis complications. BT develops in 25-30% of cirrhotic patients. BT results in high systemic inflammation, increased portal pressure, and deterioration of hemodynamics and hemostasis. BT plays a vital role in developing complications in patients with cirrhosis, including infection, spontaneous bacterial peritonitis, hepatic encephalopathy, hepatorenal syndrome, acute-on-chronic liver failure, and increased mortality (Figure 6). The degree of BT increases with the severity of liver disease. BT, as evaluated by endotoxemia (plasma endotoxin level > 5.7 pg/ml), is found in 27%, 85%, and 41% of patients with chronic hepatitis, chronic hepatitis with acute exacerbation, and cirrhosis, respectively (123). Cirrhotic patients with BT had a higher Child-Pugh score than patients without BT. Child-Pugh score is the independent predictor for BT (32). Several mechanisms are related to increased BT in cirrhosis patients, including intestinal bacterial overgrowth, increased intestinal permeability, and immunological impairment. Figure 6 shows the mechanisms and consequences of BT in cirrhosis.

Small intestinal bacterial overgrowth (SIBO) is a condition in which an increased number and/or type of bacteria in the small intestine (124). SIBO is more prevalent in patients with cirrhosis than healthy controls, particularly those with severe liver dysfunction and prior episodes of SBP and/or HE (125, 126). The prevalence of SIBO in cirrhotic patients varies from 48% to 73% (127, 128). SIBO is one of the key factors that increase BT, as demonstrated in the experimental models (129, 130). Moreover, cirrhotic patients exhibited reductions in microbial diversity and cirrhosis-specific profiles (131, 132). These profiles appear to be predominated by *Fusobacteria,*

Proteobacteria, Enterococcaceae, and *Streptococacceae* with a relative decrease in *Bacteroides, Ruminococcus, Roseburia, Veillonellaceae,* and *Lachnospiraceae* (133, 134). SIBO and dysbiosis in cirrhosis have been linked to reduced small-bowel motility, gut transit time, decreased enterohepatic recirculation, and low secretion of bile acid and gastric acid (135). Hypo- and achlorhydria with or without acid-suppressive medicines are associated with SIBO in cirrhosis (125, 135).

Increased intestinal permeability

The gut barrier consists of a mucinous component secreted by intestinal epithelial cells and intestinal epithelium, which forms a layer that prevents the passage of the bacterial endotoxin and products (36). Intestinal epithelial cells produce mucus, which includes a thick layer over the gut mucosa, preventing bacterial entry. Mucous secretions contain a high concentration of immunoglobulin A, eradicating toxins and pathogens and preventing their attachment and colonization (33). Bile acid also contributes to gut permeability by influencing the mucosa and neutralizing endotoxin (36). In cirrhosis, the microcirculation in the gut mucosa changes, leading to a decrease in mucosal vascular supply, which causes congestion, edema, ischemia, widening intercellular spaces, and reduced mucosal permeability (136). Moreover, cirrhotic patients have decreased intestinal bile acid concentration. A previous study showed an increased gut permeability in patients with cirrhosis, particularly those with a prior history of SBP, HE, or sepsis (137-139). Oxidative stress to the gut mucosa, endotoxemia, and increased nitric oxide and pro-inflammatory cytokines may increase gut permeability (140).

Immunological impairment

The gut is an active organ of the immune response, containing all white blood cells responsible for adaptive and innate immune systems. In cirrhosis, local and systemic immunity is impaired, such as low complement levels, low secreted pattern recognition receptors (PRRs), and decreased immune cells function, promoting the development of BT (136).

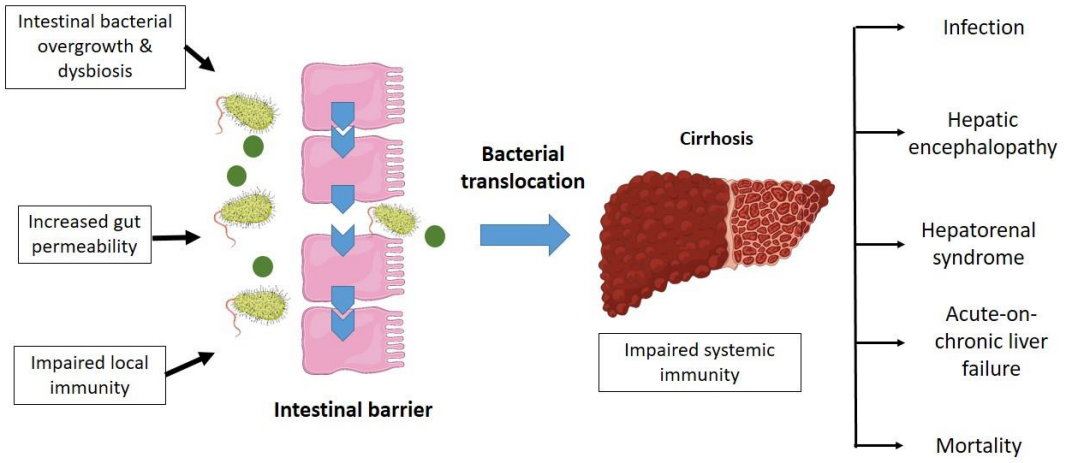

Figure 6 Mechanisms and consequences of BT in cirrhosis: *Cirrhotic patients have intestinal bacterial overgrowth, poor gut permeability and immune dysfunction, which results in bacterial translocation, causing systemic inflammation and the development of cirrhotic complications.*

Biomarkers of pathological bacterial translocation

Bacterial DNA

Over the past few decades, several biomarkers have been studied to improve the accuracy of pathogen detection and identification in diagnostic bacterial microbiology (141-143). The broad-range nucleic amplification technique and DNA sequencing of the 16s ribosomal RNA (rRNA) gene result in the recovery and subsequent categorization of bacterial species, becoming one of the most recent widely used tests (143-145). All bacteria have the 16s ribosomal gene, which encodes

16s rRNA responsible for converting genetic messages to cell function by translating mRNA to proteins. The ~1,500 base pair (bp) 16s rRNA gene comprises highly conserved nucleotide sequences interspersed with nine variable regions (V1-9) (Figure 7). The conserved areas have a slow rate of evolution and indicate the phylogenetic relationship among bacterial species, while hypervariable regions reflect sequence diversity among different species (46, 47). The relation of conserved and variable regions provides the utility of the 16s rRNA gene in identifying and detecting all species of bacteria. As a result of the invention and increasing availability of PCR and DNA sequencing, the 16s rDNA sequencing has been used in several applications in research purposes as well as clinical microbiology, including bacterial detection, identification of strains isolated in culture, identification of slow-growing or unculturable bacteria, detection of new species or genera of bacteria and the use for phylogenetic studies (48).

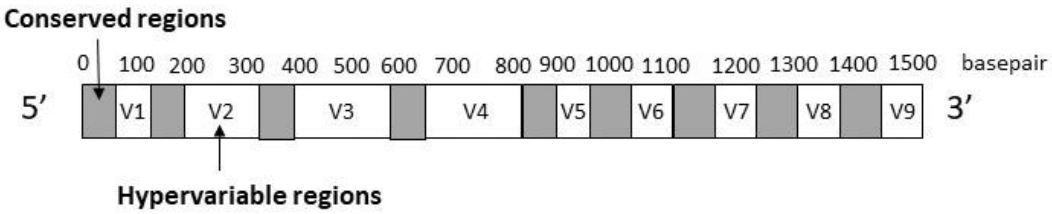

Figure 7 Structure of 16s ribosomal RNA gene (146)

Regarding BT, the detection of 16s bactDNA fragments in blood or ascitic fluid is a current BT parameter. Its identification by the polymerase chain reaction (PCR) is related to systemic inflammation and poor outcome in patients with cirrhosis. In cirrhosis, 16s bactDNA was detected in 34% of patients with non-infected ascites and 100% in all patients with spontaneous bacterial peritonitis. No patient receiving norfloxacin exhibited bactDNA translocation. Serum 16s bactDNA concentration is

correlated with serum TNF-α, IL-12, IFN-γ, and nitric oxide levels in patients with bactDNA translocation or spontaneous bacterial peritonitis (37). Cirrhotic patients with the presence of 16s bactDNA had a significantly lower mean arterial pressure and systemic vascular resistance than patients with the absence of 16s bactDNA. BactDNA translocation is associated with systemic circulatory abnormalities, intrahepatic endothelial dysfunction, and increased systemic inflammatory state (36). In cirrhotic patients with non-infected ascites, the presence of 16s bactDNA was associated with increased one-year mortality than patients with negative 16s bactDNA, and the leading cause of death was acute-on-chronic liver failure (147). Consistent with another study, the 16s bactDNA translocation in patients with refractory ascites was associated with impaired cardiovascular and kidney functions and an increased risk of hepatorenal syndrome and death (147).

Lipopolysaccharide (LPS)

LPS, or endotoxin, is a lipopolysaccharide found in the cell walls of Gram-negative bacteria. It comprises of a lipid and polysaccharide molecule. Plasma LPS levels in patients with alcoholic cirrhosis were significantly higher than in patients with non-alcoholics, and the levels appeared to be related to pro-inflammatory cytokine levels (TNF-α levels) and severity of liver disease (Child-Pugh) (148). LPS level is affected by various factors, including the density of LPS transporter, antibodies, high-density lipoproteins, and physiological variables. LPS has a short half-life of 2-3 hours which is a major drawback of its utility as a surrogate marker of BT (149). Moreover, the difficulty in measuring LPS by the Lymulus Amebocyte Lysate test was not originally designed for quantified LPS in biological samples. In addition, sample collection was needed to use endotoxin-free systems.

Lipopolysaccharide-binding protein (LBP)

LBP is a soluble acute-phase protein (molecular weight 58-60 KDa) produced by hepatocytes and is released into the bloodstream during acute-phase stimulation from endotoxin or acute trauma. In addition, gut, lung epithelial cells, and gingival epithelial cells have been found to produce LBP (150, 151). LBP is released into the bloodstream within 1 hour after inducing endotoxemia in rats (152). Its average level in human serum is 5-15 μg/L, which can rise to 30 times during the acute phase response (153). LBP binds to lipid A moiety of bacterial lipopolysaccharide (LPS) to stimulate immune responses by transferring LPS to cell surface pattern recognition receptors (PRRs), e.g., CD14 (154). The LPS-LBP-CD14 complex binds to Toll-like receptor4 (TLR4) in monocytes/macrophages and induces pro-inflammatory cytokines, including TNF-α and IL-6 (155). Moreover, LBP can bind and modulate the effect of lipoteicholic acid (LTA), the products of gram-positive bacterial cell walls, and the surface of whole bacteria (156, 157). The LBP knockout mice could not develop meningitis if treated with a pneumococcal cell wall preparation (158). Because of its prolonged half-life (2-3 days), LBP levels remain in serum for a long period after an event of bacteremia, providing its benefit over LPS (half-life 1 hour), and LBP is a relatively reliable biomarker for the diagnosis of BT (153). LBP levels indicate long-term bacterial and endotoxins exposure (159).

Plasma LBP was markedly higher in cirrhotic patients with ascites than those without healthy controls. High plasma LBP levels were found in 42% of cirrhotic patients with ascites. Ascitic cirrhotic patients with marked immune and hemodynamic derangements had higher LBP levels (> 9.62 μg/ml) when compared to those with normal LBP levels or healthy controls, suggesting increased BT in patients with clinically significant portal hypertension (159). Furthermore, treatment with norfloxacin for four weeks in ascitic cirrhotic patients with increased LBP was related to normalized

LBP and improvement of pro-inflammatory and hyperdynamic circulatory status, implying that enteric bacteria may be involved in this process (159). Interestingly, LPS was positive in one-third of patients with high LBP, suggesting that LPS is not a good marker for BT. Serum and ascitic fluid LBP levels show a positive correlation with surrogate markers of inflammation (160). Higher LBP levels in these patients were associated with higher TNF-α, IL-6, and soluble CD14 levels. On admission, high serum LBP (≥ 13.49 Log ng/ml) was associated with increased 90-day mortality in decompensated cirrhotic patients without infection at baseline. The cause of death was HE, hepatorenal syndrome, variceal bleeding, and infection (160).

Bacterial translocation and hepatic encephalopathy

As mentioned, increasing evidence supports the new theory of the relation between BT and the development of cirrhotic complications, including HE. Modifying gut flora by probiotics intake in cirrhotic patients improves MHE and decreases venous endotoxin and ammonia levels at one month (161). Chronic hyperammonemia leads to impaired cognition and increases the sensitivity to LPS. Injection of LPS into normal and hyperammonemic rats showed increased cytokine production in both groups, while the cognitive impairment in hyperammonemic rats was greater and longer than in normal rats (162). There are inconsistent results on BT markers and HE levels in patients with cirrhosis in the prior two studies. Jain *et al.* showed that arterial ammonia, inflammatory cytokines, and serum endotoxin increased and correlated with HE (163). In contrast, Kimer *et al.* found that blood ammonia, endotoxins, or markers of systemic inflammation were not associated with minimal HE (164).

No previous studies demonstrate the diagnostic performance of plasma bactDNA level and HE in patients with cirrhosis. The evidence on the level of LBP and bactDNA, venous ammonia, and serum inflammatory markers in cirrhotic patients with and without HE as well as the correlation between these factors and the severity of

HE, is lacking. We conducted the study to evaluate the diagnostic performance of plasma bactDNA for the presence of HE in patients with cirrhosis. In addition, the correlation between plasma bactDNA levels and the PHES score, LBP levels, serum TNF-, IL-6, and venous ammonia levels in cirrhotic patients with HE.

CHAPTER III

RESEARCH METHODOLOGY

Development and validation of normative data for the PHES in a healthy Thai population

Participants

Healthy volunteers

Healthy volunteers were invited to participate in this study between May 2021 and April 2022. We enrolled them from a health check-up clinic or from caregivers who came with patients at liver clinic at Chulalongkorn University Hospital. Healthy volunteers were defined as participants aged 18 to 85 years who did not meet the following criteria: (1) Chronic liver diseases; (2) Neuropsychological diseases, or other diseases that can decrease cognitive function; (3) Use of psychoactive medications; (4) Alcohol consumption greater than 50 g/day for males and 20 g/day for females; and (5) Impaired ability to read and write. History taking was used to identify these factors. The screening form for healthy volunteer enrollment was shown in Table 7.

Table 7 Screening form for healthy volunteers

Parameter	
Sex	☐ 1) Male ☐ 2) Female
Age	..... years
Education year	..... years
Chronic liver disease (Hepatitis B, Hepatitis C, Fatty liver, Cirrhosis)	☐ 1) Yes ☐ 2) No
Neuropsychological diseases (eg. Stroke, dementia)	☐ 1) Yes ☐ 2) No
Use of psychoactive medications	☐ 1) Yes ☐ 2) No

Alcohol consumption (> 50 g/day for males and 20 g/day for females)	☐ 1) Yes ☐ 2) No
Impaired ability to read and write	☐ 1) Yes ☐ 2) No

Healthy subject provided written informed consent. The study protocol was approved by the local Institutional Review Board of Faculty Medicine, Chulalongkorn University (IRB No. 0171/65). The study protocol complied with the ethical principles of the Helsinki Declaration and followed the Good Clinical Practice guidelines.

Psychometric hepatic encephalopathy score

The Thai version of five paper-pencil tests of the psychometric hepatic encephalopathy tests, including NCT-A, NCT-B, DST, SDT, and LTT (time and errors) (Figure 8), was administered to all the enrolled healthy controls and patients in the same order. The forms of the PHES battery were kindly given by Professor Karin Weissenborn (Figure 5) from the Hannover Medical School, Germany, and modified into Thai by Dr. Monton Wongwandee (Department of Medicine, Srinakharinwirot University, Nakhon Nayok, Thailand). The alphabet in the original version of NCT-B was replaced with the Thai alphabet in the same sequence due to incompatibilities between the English and Thai alphabets. All subjects finished PHES after a full explanation, demonstration, and training in an identical series of subtests. The tests were conducted one-to-one in a quiet room with sufficient lighting. A specially trained nurse and research assistant supervised the participants in completing these tests.

All PHES exams were rated by one rater (KT). NCT-A, NCT-B, and SDT results were evaluated in seconds, including the time necessary to correct any errors, while the DST result was measured in points of corrected pairs. In LTT, results were calculated as the sum of total time and error (107). The error points were assigned every time the drawn line touched (1 point) or crossed the boundary line (2 points)

(107). The findings from each subtest are translated into scores ranging from +1 to - 3 based on how many standard deviations of test value differ from the mean among the enrolled healthy subjects. The results (NCT-A, NCT-B, SDT, and LTT) within ± 1 standard deviation (SD) from the mean of the control group were scored as 0 points. The scores for results ranging from +1 and +2SD, +2SD and +3SD, and worse than +3SD were -1, -2, and -3 points, respectively. Those that performed better than mean -1SD earned +1 point (79). The result of DST within ± 1SD from the mean of the control group was scored as 0 points. The scores for results ranging from -1 to -2SD, -2SD to -3SD, and worse than -3SD were -1, -2, and -3 points, respectively. A result better than mean -1SD was scored as +1. The final PHES was calculated by adding the results of five subtests, ranging between +5 and -15. The normative data of PHES was determined at the mean – 2SD.

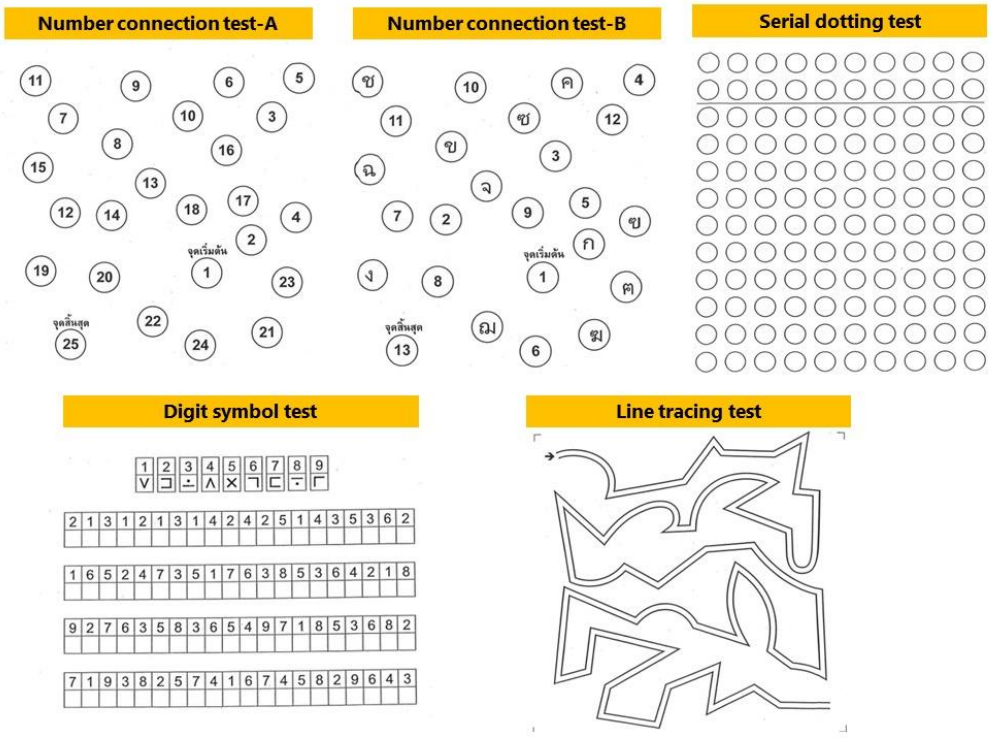

Figure 8 Thai version of the Psychometric Hepatic Encephalopathy Test

Clinical study

The difference in plasma 16s ribosomal bactDNA between cirrhotic patients with and without hepatic encephalopathy.

Participants

Inclusion criteria

1. Patients with cirrhosis diagnosed by radiological findings.

2. Age 18-80 years

3. Patients with written informed consent.

Exclusion criteria

1. Consumption of any antibiotics or psychotropic drugs during the preceding two weeks. Psychotropic drugs are identified based on the list of the Thai Food and Drug Administration, Ministry of Public Health (https://www.fda.moph.go.th/sites/Narcotics/List_of_Narcotic/PHYCHO_list_25.04.2019).

2. History of any bacterial infections during the past two weeks

3. Presence of active neurological or psychiatric diseases diagnosed by the physician during the past two weeks

4. Previous transjugular intrahepatic portosystemic shunt (TIPS) or shunt surgery

5. Alcohol misuse in the previous three months

Data collection

Cirrhotic patients at the Liver Clinic or inpatient wards at King Chulalongkorn Memorial Hospital who do not meet the exclusion criteria were enrolled. The clinical

history, patient baseline characteristics, and physical examination are recorded. All the patients are evaluated for WHC. Patients without overt HE was assessed for PHES battery. Laboratory parameters, including complete blood count, liver function test, serum creatinine, prothrombin time, and venous ammonia, are evaluated in all patients. All patients are informed consent about the risks and benefits before deciding to participate in the study.

At enrollment, one EDTA-anticoagulated whole blood (10 ml) and one clotted blood (10 mL) were collected, centrifuged, aliquoted into cryotubes, and stored at –80 °C. Serum LBP, soluble CD14, IL-6, TNF-α, venous ammonia level, and plasma 16s ribosomal bactDNA level were further evaluated. The study protocol and patient consent form were approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB 0171/65). All participants or their legitimate representatives provided written informed consent to participate in the study. The study protocol adhered to the ethical principles of the Helsinki Declaration and followed the Good Clinical Practice recommendations. The study protocol was approved at the Thai Clinical Trial Registry (TCTR20220528002).

Bacterial DNA quantification by real-time PCR

Bacterial DNA was extracted from 400 µL of plasma under complete aseptic conditions using the MagDEA® Dx reagents and the automated sample preparation system (Precision System Science Co., Ltd., Chiba, Japan). A real-time polymerase chain reaction (PCR) condition for the broad-range detection of the 16s ribosomal RNA gene of bacteria was adapted from Jordan *et al.* study (165). Briefly, primers directed against the V7-V9 variable region of the 16S gene (forward: RW01; 5'-AACTGGAGGAAGGTGGGGAT-3', reverse: DG74.R; 5'-AGGAGGTGATCCAACCGCA-3') were mixed with a custom fluorescent probe (5'-6-FAM- TACAAGGCCCGGGAACGTATTCACCG-BHQ-3'; Integrated DNA Technologies Pte. Ltd., Singapore) at final concentrations of

0.3 mM and 0.05 mM, respectively. This was combined with 5 µL of the 2X Maxima Probe no ROX qPCR Master Mix (Thermo Fisher Scientific Baltics UAB, Lithuania), 1 µL of Uracil-DNA Glycosylase (Thermo Fisher Scientific Baltics UAB, Lithuania), 1 µL of 10X buffer Uracil-DNA Glycosylase (Thermo Fisher Scientific Baltics UAB, Lithuania), 1.3 µL of PCR-grade water and 1 µL of DNA template, to give a final reaction volume of 10 µL. A 50-cycle PCR was run in a Light Cycler® 480 System (Roche Diagnostics, Switzerland) using the following conditions; one cycle at 50 °C for 2 minutes (min) and 95 °C for 10 min, 50 cycles at 95 °C for 15 seconds and 60 °C for 1 min.

Microbial DNA from *E.coli* (MBD0013, Sigma-Aldrich) was used with a serial 10-fold dilution (0.003 pg/mL to 3,000 pg/mL) and negative control to obtain a standard curve. The standards and samples were tested in duplicate, and the mean was calculated. Standard curves were generated, and the 16s bactDNA concentrations were determined in the Prism version 9.0 software (GraphPad, La Jolla, CA). The 16s bactDNA levels were identified as pg/ml of collected whole blood.

Measurement of blood ammonia level

To determine blood ammonia levels, venous blood sample was collected into heparinized Vacutainer tubes following standard operating procedures. The sample was then placed immediately on ice and transferred to the laboratory, where it was processed and analyzed within 30 minutes. The enzymatic assay evaluated Venous ammonia level using the glutamate dehydrogenase reaction with reagents obtained from Roche Diagnostics (Indianapolis, Indiana) according to the manufacturer's protocol on a Roche Diagnostics Hitachi 917 analyzer.

Measurement of inflammatory cytokines and endotoxin markers

Bacterial translocation markers, including LBP (ab279407) and soluble CD14 (ab2089836) and the studied cytokines, including IL-6 (ab178013) and TNF-**α** (ab181421), were determined in stored serum samples using the Human SimpleStep

enzyme-linked immunosorbent assay® (ELISA®) kit (Abcam, UK). The calculated minimal detectable dose (MDD) of serum LBP, sCD14, IL-6, and TNF-α were 6.79 pg/ml, 4 pg/ml, 1.6 pg/ml, and 4.32 pg/ml, respectively. All studied cytokines, and endotoxin markers were tested according to the manufacturer's instructions.

Statistical analysis

Categorical variables were expressed as numbers and percent, while continuous variables were expressed as median and interquartile range. Continuous variables were analyzed for normality using the Shapiro-Wilk test. Normally distributed data were expressed as mean (standard deviation) and compared groups using the independent t-test, whereas non-normally distributed data were expressed as median (interquartile range) and compared groups using the Mann-Whitney U test. Pearson's chi-square or Fisher's exact test was used for categorical variables. Spearman's correlation coefficient was used to investigate the correlation among PHES score, plasma 16s ribosomal bactDNA level, inflammatory cytokines, and venous ammonia level. Statistical analysis was performed using SPSS software (version 22, IBM Corporation, Armonk, NY, USA), and 2-sided p values < 0.05 were considered statistically significant.

One-way analysis of variance (ANOVA) was used to determine if there were any statistically significant differences between the means of three independent groups to avoid the inflation of the p-value. However, when the analysis revealed a p-value < 0.05, it could not imply that the three groups differed from each other. It only provided information that the mean of the three groups might differ and at least one group might show a difference. It did not provide details on which group differed from which other groups. Post-hoc analysis was required to determine which group differed from which other groups. Post-hoc analysis was performed using the Turkey test, which was corrected for p-value inflation. Before performing, one-way ANOVA analysis, test of

assumptions were required before one-way ANOVA analysis, including normally distributed for each category of the independent variable, homogeneity of variances and no significant outliers. The Shapiro-Wilk test was used to determine normality, and the Levene's test was used to determine variance homogeneity.

Sample size calculation

The following formula is used for the sample size calculation.

$$N = \frac{2x\,(Z_{\alpha/2} + Z_{\beta})^2}{d^2} \times \frac{\pi}{3}$$

Where $Z_{\alpha/2}$ is the critical value of Normal distribution at $\alpha/2$.

A confidence level of 95%, α is 0.05 and the critical value is 1.96

Z_{β} is the critical value of the Normal distribution at β. For a power of 80%, β is 0.2 and the critical value is 0.84

$\pi/3$ is the asymptotic relative efficiency adjusting for non-parametric test.

$\pi/3$ = 3.1416/3 (Lehman EL.) (166)

d is the difference you would like to detect. Estimated medium effect size = 0.5

$$\text{n/group} = \frac{2\,(1.96+ 0.84)^2}{(0.5)^2} \times \frac{3.1416}{3} = 66$$

Ethical considerations

The following Belmont Report Ethical Principles and Guideline for the Protection of Human Subjects of Research are put into place for the research period:

1. Respect for persons: respecting the autonomy of all people, treating them with decency and respect, and providing for informed consent

2. Beneficence: the Philosophy of "Do no harm" while maximizing benefits for the study effort and reducing harm to research participants

3. Justice: ensuring that acceptable, non-exploitative, and well-thought-out processes are administered fairly and equally — the equitable distribution of expenses and rewards to potential study participants.

CHAPTER IV

RESULTS

Phase 1: Developmental phase

Optimization of the protocol for quantification of 16s ribosomal bactDNA from a human blood sample by real-time PCR technique

Because we would be concentrating our efforts on detecting the presence of bactDNA in patients with bacterial translocation who may have a tiny amount of bacteria in their blood. Therefore, in the first part of our project, we aimed to optimize and maximize the protocol for measuring 16s ribosomal bactDNA to detect the lowest possible concentration of bactDNA from a human blood sample using real-time PCR technique. The type and amount of the patient's blood specimen, primer, and probe sequence were investigated. Figure 9 summarizes the experimental design to optimize the 16s ribosomal bactDNA level protocol.

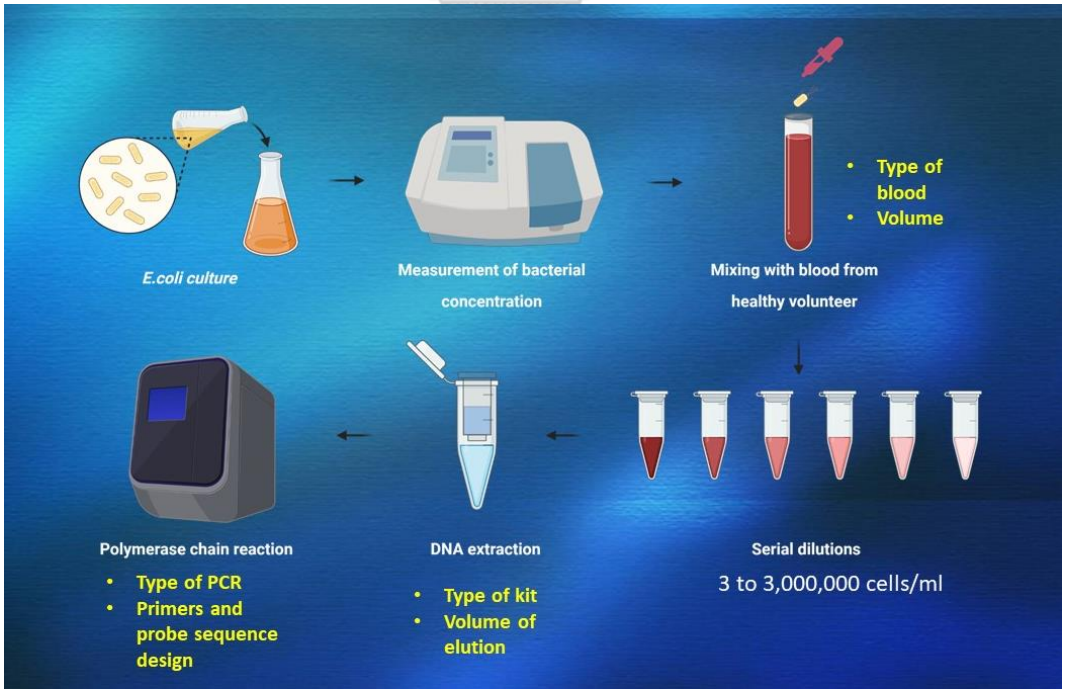

Figure 9 The method for optimization of the 16s ribosomal bactDNA assay

Type of blood sample

BactDNA can be assessed in all types of blood components, including whole blood, plasma and serum. However, in theory, whole blood contains a large amount of human DNA that could interfere with primers and probes binding during PCR. Furthermore, whole blood is prone to inhibit PCR reaction by several inhibitors such as iron, immunoglobulins, and heparin, resulting in a decreased detection capacity of the PCR assay. Serum is the liquid that persists after the blood has clotted. In contrast, plasma is the liquid that remains when clotting is inhibited with the addition of anticoagulants. Hence, the serum should contain less bactDNA than plasma because the spinning process will eliminate bacteria that adhere to blood cells. Therefore, we designed the first set of an experiment to assess the capabilities of bactDNA level measurements in three different types of human blood, including whole blood, plasma, and serum.

Escherichia coli (ATCC 11775) cells were cultured in the 5 ml lysogeny broth (LB) for 12 hours at 37 °c with agitation. The concentration of *E. coli* in this suspension was measured by a spectrophotometer. The optical density measurements (OD) at a wavelength of 600 nm (OD600) is a widely accepted method for estimating the number of bacterial cells in a liquid suspension (167). The amount of lysate of *E. coli* was calculated to get the final concentration of 1,000 and 100,000 cells/ml when mixing with 5 ml of human blood. The concentration of 1,000 cells/ml and 100,000 cells/ml were selected because they were previously considered to represent the minimum and maximum concentration measured by PCR assay during bloodstream infection (168). Then, the bacterial lysates were spiked into 5-ml EDTA-anticoagulated and 5-ml clot blood samples from a healthy volunteer donor to mimic the patient's sample. Whole blood, serum, and plasma containing *E. coli* and sterile water as negative control were prepared. Subsequently, DNA extraction and quantification of 16s

ribosomal bactDNA levels by the real-time PCR method were performed using 200 uL from each sample. Figure 10 concluded the method of this experiment.

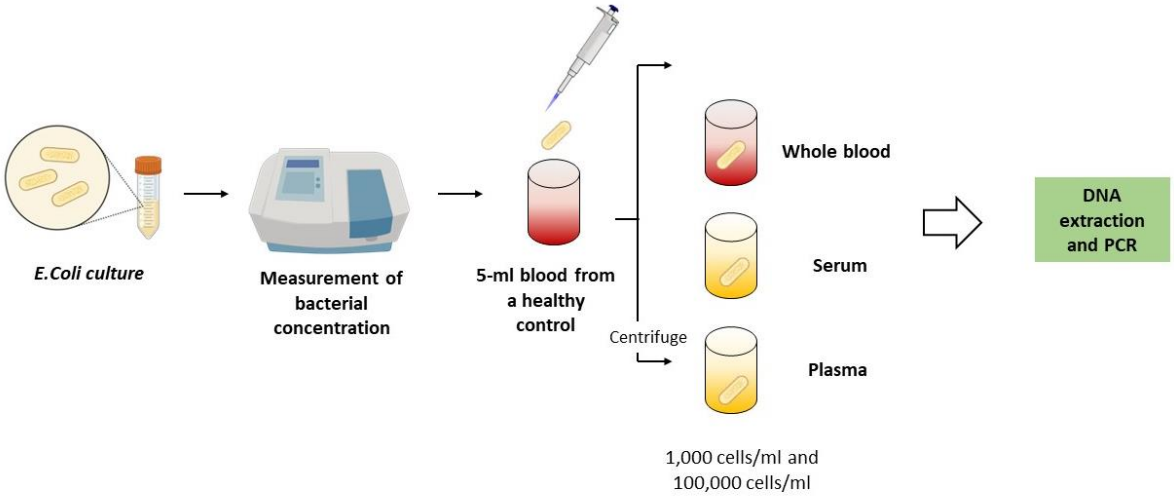

Figure 10 Experimental design to optimize the protocol for quantification of 16s bactDNA based on types of blood

Table 8 shows the real-time PCR Ct values and bactDNA concentrations in whole blood, serum, and plasma containing *E.coli* with concentrations of 1,000 and 100,000 cells/ml. The Ct values and bactDNA level were comparable between plasma and whole blood in both concentrations. While the ability to quantify bactDNA in both plasma and whole blood concentrations. However, the capacity to detect bactDNA in serum was less than that of plasma and whole blood, as evidenced by lower Ct values and bactDNA levels in both concentrations. In addition, to avoid the previously mentioned limitation of using whole blood. We concluded that patient plasma should be used to measure 16s ribosomal bactDNA concentrations.

Table 8 Real-time PCR Ct values and bactDNA concentrations in whole blood, serum, and plasma containing E. coli

	Serum		Plasma		Whole blood	
	Ct	**bactDNA (pg/ml)**	**Ct**	**bactDNA (pg/ml)**	**Ct**	**bactDNA (pg/ml)**
E.coli 100,000 cells/ml	26.02	2.23	16.74	145.0	16.70	143.0
E.coli 1,000 cells/ml	29.06	0.33	19.74	27.15	19.78	26.25
Negative control	Negative	Negative	Negative	Negative	Negative	Negative

Amount of patient plasma

In the next set of the experiment, we intended to optimize the amount of plasma to maximize the detection sensitivity. The experiment was designed with the following steps. The lysate of *E. coli* culture was spiked in 800 uL of blood from a healthy control at concentrations of 3, 30, 300, 3,000, 30,000, 300,000, and 3,000,000 cells/ml. Then, the plasma from each concentration was divided into 200 µL and 400 µL. DNA extraction was performed. The plasma from each concentration was then separated into 200 µL and 400 µL portions. All samples underwent DNA extraction and real-time PCR. Figure 11 summarized the experimental method.

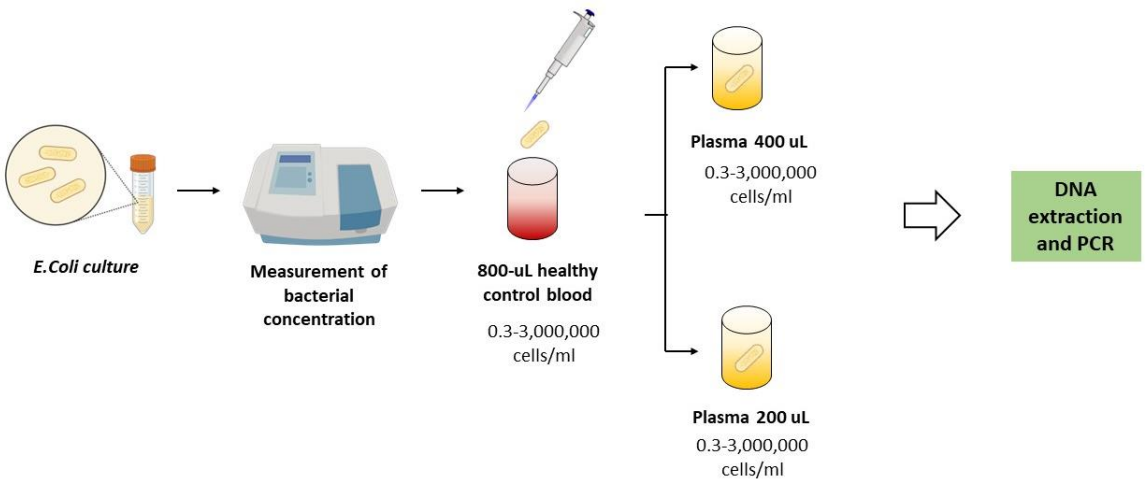

Figure 11 Experimental design to optimize the protocol for quantification of 16s bactDNA based on plasma volume.

Detecting bactDNA with 400 µL of plasma was more sensitive than with 200 µL. Using the 200 µL and 400 µL of plasma, the lowest detection limit of bactDNA concentration was 30 cells/ml and 0.3 cells/ml, respectively. The PCR Ct value from different *E.coli* concentrations isolated from 200 µL and 400 µL of plasma was shown in Table 9. Therefore, 400 µL of patient plasma was the optimal volume of measurement of 16s ribosomal bactDNA assay.

Table 9 The PCR Ct value from different E. coli concentrations isolated from 200 and 400 uL of plasma

Bacterial concentration (cells/ml)	PCR Ct value from 200-uL plasma	PCR Ct value from 400-uL plasma
3,000,000	18.53	17.40
300,000	21.67	19.71
30,000	25.72	24.03
3,000	29.26	26.27
300	32.88	30.53
30	38.95	34.35

3	-	37.02
0.3	-	38.90

Probe and PCR primers of PCR assay

In the next set of experiments, we aimed to determine the ability of broad-range real-time PCR-based assays employing different broad-range primer pairs and probes targeting the 16s ribosomal RNA genes. Three primer pairs and probes are shown in Table 10. Microbial DNA from *E. coli* (MBD0013, Sigma-Aldrich) was used with a serial 10-fold dilution (0.003 pg/mL to 3,000 pg/mL) and negative control to obtain a standard curve utilizing three different primers and probes at the same time. The amount and concentration of the primers and probes as well as PCR conditions, were similar among three conditions.

Table 10 Oligonucleotide primers and probe sequence used for real-time PCR assays.

Set of experiments	Primer pairs	Probe
A (169)	Forward: 5'-AGTTTGATCMTGGCTCAG-3' Reverse: 5'-GGACTACHAGGGTATCTAAT-3'	5'-FAM-CGTATTACCGCGGCTGCTGGCAC-BHQ1-3'
B (170)	Forward: 5'-AGTTTGATCMTGGCTCAG-3' Reverse: 5'-GWATTACCGCGGCKGCTG-3'	5'-FAM-GCTGCCTCCCGTAGGAGT-BHQ1-3'
C (165)	Forward: 5’-AACTGGAGGAAGGTGGGGAT-3’ Reverse: 5’-AGGAGGTGATCCAACCGCA-3’	5’-6-FAM-TACAAGGCCCGGGAACGTATTCACCG-BHQ-3’

The results of real-time PCR graph of E. coli concentrations from 3 experiments were shown in Figure 12 (A, B, C). PCR assay experiment A and B exhibited abnormal amplification plots (Figure 12A and 12B). There was no amplification achieved from these conditions. The possible explanations might be inhibitor presence, non-optimized buffer composition, non-optimized thermal cycling conditions, degraded template material, or poor probe quality. While Figure 12C illustrates a typical real-time PCR amplification plot generated from serial dilutions of a target *E. coli* DNA using probes and primer of experiment C indicating the good yield of this PCR protocol.

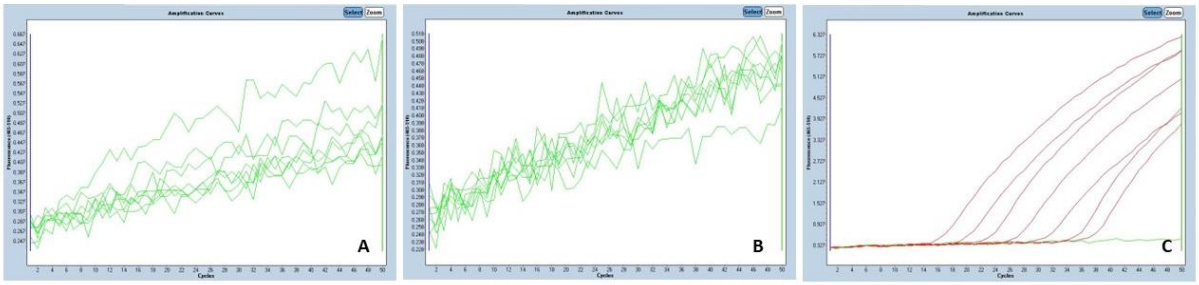

Figure 12 The real-time PCR graph of E. coli concentrations from three conditions

Final protocol for plasma 16S ribosomal RNA from human plasma sample

The final real-time PCR protocol optimized from all experiments from the first part of our study was summarized.

DNA extraction

BactDNA was extracted from 400-µl of plasma under complete aseptic conditions using the MagDEA® Dx reagents and the fully automated nucleic acid systems (Precision System Science Co., Ltd., Chiba, Japan). DNA was eluted in 50 µl of buffer following a 5-min incubation. Extracts were stored at -20°C before analysis.

Real-time PCR

Real-time PCRs were carried out using the LightCycler480 System (Roche) in optical 96-well plates. Table 11 showed the standard master mix reagents.

Table 11 Real-time PCR reagents

Reagent	**Per well (µl)**
2X Maxima Prob qPCR master mix, no ROX (Thermo Scientific) [ward medic]	5.0
10µM forward primer*	0.3
10µM reverse primer**	0.3
5µM TaqMan probe***	0.1
1U/µl Uracil-DNA Glycosylase (Thermo Scientific) [ward medic]	1.0
10x buffer Uracil-DNA Glycosylase (Thermo Scientific) [ward medic]	1.0
Distilled water	1.3
DNA template #	1.0
Total	10

The PCR probe, forward and reverse primers of 16S ribosomal RNA gene were summarized in Table 12.

Table 12 Nucleic sequences of PCR probe and primer

Primer or probe name	Primer sense	Sequence
Primers		

RW01*	Forward	5'-AGTTTGATCMTGGCTCAG-3'
DG74.R**	Reverse	5'-GWATTACCGCGGCKGCTG-3'
Probe* **		
-	-	5'-FAM-GCTGCCTCCCGTAGGAGT BHQ1-3'

A 50-cycle PCR was run in a Light Cycler® 480 System (Roche Diagnostics, Switzerland) using the following cycling conditions (Table 13).

Table 13 PCR cycling conditions.

Step	Temperature °C	Time	Number of cycles
UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	50
Annealing/ Extension	60	1 min	

A serial 10-fold dilution of microbial DNA (0.003–3,000 pg/mL) from *E. coli* (MBD0013, Sigma-Aldrich) and negative control were used to generate a standard curve. The standards and samples were tested in duplicate, and the mean was calculated. Standard curves were generated, and the 16s bactDNA concentrations were determined using the Prism software, Version 9.0 (GraphPad, La Jolla, CA). The 16s bactDNA levels were expressed as pg/mL of collected whole blood. Figure 12c showed the PCR amplification plots and standard curve of bactDNA concentration, respectively. The lowest limit of detection of the assay was 0.003 pg/ml.

The feasibility of using the 16s ribosomal bactDNA level to detect various bacterial strains from positive blood culture samples.

To further investigate the plasma 16s ribosomal bactDNA assay as a generic platform that can comprehensively detect the presence of different bacterial organisms. Twenty positive blood cultures were obtained from patients with various clinical symptoms and signs of infection. The suspension from the positive hemoculture bottles were proceeded to quantify bactDNA according to our protocol.

The majority of the patients (90%, n=18) presented with acute fever. The most common diagnosis was urinary tract infection (25%, n=5), primary bacteremia (20%, n=4), and pneumonia/respiratory tract infection (20%, n=5). Gram-negative bacteria were found in half of the hemoculture samples. *Staphylococcus species* (40%, n=8) and *Escherichia coli* (25%, n=5) were the most commonly identified organisms using the hemoculture approach. BactDNA can be detected in all of the samples by utilizing the universal probe of the 16s ribosomal bactDNA assay, suggesting the feasibility of this assay in the broad-range detection of pathogenic bacteria. The mean of bactDNA level from all enrolled positive hemoculture samples was 144,501.1 ± 292,515.7 pg/ml. BactDNA concentrations ranged from 0.5 to 947,500 pg/ml. Clinical presentation, diagnosis, identifiable bacteria, and bactDNA level of each patient were shown in Table 14.

Table 14 Patient's presenting symptoms, diagnosis, identified bacteria and bactDNA level.

Patient number	Presentation	Diagnosis	Bacterial identification from hemoculture	Gram pos/neg	BactDNA level (pg/ml)
1	Fever with drowsiness 1 day	Septicemia	*Escherichia coli*	Neg rod	1,762.5
2	Fever with dyspnea 3 days	Severe sepsis, respiratory failure	*Serratia marcuscens*	Neg rod	13,375
3	Fever after right thoracotomy and explore lap with revised colonic conduit	Catheter-related bloodstream infection	*Acinetobacter Baumannii*	Neg rod	0.5
4	Fever with drowsiness 1 day	Acute pyelonephritis	*Escherichia coli*	Neg rod	393,750
5	Fever with dyspnea 3 days	Tracheobronchitis	*Klebsiella pneumoniae* (CRE)	Neg rod	947,500
6	Dyspnea and hematuria 1 day	Parafinoma with urinary tract infection	*Salmonella group D*	Neg rod	626,250
7	Abdominal pain 1 day	Acute cholecystitis	*Escherichia coli*	Neg rod	825,000

8	Fever during hemodialysis for 2 hours	Infected renal cyst and septicemia	*Escherichia coli*	Neg rod	3,300
9	Fever with drowsiness for 1 day	Urinary tract infection	*Escherichia coli*	Neg rod	54,125
10	Fever with diarrhea for 2 days	Septicemia	*Escherichia coli*	Neg rod	287.5
11	Fever for 1 day	Urinary tract infection	*Staphylococcus warneri*	Pos cocci	642.5
12	Fever dyspnea and chest pain for 1 day	IE with severe AR and respiratory failure	*Staphylococcus lugdunensis*	Pos cocci	3,962
13	Fever 2 days	Cholangitis	*Streptococcus gallolyticus subsp pasteurianus*	Pos cocci	12,500
14	Fever with dyspnea for 2 days	Pneumonia	*Staphylococcus aureus*	Pos cocci	468.8
15	Fever with dyspnea for 2 days	Croup	*Staphylococcus warneri*	Pos cocci	563.8

16	Fever with dyspnea for 2 days	Pneumonia	*Staphylococcus aureus*	Pos cocci	2,812.5
17	Fever with left cheek swelling for 2 days	Parotic abscess	*Staphylococcus aureus*	Pos cocci	2,625
18	Fever with cough for 3 days	Pneumonia	*Staphylococcus capitis*	Pos cocci	966.3
19	Fever during admission	Complicated urinary tract infection	*Coagulase negative Staphylococcus*	Pos cocci	112.7
20	Fever with right foot swelling for 1 day	Infected diabetic foot	*Streptococcus agalactiae*	Pos cocci	18

AR; aortic regurgitation, IE; infective endocarditis, Neg; negative, Pos; positive

Psychometric hepatic encephalopathy score for the diagnosis of minimal hepatic encephalopathy in Thai cirrhotic patients

PHES standardization in healthy controls

To obtain a normative database of PHES in Thais, 194 healthy volunteers were included in this study. The control group consisted of 126 women (64.9%) with a mean age of 47.1 ± 15.6 years (range 18-79 years). The mean formal education was 13.8 ± 4.1 years (3-18 years). The distribution of healthy volunteers classified by age group was summarized in Table 15.

Table 15 Distribution of healthy volunteers according to age group

	Gender (male/female)	Education (years)
18-30 years (n=31)	14 (45.2%)/ 17 (54.8%)	15.5 ± 2.3
30-40 years (n=40)	12 (30%)/ 28 (70%)	14.4 ± 3.2
40-50 years (n=30)	12 (40%)/ 18 (60%)	14.4 ± 3.9
50-60 years (n=47)	16 (34%)/ 31 (66%)	13.6 ± 4.3
60-79 years (n=46)	14 (30.4%)/ 32 (69.6%)	11.9 ± 5.1

The results of NCT-A, NCT-B, SDT, LTT, and DST were 37.2 ± 8.4 seconds (s), 89.2 ± 14.2 s, 73.9 ± 13.3 s, 110.9 ± 12.5, and 45.9 ± 10.9 points, respectively. All five tests had a strong correlation with age and education years. There was no relationship between gender and overall test performance. Pearson's correlation between PHES test results and studied variables was shown in Table 16.

For the determination of the Thai norms for the PHES, the Kolmogorov-Smirnov test of normality revealed the normal distribution only of the DST. Other tests that did not fit into a normal distribution were converted using logarithm (log) for NCT-A, NCT-B, SDT, and log-log for LTTsum. After transformation, these tests achieved the normal distribution. These tests attained normal distribution after transformation. Multiple linear regression models were used to create the prediction equation for each subtest depending on age and education level (Table 17). The normal values were calculated as the values of age-dependent mean and of deviations of -1, +1, +2, +3 SDs for NCT-A, NCT-B, SDT, and LTTsum or +1, -1, -2, -3 SDs for DST from the mean value.

In the healthy participants, the mean PHES score was – 0.26 ± 2.28 (-8 to +10) points. The normal range of PHES was determined at > -5 points. The pathological cutoff was calculated to be mean - 2SD. As a result, MHE was identified when the score was ≤ -5 points. The PHES score was significantly correlated with age (r =-0.62,

p<0.001) and education (r = 0.82, p<0.001), but not with gender (r = 0.12, p = 0.09). Furthermore, there was no difference in scores between men and women (p = 0.11).

Table 16 Correlation between the results of PHES and studied factors in healthy subjects.

	Age	Education years	Gender
NCT-A	r = 0.32, p < 0.001	r = - 0.44, p < 0.001	r = - 0.12, p = 0.11
NCT-B	r = 0.38, p < 0.001	r = - 0.33, p < 0.001	r = 0.04, p = 0.55
SDT	r = 0.56, p < 0.001	r = - 0.38, p < 0.001	r = 0.01, p = 0.99
LTT	r = 0.24, p < 0.001	r = - 0.25, p < 0.001	r = 0.03, p = 0.69
DST	r = -0.32, p < 0.001	r = 0.56, p < 0.001	r = -0.04, p = 0.55

NCT-A, Number connection test-A; NCT-B, number connection test-B; SDT, serial dotting test; LTT, line tracing test; DST, digital symbol test; PHES, psychometric hepatic encephalopathy score

Table 17 Predictive equations of psychometric hepatic encephalopathy score of each sub-test

Test	Equation	SD
Log (NCT-A)	1.524 + (0.004 * age) – (0.014 * education year)	0.15
Log (NCT-B)	1.952 + (0.002 * age) – (0.011 * education year)	0.17
Log (SDT)	1.926 + (0.002 * age) – (0.014 * education year)	0.15
Log-log (LTT-sum)	0.310 + (0.00032 * age) – (0.001 * education year)	0.03
DST	49.561 – (0.492 * age) + (1.412 * education year)	9.7

NCT-A, Number connection test-A; NCT-B, number connection test-B; SDT, serial dotting test; SD, standard deviation; LTT, line tracing test; DST, digital symbol test

In the healthy controls, the mean PHES score was – 0.26 ± 2.28 (-8 to +10) points. The normative range of PHES was established at > -5 points. The pathological

cutoff was defined to be mean - 2SD. As a result, MHE was identified when the score was ≤ -5 points. The PHES score was significantly correlated with age (r =-0.62, p<0.001) and education (r = 0.82, p<0.001), but not with gender (r = 0.12, p = 0.09). Moreover, there was no difference in scores between men and women (p = 0.11).

Phase 2 Clinical Study

The difference of 16s ribosomal bacterial DNA and hepatic encephalopathy in patients with cirrhosis

In the second part of the clinical study, we aimed to investigate the correlation between plasma 16s ribosomal bactDNA and the levels of serum inflammatory cytokines, venous ammonia, and PHES score in cirrhotic patients with and without HE.

Patient baseline characteristics

Of the 305 patients with liver cirrhosis, 294 patients were consecutively enrolled. Eleven patients were excluded because they met one or more exclusion criteria. The flowchart of study enrolment is shown in Figure 13. The patients consisted of 121 women (42.2%) with a mean age (standard deviation: SD) of 58.8 (13.6) years. The most common etiology of liver cirrhosis was chronic Hepatitis B virus (n=87, 29.6%), followed by alcohol-related liver disease (n=79, 26.9%). The mean Child-Pugh and MELD scores were 7.2 ± 2.6 and 13.8 ± 7.7, respectively. A total of 150 patients (51.0%) were diagnosed with HE (22 MHE, 70 grade 1 HE, 42 grade 2 HE, 13 grade 3 HE, and 3 grade 4 HE). Covert and overt HE was detected in 92 (31.3%) and 58 (19.7%) patients, respectively. Baseline characteristics and laboratory parameters classified by the presence of HE and severity of HE were shown in Table 18 and Table 19, respectively. Patients with HE were older and had higher levels of white blood cell count, INR, total bilirubin, aspartate aminotransferase, creatinine, Child-Pugh score and MELD score than those without HE. Whereas hemoglobin, platelet counts, albumin and serum sodium were lower in patients with HE than those without HE. All the patient characteristics

were not different between patients without HE and with covert HE except for Child-Pugh score and MELD score, which were significantly higher in those with covert HE. While patients with overt HE had significantly higher levels of WBC, neutrophil-lymphocyte ratio (NLR), Child-Pugh and MELD scores than those with covert HE.

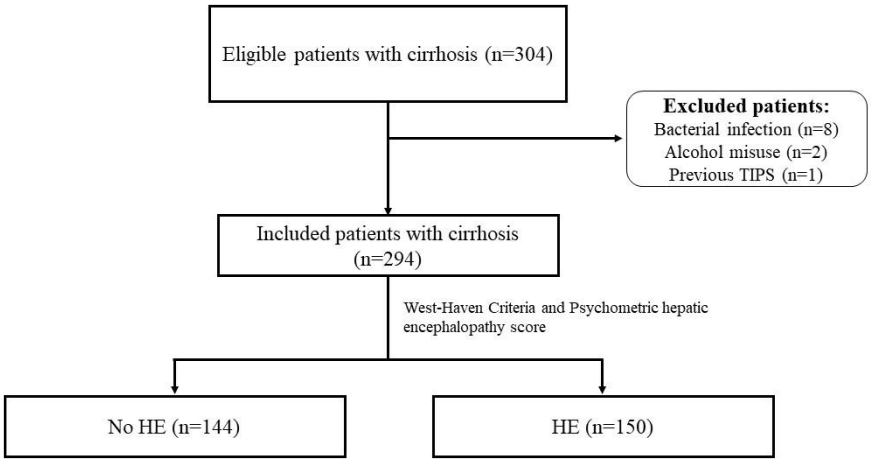

Figure 13 Flow chart of patient enrollment

Table 18 Patient's characteristics and laboratory parameters

	All patients (n=294)	No HE (n=144)	HE (n=150)	P-value
Age, years	58.8 ± 13.6	55.8 ± 12.6	61.4 ± 14.0	0.001
Male, n (%)	173 (58.8%)	80 (55.6%)	93 (62.0%)	0.26
Hb, g/dL	11.4 ± 2.8	12.1 ± 2.3	10.6 ± 3.0	<0.001
WBC	7,085.9 ± 5876.6	6,850.1 ± 6,559.1	7,381.8 ± 5,188.5	0.13
Neu/Lym ratio	4.5 ± 6.3	3.2 ± 3.7	5.8 ± 7.9	<0.001
Platelet, ($x10^9$/litre)	151.7 ± 93.3	175.7 ± 101.3	130.5 ± 80.1	<0.001
TB, mg/dL	4.2 ± 7.9	1.5 ± 1.6	6.6 ± 10.3	<0.001
AST, U/L	80.6 ± 181.4	61.9 ± 195.8	99.2 ± 166.9	<0.001
ALT, U/L	52.8 ± 130.4	52.6 ± 150.5	53.6 ± 109.8	0.62
Albumin, g/dL	3.4 ± 0.8	3.7 ± 0.7	3.0 ± 0.7	<0.001
INR	1.47 ± 0.74	1.28 ± 0.35	1.6 ± 0.9	<0.001
Creatinine, mg/dL	1.09 ± 1.01	0.90 ± 0.47	1.3± 1.3	0.02
Sodium, mEq/L	136.4 ± 4.9	137.7 ± 3.2	135.3 ± 5.7	0.001
Child-Pugh Class				
- A	164 (55.8%)	112 (77.8%)	52 (34.7%)	<0.001
- B	68 (23.1%)	26 (18.1%)	42 (28.0%)	0.04
- C	62 (21.1%)	6 (4.2%)	56 (37.3%)	<0.001
Child-Pugh score	7.2 ± 2.6	6.0 ± 1.6	8.4 ± 2.9	<0.001
MELD	13.8 ± 7.7	10.8 ± 4.1	16.6 ± 9.0	<0.001

AST; aspartate aminotransferase, ALT; alanine aminotransferase, Hb; hemoglobin, Neu/Lym ratio; neutrophil-lymphocyte ratio, TB; complete bilirubin, WBC; white blood cell count.

Table 19 Baseline patient characteristics classified according to the severity of hepatic encephalopathy.

	No HE (n=144)	Covert HE (n=92)	Overt HE (n=58)	p-value
Age, years	55.8 ± 12.6	61.6 ± 13.2	61.2 ± 13.6	0.004
Male, n (%)	80 (55.6%)	56 (60.9%)	37 (63.8%)	0.50
Hb, g/dL	12.2 ± 2.3	11.3 ± 2.7	9.5 ± 3.0	<0.001
WBC, (cells/μL)	6,850.1 ± 6,559.1	5,885.0 ± 2,521.2	9,859.4 ± 7,188.2**##	<0.001
Neu/Lym ratio	3.2 ± 3.7	4.0 ± 7.1	8.6 ± 8.4 @@	<0.001
Platelet, ($x10^9$/liter)	175.7 ± 101.3	129.3 ± 68.9	132.4 ± 95.7	<0.001
TB, mg/dL	1.5 ± 1.6	3.8 ± 6.9	11.0 ± 12.9	<0.001
AST, U/L	61.9 ± 195.8	62.8 ± 80.4	156.3 ± 238.0	<0.001
ALT, U/L	52.6 ± 150.5	37.8 ± 42.4	78.2 ± 165.7	0.20
Albumin, g/dL	3.7 ± 0.7	3.3 ± 0.7	2.7 ± 0.7	<0.001
INR	1.28 ± 0.35	1.41 ± 0.66	1.95 ± 1.20	<0.001
Creatinine, mg/dL	0.90 ± 0.47	1.26 ± 1.54	1.22 ± 0.83	0.06
Sodium, mEq/L	137.7 ± 3.2	136.7 ± 4.8	133.4 ± 6.2	<0.001
Child-Pugh Class - A - B - C	 111 (77.1%) 26 (18.1%) 7 (4.9%)	 51 (55.4%) 26 (28.3%) 15 (16.3%)	 2 (3.4%) 16 (27.6%) 40 (69.0%)	<0.001
Child-Pugh score	6.0 ± 1.6	7.1 ± 2.3@@	10.6 ± 2.2**##	<0.001
MELD	10.8 ± 4.1	13.5 ± 7.4@	21.5 ± 9.1**##	<0.001

*p<0.05 compared with overt HE and no HE, **p<0.001 compared with overt HE and no HE, @ p<0.05 compared with covert HE and no HE, @@ p<0.001 compared with covert HE and no HE, # p< 0.05 compared with overt HE and covert HE, ## p< 0.001 compared with overt HE and covert HE

AST; aspartate aminotransferase, ALT; alanine aminotransferase, Hb; hemoglobin, Neu/Lym ratio; neutrophil-lymphocyte ratio, TB; complete bilirubin, WBC; white blood cell count.

Plasma 16s ribosomal bactDNA, LBP, IL-6, TNF-α, and venous ammonia levels between cirrhotic patients with and without HE

Overall, bactDNA translocation was detected in 106 (36.1%) patients with cirrhosis. The prevalence of bactDNA translocation was found in 31.3% (n=45), 35.9% (n=33), and 48.3% (n=28) in cirrhosis CTP class A, B, and C patients, respectively. Patients with HE tended to have more bactDNA translocation (40.7% vs. 31.3%, p=0.09)(Figure 14), and bactDNA level (622.4 ± 5,216.2 vs. 76.9 ± 522.9, pg/μL, p=0.13) than those without HE although the difference was not statistically significant. Similar to other bacterial translocation markers, serum LBP (12,543.1 ± 8,680.7 vs 10,788.5 ± 7.399.4 ng/ml, p = 0.07) and sCD14 (3,113 ± 1,993.5 vs 2,761.1 ± 1,429.6 ng/ml, p=0.16) tended to be higher in patients with HE than those without HE. In contrast, levels of serum TNF-α (12.4 ± 18.5 vs 7.7 ± 8.9 pg/ml, p=0.01), IL-6 (66.8 ± 215.7 vs 10.9 ± 25.6 pg/ml, p<0.001) and ammonia (80.8 ± 39.5 vs 62.2 ± 26.8 μg/dL, p<0.001) were substantially higher in HE patients than non-HE patients. Figure 15 showed the level of these tests in cirrhotic patients based on the presence of HE.

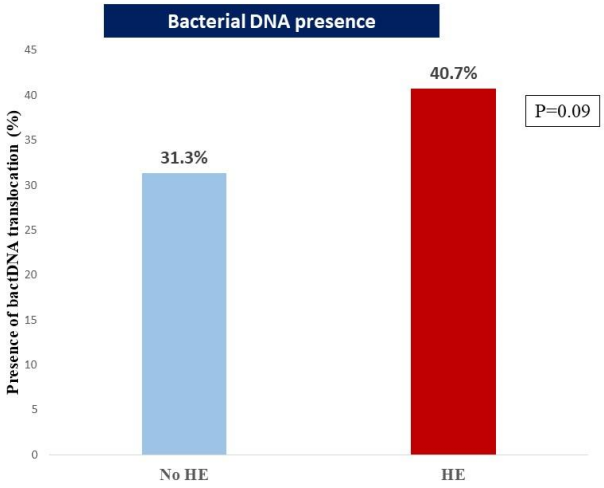

Figure 14 Presence of bacterial DNA in patients with hepatic encephalopathy

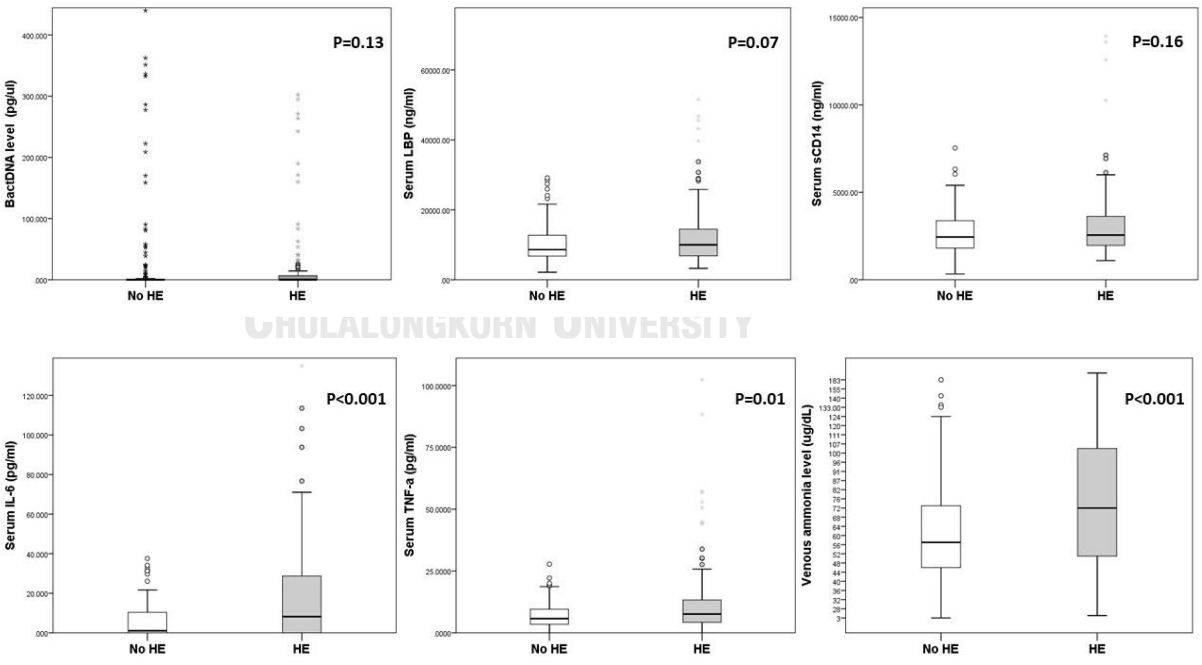

Figure 15 Levels of bacterial DNA and translocation markers, inflammatory cytokines and venous ammonia

The difference in plasma 16s ribosomal bactDNA, LBP, IL-6, TNF-α and venous ammonia levels between cirrhotic patients based on the severity of HE

To explore the relationship between BT, systemic inflammation, ammonia level, and HE severity, the studied biomarkers were analyzed in patients without HE, covert HE, and overt HE. BactDNA levels (381.7 ± 1,572.2 vs. 76.9 ± 522.9 pg/uL, p<0.001) and serum LBP (13,480.0 ± 9,469.3 vs. 10,788.5 ± 7,399.4 ng/ml, p=0.01) were significantly higher in patients with overt HE compared to those without HE indicating that BT was greater in patients with overt HE. Furthermore, patients with overt HE exhibited higher serum TNF-α (12.7 ± 15.5 vs. 7.7 ± 8.9, p=0.04) and IL-6 levels (105.9 ± 302.2 vs. 10.9 ± 25.6, p<0.001) than those without HE, showing a higher pro-inflammatory state. In contrast, patients with covert HE tended to have higher levels of bactDNA, serum LBP, sCD14, and IL-6 than those without HE, but this did not reach statistical significance. Furthermore, bactDNA, LBP, and studied cytokines were comparable between patients with overt and covert HE. Ammonia level was associated with the severity of HE; patients with overt HE had higher ammonia levels compared to those with covert HE (92.7 ± 41.3 vs. 73.0 ± 36.4 ug/dL, p<0.001) or no HE (92.7 ± 41.3 vs. 62.2 ± 26.8 ug/dL, p<0.001). Table 20 showed the levels of bactDNA and the studied inflammatory biomarkers based on the degree of HE.

Table 20 Bacterial translocation and inflammatory biomarkers based on the severity of hepatic encephalopathy.

	All patients (n=294)	No HE (n=144)	Covert HE (n=92)	Overt HE (n=58)	p-value
BactDNA presence	106 (36.1%)	45 (31.3%)	33 (35.9%)	28 (48.3%)*	0.07
BactDNA level, pg/uL	355.2 ± 3747.6	76.9 ± 522.9	774.1 ± 6,553.0	381.7 ± 1572.2*	0.06
TNF-α, pg/ml	10.1 ± 14.7	7.7 ± 8.9	12.2 ± 15.6@	12.7 ± 15.5*	0.04
IL-6, pg/ml	39.2 ± 156.9	10.9 ± 25.6	41.6 ± 129.4@	105.9 ± 302.2**	<0.001
sCD14, ng/ml	2,941.1 ± 1,746.3	2,761.1 ± 1,429.6	3,086.8 ± 2,195.9	3,156.8 ± 1,639.3	0.22
LBP, ng/ml	11677.8 ± 8108.2	10,788.5 ± 7,399.4	11,939.3 ± 8,129.8	13,480.0 ± 9,469.3*	0.05
Ammonia, ug/dL	71.6 ± 35.0	62.2 ± 26.8	73.0 ± 36.4@	92.7 ± 41.3 #, **	<0.001

*p<0.05 compared with overt HE and no HE, **p<0.001 compared with overt HE and no HE, @ p<0.05 compared with covert HE and no HE, @@ p<0.001 compared with covert HE and no HE, # p< 0.05 compared with overt HE and covert HE, ## p< 0.001 compared with overt HE and covert HE.

Patients with bactDNA translocation exhibited significantly greater white blood cell count, serum IL-6, and LBP than patients without bactDNA translocation. There were no differences in Child-Pugh, MELD score, TNF, sCD14, or ammonia between both groups of cirrhotic patients (Table 21).

Table 21 Baseline characteristics and inflammatory mediators in patients with and without bacterial DNA translocation

	Absence of bactDNA translocation (n=188)	Presence of bactDNA translocation (n=106)	p-value
Age, years	58.0 ± 13.7	59.9 ± 13.4	0.07
Male, n (%)	115 (61.2%)	58 (54.7%)	0.28
Hb, g/dL	11.4 ± 2.9	11.3 ± 2.7	0.31
WBC	6,611.4 ± 4,695.7	8,070.4 ± 7,578.5	0.04
Neu/Lym ratio	4.4 ± 5.4	4.7 ± 7.8	0.73
Platelet	150.7 ± 91.6	155.3 ± 97.3	0.55
TB, mg/dL	3.7 ± 7.3	4.9 ± 8.8	0.82
AST, U/L	66.4 ± 98.6	106.8 ± 272.9	0.34
ALT, U/L	40.7 ± 50.6	75.2 ± 206.7	0.67
Albumin, g/dL	3.4 ± 0.8	3.3 ± 0.8	0.14
INR	1.4 ± 0.8	1.5 ± 0.7	0.55
Creatinine, mg/dL	1.1 ± 0.9	1.1 ± 1.2	0.82
Sodium, mEq/L	136.5 ± 4.5	136.1 ± 5.6	0.06
Child-Pugh Class - A - B - C	 103 (54.8%) 50 (26.6%) 35 (18.6%)	 61 (57.5%) 18 (17%) 27 (25.5%)	 0.08 0.67 0.09
Child-Pugh score	7.2 ± 2.5	7.3 ± 2.8	0.08
MELD	13.5 ± 7.6	14.5 ± 7.8	0.94

TNF-α, pg/ml	9.3 ± 10.6	11.5 ± 20.0	0.82
IL-6, pg/ml	20.4 ± 57.3	71.0 ± 243.6	0.009
sCD14, ng/ml	2,859.1 ± 1,579.1	3,086.5 ± 2,008.9	0.63
LBP, ng/ml	10,609.6 ± 6,814.6	13,580.4 ± 9,756.7	0.003
Ammonia, ug/dL	71.4 ± 35.2	71.9 ± 34.8	0.92

AST; aspartate aminotransferase, ALT; alanine aminotransferase, BactDNA; 16s ribosomal bacterial deoxyribonucleic acid, Hb; hemoglobin, IL-6; interleukin-6, LBP; lipopolysaccharide-binding protein, Neu/lym ratio; neutrophil to lymphocyte ratio, sCD14; soluble CD14, TB; total bilirubin, TNF-α; tumor necrosis factor-alpha, WBC; white blood cell count

Correlation between plasma 16s ribosomal bactDNA, serum LBP, IL-6, TNF-α, and venous ammonia level in cirrhotic patients.

The correlation between plasma 16s ribosomal bactDNA levels, inflammatory cytokines and ammonia was investigated. Among the bacterial translocation biomarkers, plasma bactDNA levels showed a weakly positive correlation with serum LBP (r=0.25, p<0.001) and sCD14 (r=0.13, p=0.03), WBC (r=0.14, p=0.02) and ALT level (r=0.14, p=0.02). Moreover, levels of plasma bactDNA exhibited a weak positive correlation with venous ammonia (r=0.13, p=0.03), serum TNF-α (r=0.17, p=0.004) and IL-6 (r=0.32, p<0.001). On the other hand, bactDNA levels had no significant correlation with total bilirubin, albumin, INR, Child-Pugh, or MELD score.

Correlation between plasma 16s ribosomal bactDNA, serum LBP, IL-6, TNF-α, venous ammonia level and PHES in cirrhotic patients with covert HE.

In 236 patients without overt HE, the correlation between PHES, the studied biomarkers and disease severity was evaluated (Table 22). The PHES showed a weak negative correlation with total bilirubin (r = -0.24, p=0.003), INR (r = -0.24, p=0.004),

Child-Pugh score (r = -0.26, p = 0.001), MELD score (r = -0.27, p=0.001) and a weak positive correlation with serum albumin (r = 0.29, p<0.001). In contrast, there was no significant correlation between bactDNA, serum LBP, sCD14, TNF-α, IL-6, ammonia level and the PHES score (p>0.05).

Table 22 Correlation between the PHES score and markers of bacterial translocation, inflammation and disease severity in cirrhotic patients without overt HE

	Correlation coefficient (r)(Pearson)	p-value
White cell counts ($x10^9$/L)	0.07	0.42
Total bilirubin (mg/dL)	-0.24	0.003
AST (U/L)	-0.09	0.24
ALT (U/L)	0.04	0.60
Albumin (mg/dL)	0.29	< 0.001
INR	-0.24	0.004
Child-Pugh score	-0.26	0.001
MELD score	-0.27	0.001
BactDNA level (pg/μL)	-0.02	0.79
TNF-α, pg/ml	-0.12	0.15
IL-6, pg/ml	-0.14	0.09
sCD14, ng/ml	0.05	0.53
LBP, ng/ml	0.06	0.46
Ammonia, ug/dL	-0.10	0.20

CHAPTER V

DISCUSSION

The current study optimized the protocol for quantification of 16s ribosomal bactDNA from human blood samples by real-time polymerase chain reaction technique. Furthermore, we determined the normative data of the PHES in the healthy Thai population. In addition, our study investigated the association between bactDNA translocation, serum LBP, IL-6, TNF-α, sCD14, ammonia levels, and HE severity. The main findings of the current study revealed that bactDNA translocation was more prevalent in advanced liver disease, with prevalence rates of 31.3%, 35.9%, and 48.3% in cirrhotic patients with CTP classes A, B, and C, respectively. In addition, patients with overt HE had more bactDNA translocation and higher serum LBP, TNF-α, IL-6, and ammonia levels than those without HE, indicating a relationship between BT, systemic inflammatory state, and overt HE. In cirrhotic patients, the levels of bactDNA had a positive correlation with WBC, TNF-α, IL-6, and ammonia. Furthermore, the optimal cutoff of PHES for diagnosing MHE is ≤−5 points in Thai cirrhotic patients.

Over the last decade, several studies have shown the efficacy of real-time PCR for the diagnosis of sepsis. Several protocols for quantifying total bacterial 16s ribosomal DNA in plasma (165, 170) have been published, mostly validated in patients with sepsis. There were many differences in these assay protocols, such as the type and amount of human sample, the nucleic sequence of primer and probe in the real-time PCR method. The benefit of real-time PCR is that it provides for quantitative assessments, allowing for determining the bactDNA load related to the severity of infection. Measurement of 16s ribosomal DNA of bacteria in plasma using quantitative real-time PCR is one of the promising methods for the diagnosis of BT. Therefore, we developed and optimized the protocol for quantification of 16s ribosomal bactDNA in the first part of our research. The optimized protocol aimed to maximize the lowest

detection limit for detecting 16s ribosomal bactDNA from non-infectious human blood. We demonstrated that the lowest limit of detection of 16S ribosomal bactDNA in our protocol was 0.003 pg/ml. In addition, the assay is able to detect broad-range gram-positive and gram-negative pathogenic bacteria from human sample specimens. During bloodstream infection, the bactDNA load was 10^3 – 10^4 genome copies per ml or approximately 5-50 x 10^6 pg/ml (168). Therefore, the detection range of 16s ribosomal bactDNA from our protocol should be sufficient to apply for patients with BT who had lower bactDNA load in their blood.

To maximize the lowest detection limit for the 16s ribosomal bactDNA, our protocol suggests extracting DNA from 400 µL of patient plasma. The extraction of the 16s ribosomal bactDNA from human serum yields the smallest amount of DNA because the centrifugation process removes bacteria that bind to blood cells. Although our study found no difference in the yield of 16s ribosomal bactDNA concentration between whole blood and plasma. We prefer plasma because whole blood contains substantial human DNA that could disturb PCR primers and probes. In addition, whole blood had various PCR inhibitors, including iron, immunoglobulins, and heparin, which reduced the detection capacity of the PCR assay. Keeping whole blood in the refrigerator for a long time may cause extravascular hemolysis and the development of circulating non-transferring-bound iron, which may interfere with the PCR assay (171, 172). The disadvantages of real-time PCR of bactDNA measurement include contamination and may result in false-positive results. Contamination can occur due to DNA from the environment or PCR reagents, despite using sterile techniques or nucleic-free substances. Using the negative control was used for quality controls.

Recently, there has been growing evidence that BT and subsequent systemic inflammation are novel drivers leading to the development or progression of complications in patients with cirrhosis (173). Moreover, systemic inflammation

collaborates synergistically with traditional mechanisms involved in developing cirrhotic complications. Systemic transmission of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), released by the diseased liver, interact with innate immune cells and release pro-inflammatory molecules. This cascade of events leads to acute decompensation and organ dysfunctions (174). The findings of our study supported this theory. We found that non-bacterial infected cirrhotic patients with bactDNA translocation had greater WBC, IL-6 and LBP levels than those who did not have bactDNA translocation, indicating a more inflammatory state. Systemic inflammation may impair organ function through three possible mechanisms. First, nitric oxide-mediated enhancement of the underlying splanchnic vasodilation, leading to overactivation of the endogenous vasoconstrictor systems resulting in organ hypoperfusion and dysfunction, particularly renal function impairment. Second, systemic inflammation can cause tissue injury through the immune system, a process called immunopathology. Third, systemic inflammation may induce significant metabolic derangements, including the reallocation of nutrients to stimulate immune activation, reducing the consumption of nutrients in peripheral organs (174). Moreover, a simultaneous rise in plasma soluble proinflammatory cytokines, immunosuppressive compounds, and immune-incompetent myeloid cells, indicating immunoparesis, enhances the effect on organ dysfunction. Patients with acute decompensation, defined as acute development of ascites, HE, gastrointestinal bleeding, bacterial infection, or any combination of these complications, had higher plasma inflammatory markers than those with compensated cirrhosis. Systemic inflammation was associated with the number of decompensations upon admission (58, 175).

Regarding BT, systemic inflammation and complication of cirrhosis, the presence of bacterial infection independently predicts the risk of rebleeding and death

in patients with variceal bleeding (176-178). Additionally, prophylactic use of antibiotics reduces the incidence of bacterial infection, the risk of rebleeding and overall mortality (179, 180). A recent single-centre case-control study showed that variceal bleeders had significantly lower levels of anti-endotoxin immunoglobulin M antibody (IgM anti-endotoxin), nitric oxide and transforming growth factor-beta (TGF-β) but higher levels of fatty acid binding protein 2 (FABP2), a gut barrier integrity marker, and IL-6 levels. In addition, Child-Pugh score, IgM anti-endotoxin and TGF-β were independent predictors of variceal bleeding, demonstrating that BT and gut barrier disruption was directly associated with variceal bleeding risk (181). BactDNA translocation was associated with higher levels of ascitic fluid and serum pro-inflammatory cytokines in cirrhotic patients without bacterial infection (37). The presence of bactDNA in patients with refractory ascites was related to cardiovascular and renal dysfunction, a greater risk of hepatorenal syndrome and death (182). BactDNA was detected in patients with spontaneous bacterial peritonitis (SBP) more frequently than those with non-infected ascites (100% vs 34%). BactDNA translocation and cytokine responses were suppressed with norfloxacin treatment in patients with SBP (37). There is conflicting evidence on whether intestinal decontamination by antibiotics improves patients with cirrhosis. Kimer N. *et al.* study reported that treatment with rifaximin did not improve systemic hemodynamics or lower HVPG in cirrhotic patients with ascites (183). In contrast, a previous randomized controlled trial study showed that norfloxacin improved mean arterial pressure, reduced cardiac output, systemic vascular resistance and hepatic venous pressure gradient (HVPG), resulting in a reversal of the hyperdynamic circulatory state (184). For patients waiting for LT, rifaximin was associated with a decrease in hospitalization related to SBP, ascites and variceal bleeding (185).

In terms of HE, systemic inflammation increases blood-brain barrier permeability and neuroinflammation of the central nervous system (186, 187). In

addition, systemic inflammation modulates the cerebral effect of ammonia in cirrhosis (30). Previous studies on the influence of BT on HE yielded inconclusive results. An Indian cohort study by Jain *et al.* revealed that serum endotoxin, inflammatory mediators, and arterial ammonia levels correlated with HE severity, increased as the HE stage progressed, and were higher in patients with minimal HE than those without HE (163). In contrast, another Danish study found that serum endotoxin, LBP, inflammatory cytokines, and arterial ammonia levels did not differ between patients with and without minimal HE (164). The current study found that cirrhotic patients with overt HE had a significantly higher proportion of detectable bactDNA, higher levels of inflammatory mediators and venous ammonia than those without HE. Although bactDNA translocation was associated with overt HE, the presence of bactDNA, serum LBP and sCD14 levels were comparable in patients with covert HE and those without HE. Furthermore, the PHES did not correlate with BT markers in cirrhotic patients without overt HE. This suggests that the degree of BT and inflammation is important for inducing the obvious clinical manifestation of HE. We hypothesized that bacterial translocation is one of the important factors for developing HE. However, this effect has been modulated by systemic inflammation and ammonia. Systemic inflammation may compromise the blood-brain barrier resulting in neuropsychological response to bacterial component or ammonia. More study is needed to fully understand this finding. These discrepancies among studies could be explained by differences in the severity and aetiology of cirrhosis and the type of BT indicators used. This study provides new insights into the pathogenesis of HE, particularly those related to BT, enabling novel advancements in treatment and care.

To the best of our knowledge, the current study is the first study that provides PHES normative values in healthy Thai subjects. The optimal cutoff of PHES for identifying MHE is ≤ -5 points in Thai cirrhotic patients. The cutoff of PHES for

determining MHE, a cutoff ≤ -4, has been set in various countries, including German (79), Italy(95), China (104), and Turkey (103), and has been suggested as national norms (188). In contrast, studies in Polish (189), Indian (190), and Korean (105) cohorts found that a PHES score ≤ -5 constituted a diagnostic threshold for MHE. According to our findings, the PHES cutoff value among Thais is -5. The differences in details between test versions could explain why the results are inconsistent. First, the scoring systems for the LTT results and the range of total PHES scores were different. The German and Korean versions employed two independent results (LTTerror and LTTtime) with a PHES score range of + 6 to -18. Whereas the Italian, Chinese, Turkish, Polish, and the current versions used the sum (LTTsum) of time spent on the test plus error score with a PHES score range of + 5 to -15. LTTsum was selected for the current study because it is pragmatic, simple to use in the clinic, and has already been validated (107). Second, the distribution and size of numbers and letters differed between versions. NCT-B was replaced with the figure connection test in the Indian study due to the substantial proportion of non-alphabetized patients (191). Furthermore, the German alphabet in the NCT-B has been substituted by the alphabets of each country's original language, such as Korean, Chinese, and Thai alphabets (104, 105). Third, normative data are gathered differently (age and education-adjusted values in Italy, China, Korea, Poland, and our study vs age-adjusted values in Germany and India).

Our study has some limitations. First, there were a small number of patients with overt HE. However, to our knowledge, this is the first study to assess plasma bactDNA in patients with HE. Second, the variability of ammonia testing is high. Arterial ammonia may be preferable to venous ammonia (192, 193). However, venous ammonia was used in this study because it is far more convenient for patients and physicians than arterial ammonia, making it more likely to be used in clinical practice. Furthermore, both venous and arterial ammonia were associated with HE severity (23).

Third, the test of normality and homogeneity of variance have not been done before one-way ANOVA analysis.

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AWARD RECEIVED

1. First Class Honors, Chulalongkorn University, 2005
2. Young Investigator Award, The European Association Study for The Study of The Liver (EASL), International Liver Conference, 2013-2015, 2019, 2023
3. Winner of Research Award from the 9th Annual Meeting of Thai Association For The Study Of The Liver, Rayong, Thailand, 2014
4. Honorary Mention Award, The Annual Meeting of the Gastroenterological Association of Thailand, 2014, Hua Hin, Phetchaburi, Thailand.
5. Second runner-up in Research Award from the 8th Annual Meeting of Thai Association For The Study Of The Liver, Rayong, Thailand, 2013
6. Nominee for Best & Popular Poster of the Day Award, The 29th Annual Meeting of the Royal College of Physicians of Thailand, 2013, Chonburi, Thailand.
7. Nominee for Best & Popular Poster of the Day Award, The 30th Annual Meeting of the Royal College of Physicians of Thailand, 2014, Chonburi, Thailand.
8. Research Highlights. Royal College of Physicians of Thailand 2022.