การพัฒนาวิธี Multiplex PCR สำหรับตรวจวินิจฉัยโรคสครับไทฟัส โรคติดเชื้อริคเค็ทเซีย และโรคเลปโตสไปโรสซิส

นางสาวนุชนาถ แซ่เลี่ยง

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DEVELOPMENT OF MULTIPLEX PCR FOR DIAGNOSIS OF SCRUB TYPHUS, RICKETTSIAL DISEASE AND LEPTOSPIROSIS

Miss Nutchanart Sae Liang

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
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Thesis Title	DEVELOPMENT OF MULTIPLEX PCR FOR DIAGNOSIS OF SCRUB TYPHUS, RICKETTSIAL DISEASE AND LEPTOSPIROSIS
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Field of Study	Medical Science
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นุชนาถ แซ่เลี่ยง : การพัฒนาวิธี Multiplex PCR สำหรับตรวจวินิจฉัยโรคสครับไทฟัส โรคติดเชื้อริคเค็ทเซีย และโรคเลปโตสไปโรสซิส (DEVELOPMENT OF MULTIPLEX PCR FOR DIAGNOSIS OF SCRUB TYPHUS, RICKETTSIAL DISEASE AND LEPTOSPIROSIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: พ.อ.หญิง ดร.จริยาณาฏ เกวี, 58 หน้า.

โรคสครับไทฟัส โรคเลปโตสไปโรซิส และโรคมิวรีนไทฟัส เป็นโรคติดต่อจากสัตว์สู่คนที่อาจเป็นอันตรายต่อชีวิตที่พบกระจายทั่วโลก โดยเกิดจากการติดเชื้อแบคทีเรียโอเรียนเทีย ซูซูกามูชิ เลปโตสไปร่า อินเทอร์โรแกนส์ และริคเค็ทเซีย ไทฟี ผ่านทางพาหะของโรคหรือจากการสัมผัสเชื้อโดยตรง ซึ่งลักษณะทางคลินิกเบื้องต้นของโรคสครับไทฟัส โรคเลปโตสไปโรซิส และโรคมิวรีนไทฟัส จะมีลักษณะอาการคล้ายกับกลุ่มโรคไข้ไม่ทราบสาเหตุ คือ มีไข้สูง ปวดหัว ปวดเมื่อยกล้ามเนื้อ และเบื่ออาหาร ซึ่งทำให้การวินิจฉัยนั้นเกิดความล่าช้าและอาจส่งผลให้ผู้ป่วยมีอาการรุนแรงขึ้นจนถึงขั้นเสียชีวิตได้ ถึงแม้ว่าปัจจุบันวิธีทางภูมิคุ้มกันวิทยาจะเป็นวิธีมาตรฐานที่ใช้ในการวินิจฉัยโรค แต่อย่างไรก็ตามวิธีนี้ก็ยังมีข้อจำกัด คือ ในระยะเริ่มแรกของโรคจะมีแอนติบอดีไม่เพียงพอต่อการวินิจฉัย ดังนั้นวิธีการวินิจฉัยที่เร็วและแม่นยำจึงมีความจำเป็น ในงานวิจัยนี้มีวัตถุประสงค์เพื่อที่จะพัฒนาวิธีมัลติเพล็กซ์พีซีอาร์สำหรับตรวจหาเชื้อก่อโรคสครับไทฟัส โรคเลปโตสไปโรซิส และโรคมิวรีนไทฟัส และประเมินประสิทธิภาพวิธีมัลติเพล็กซ์พีซีอาร์เทียบกับวิธีทางภูมิคุ้มกันวิทยา โดยทำการหาสภาวะที่เหมาะสมของวิธีมัลติเพล็กซ์พีซีอาร์สำหรับตรวจหาเชื้อก่อโรคสครับไทฟัส โรคเลปโตสไปโรซิส และโรคมิวรีนไทฟัส และทดสอบความจำเพาะ ปริมาณเชื้อที่สามารถตรวจพบเชื้อ และประสิทธิภาพของวิธีมัลติเพล็กซ์พีซีอาร์ ผลการทดลองพบว่าวิธีมัลติเพล็กซ์พีซีอาร์ที่พัฒนาขึ้น สามารถตรวจหาเชื้อก่อโรคทั้งสามเชื้อได้อย่างจำเพาะโดยไม่เกิดปฏิกิริยาข้ามกับเชื้อก่อโรคชนิดอื่น สำหรับประสิทธิภาพของวิธีมัลติเพล็กซ์พีซีอาร์พบว่า ความไวและความจำเพาะของวิธีมัลติเพล็กซ์พีซีอาร์เท่ากับ 100% และ 71.67% เทียบกับวิธีทางภูมิคุ้มกันวิทยา และวิธีมัลติเพล็กซ์พีซีอาร์สามารถตรวจพบโรคติดเชื้อร่วมกันระหว่างโรคสครับไทฟัสและโรคเลปโตสไปโรซิสอีกด้วย ดังนั้น วิธีมัลติเพล็กซ์พีซีอาร์ที่พัฒนาขึ้น เป็นวิธีที่มีความไวและความจำเพาะ อีกทั้งยังสามารถตรวจหาเชื้อก่อโรคได้ตั้งแต่ในระยะเริ่มแรกของโรค

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS:

NUTCHANART SAE LIANG: DEVELOPMENT OF MULTIPLEX PCR FOR DIAGNOSIS OF SCRUB TYPHUS, RICKETTSIAL DISEASE AND LEPTOSPIROSIS. ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D, CO-ADVISOR: COL.JARIYANART GAYWEE, Ph.D., 58 pp.

Scrub typhus, leptospirosis, and murine typhus are life-threatening zoonosis worldwide. The causative agent of these diseases is *Orientia tsutsugamushi*, *Leptospira interrogans*, and *Rickettsia typhi*, respectively. Transmission of these bacteria occurs through vector biting or directly bacteria contraction. Clinical features of these diseases are as acute undifferentiated fever such as high fever, headache, muscular pain, and anorexia, resulting to delay diagnosis and mortality. Although a serological laboratory test is gold standard for scrub typhus, leptospirosis, and murine typhus diagnosis, it has own limitation because inadequate antibody in the early phase. In this study, we aimed to develop the multiplex PCR on the causative agents of scrub typhus, leptospirosis, and murine typhus detection and evaluate the performance of multiplex PCR compared to the serological tests. In the experiment, we optimized the appropriate multiplex PCR for detection of tested bacteria. Subsequently, we evaluated the specificity, sensitivity, and performance of the developed multiplex PCR. Our results showed only positive detectable PCR product from tested bacteria. The sensitivity and specificity of the developed multiplex PCR was 100% and 71.67% compared to the serological tests. And also, the assay found the co-infection of scrub typhus and leptospirosis. The designed multiplex PCR assay is sensitive, specific and rapid. The assay can be used for identifying the causative agent in the early phase of these diseases.

Field of Study: Medical Science
Academic Year: 2015

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

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CHAPTER I

INTRODUCTION

Scrub typhus, leptospirosis, and murine typhus are the worldwide life-threatening, and neglected infectious diseases. These diseases are caused by *Orientia tsutsugamushi*, *Leptospira interrogans,* and *Rickettsia typhi*, respectively. Orientiae and rickettsiae are obligate intracellular bacteria; they are transmitted to human through the biting of chigger or trombiculid mite as *O. tsutsugamushi*'s vector, and an inoculation of rat flea's feces on human skin as *R. typhi*'s vector. Leptospires are gram-negative, spirochete bacteria; they are transmitted through either direct urine of infected animals or contaminated water contract (1-6). These diseases distribute in the tropical and temperature climates, especially rural, urban, and suburban areas. They have been reported that the increase of morbidity and mortality rate each year (7-9). This challenge reflects that awareness and experience of physicians and diagnostic tools for testing have been limited.

The early clinical symptoms of scrub typhus, leptospirosis, and murine typhus are as acute undifferentiated fever including headache, muscular pain, and anorexia. The clinical symptoms of these diseases range from mild, severe and to fatal (10-13). Though the first clinical appearance of scrub typhus is an eschar, which is a lesion caused by chigger biting, it is not always presents. And also, lesion-like eschar can occur in other diseases such as rickettsialpox, and anthrax (14, 15). Moreover, the presence of macular rash on the trunk, which is general clinical symptom in murine typhus, it can also form in scrub typhus (10, 16). The specific clinical symptoms of leptospirosis are biphasic fever and multi-organ failure (Weil's disease) in the severe case; resulting in lethal (17). Because clinical symptoms of these diseases are recognized as acute undifferentiated fever resulting in underdiagnosed, a rapid and

precisely laboratory diagnostic tool is necessary for certainly confirmation and efficiently treatment.

Recently, laboratory investigations for these diseases diagnosis primarily depend on antigen and antibody detection. Although antibody detection (indirect immunofluorescence assay or microscopic agglutination test) is a gold standard diagnostic tool for these diseases, it may result in false negative results in the early phase of diseases. Antigen detection is clinically desired as direct microbial organism identification. The important of antigen detection of scrub typhus, leptospirosis, and murine typhus is that it can identify the microbial in the early phase of diseases because orientiae, leptospires, and rickettsiae can be found in bloodstream in the first week of diseases (18-21). Therefore, the development of a new diagnostic tool, which base on antigen detection, is essential for the rapid and reliable diagnosis, treatment, and awareness of the diseases.

The multiplex PCR is one of molecular laboratory tests, which is a simultaneous amplification with different primer pairs in the same reaction (22). It has been used in several infections identification including bacteria, fungi, parasites, and viruses (23). The multiplex PCR is rapid and time-saving because the multiplex PCR is capable for detecting multiple microbial (22, 24). Here, we developed the multiplex PCR on *O. tsutsugamushi*, *L. interrogans* and *R. typhi* identification and evaluated a performance of the designed multiplex PCR compared to either indirect immunofluorescence assay or microscopic agglutination test. In this study, we selected three target genes including *56-kDa type-specific antigen* gene of *O. tsutsugamushi*, *LipL32* gene of *L. interrogans*, and *17-kDa antigen* of *R. typhi* for detecting each pathogen in both bacterial cell culture and human whole blood samples of acute undifferentiated fever patients.

Research questions of this study

1. Do the different primer pairs distinguish each of bacteria?
2. Does the designed multiplex PCR effectively diagnose scrub typhus, leptospirosis, and murine typhus?

Objectives of this study

1. To develop the multiplex PCR on *O. tsutsugamushi*, *L. interrogans* and *R. typhi* detection.
2. To evaluate a performance of the designed multiplex PCR compared to either indirect immunofluorescence assay or microscopic agglutination test as gold standard for diagnosing these diseases.

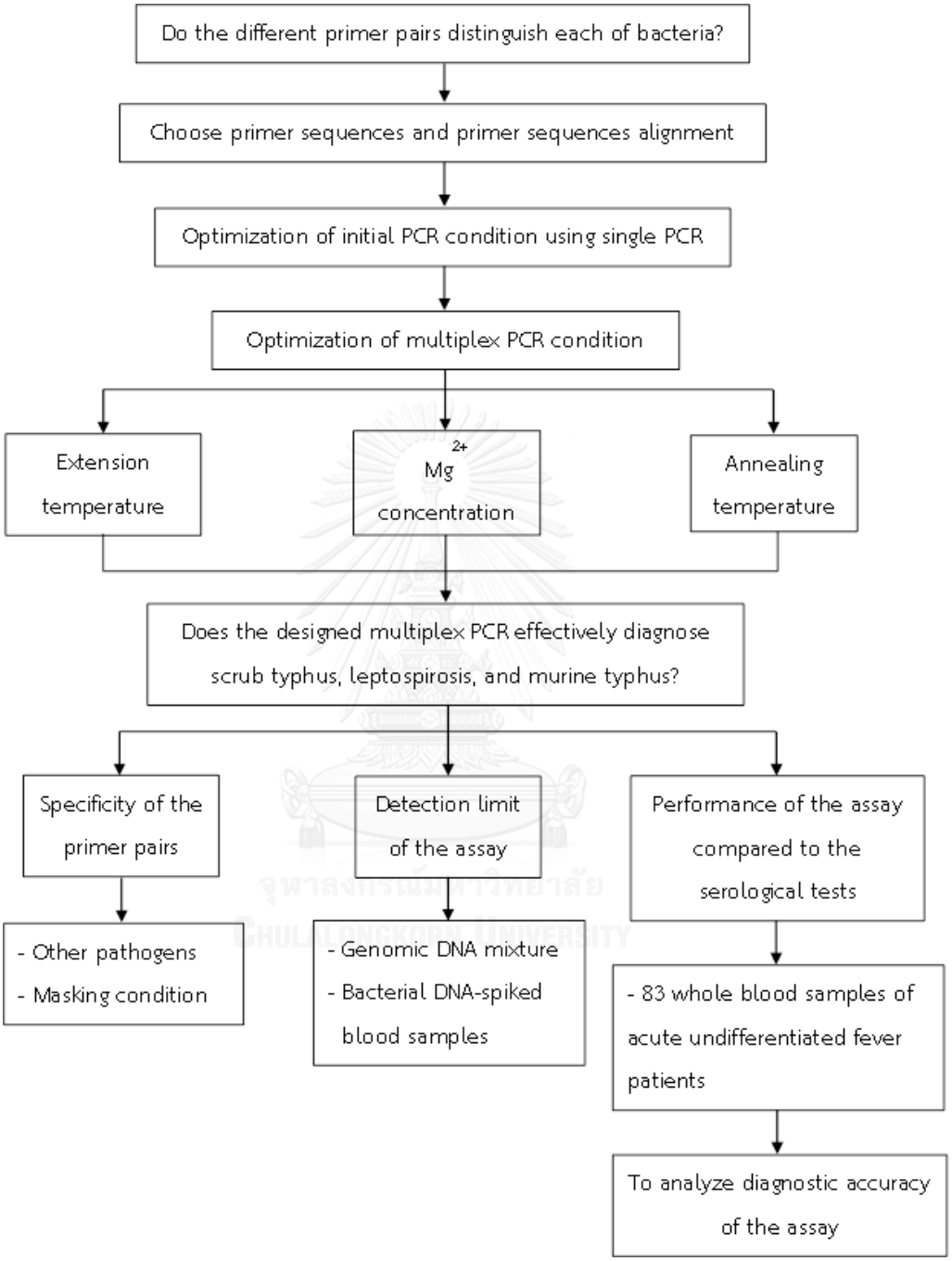
Hypothesis of this study

1. Each primer pair can distinguish *O. tsutsugamushi*, *L. interrogans*, and *R. typhi*.
2. The developed multiplex PCR effectively diagnoses scrub typhus, leptospirosis, and murine typhus from blood specimens of acute undifferentiated fever patients.

Expected outcome of this study

1. To apply the new diagnostic tool on identification of the pathogenic agents causing scrub typhus, leptospirosis, and murine typhus in the same time.
2. To obtain the diagnostic tool that reducing the time necessary to make clinical diagnoses and begin treatment.
3. To apply the knowledge from this study to develop the method for other diseases detection further.

Conceptual framework



Keyword: *Orientia tsutsugamushi*, *Leptospira interrogans*, *Rickettsia typhi*, Multiplex PCR

CHAPTER II

LITERATURE REVIEW

Acute undifferentiated fever

Acute undifferentiated fever (AUF) or acute pyrexia of unknown origin (acute PUO) is a high temperature fever (≥38°C) within 14 days as well as non-specific symptoms including headache, muscular pain, and anorexia (25). Acute undifferentiated fever is widespread in tropical and temperature regions including Thailand. It is a major concern because morbidity rate of AUF in Thailand remains increased that reported by Annual Epidemiology Surveillance Report 2013 (26).

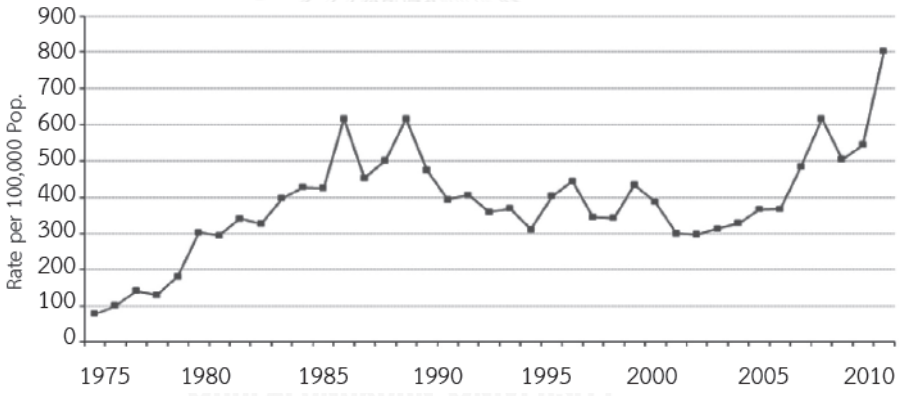


Figure 1 Reported Cases of Pyrexia in Thailand by Year, 1975-2013 was reported by Annual Epidemiology Surveillance Report (modified from (26)).

There are many previous studies reported that emergence of acute undifferentiated fever in Thailand. In 2003, Pradutkanchana and colleagues studied the etiology of acute undifferentiated fever in children after a flood in 2000. Their results showed that dengue (29.4%), leptospirosis (27.2%), and scrub typhus (1.1%) are the common cause of acute undifferentiated fever (27).

In 2004, Leelarsamee and coworkers discovered the etiologies of acute undifferentiated fever in Thailand. The result reported that leptospirosis, scrub typhus and murine typhus were acute undifferentiated fever that often found in Thailand (25).

In 2006, Suttinont and the other investigated a cause of acute undifferentiated fever in rural Thailand. Their result demonstrated that acute undifferentiated fever was identified consisting of leptospirosis (36.9%), scrub typhus (19.9%), dengue or influenza (10.7%) and murine typhus (2.8%) that found in agricultural workers. Their study suggested that leptospirosis and rickettsial infection were the major cause of acute undifferentiated fever in rural Thailand (28).

In 2014, Thipmontee and colleagues determined the trends of leptospirosis between year 2001-2002 and year 2011-2012. Their data reported that scrub typhus (24.1%) and leptospirosis (40%) were common cause of acute undifferentiated fever in 2001-2002. However, scrub typhus (28.3%) was more common cause of acute undifferentiated fever than leptospirosis in 2011-2012 (29).

Scrub typhus, murine typhus and leptospirosis

Scrub typhus is a mite-borne rickettsioes. The pathogenic agent is *O. tsutsugamushi,* which is a gram-negative obligate intracellular bacterium. Serotypes of *O. tsutsugamushi* include Karp, Kato, Gilliam, Boryon, Kawasaki, and Koroki, it is different antigenicity (21). Chigger or trombiculid mite, which is a larva of the genus *Leptotrombidium* mites, is vector of *O. tsutsugamushi*. Orientiae are accidentally transmitted to human through the biting of infected chigger (30). In general, orientiae infect in various cell type including endothelial cells, macrophages, and leukocytes. They invade endothelial cells and multiples by binary fission. Invasion of endothelial cells results in cell membrane disruption and releasing amount of orientiae into bloodstream. And also infected endothelial cells lead to propagate vasculitis in multiple organs, resulting in vessel leakage in multiple organs (30, 31).

Scrub typhus distributes in the tropical and temperature areas. The endemic area of scrub typhus is agricultural area, which is the habitat of mites. Appearance of mites defines seasonality of disease, scrub typhus is found during the end of rainy season and the beginning of winter. The incidence and mortality rate of scrub typhus have increased each year (1, 9).

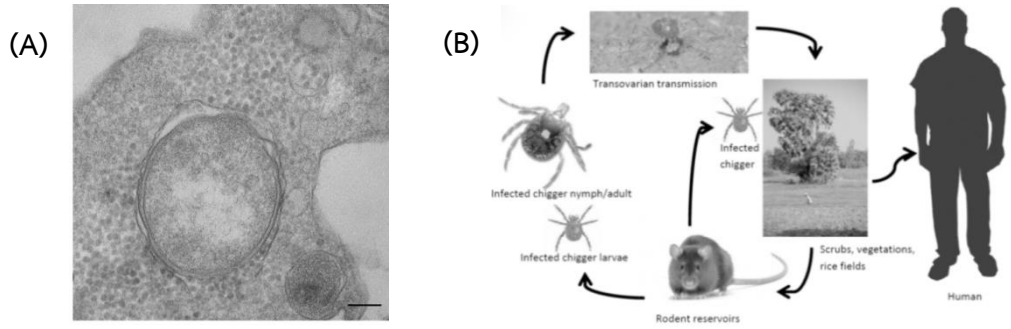


Figure 2 (A) *O. tsutsugamushi* in the cytoplasm of infected cells (modified from (31)) and (B) Life cycle of *O. tsutsugamushi* infection (modified from (19)).

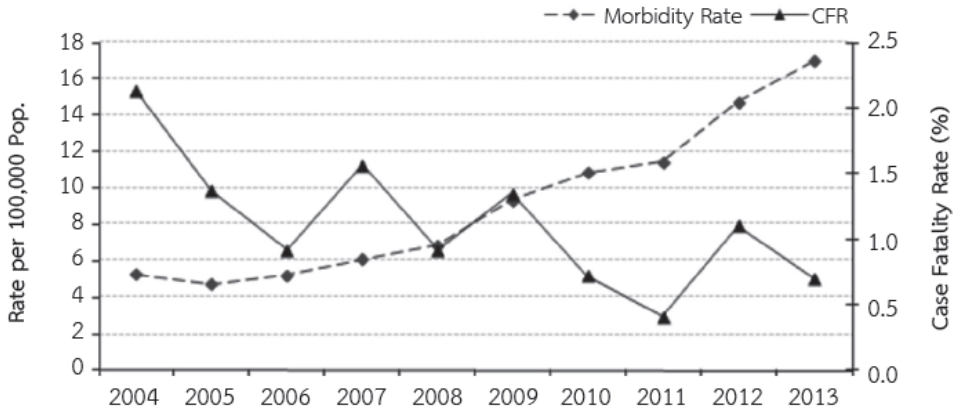


Figure 3 Reported cases of scrub typhus per 100,000 populations and case fatality rate by year Thailand, 2004 – 2013 (26).

Murine typhus is a flea-borne rickettsial disease caused by *R. typhi;* it is the member of typhus group and gram-negative obligate intracellular bacterium (16). Vector of *R. typhi* is rat flea. *R. typhi* grows in rat flea's midgut and is discarded with feces of rat flea. It is transmitted to human through contract rat flea's feces at the bite site. It invades and multiples in infected endothelial cells. Multiplying of rickettsiae leads to damage endothelial cells, releasing of large rickettsiae into blood circulation, and results in macular rash formation (32).

Murine typhus is widespread worldwide, especially tropical and temperature climate. The endemic area is urban and suburban area, which is a high population of rodents and fleas (16). Murine typhus is found in all seasons (9, 33, 34).

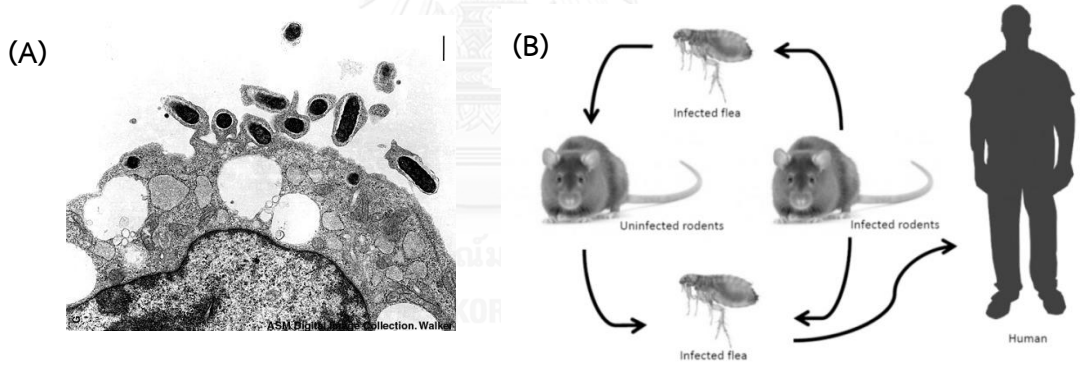


Figure 4 (A) *R. typhi* in the cytoplasm of infected cells (modified from (35)), and (B) Life cycle of *R. typhi* infection (modified from (19)).

Leptospirosis is a global zoonosis caused by *L. interrogans*, which is a pathogenic *Leptospira* genus. Leptospires are thin spirochetes, motile by periplasmic flagella, obligate aerobe with optimal temperature for growing at 28-30°C. Leptospires have double membrane containing cytoplasmic membrane and peptidoglycan cell wall (4, 5). Pathogenic leptospires can be identified based on epitopes of lipopolysaccharide (LPS) antigen included 230 serovars grouped in 23 serogroups (36). Rat is the main carrier of pathogenic Leptospires in human leptospirosis. They live in the renal tubules of carrier, colonize and are excreted with urine in the environment such as water or soil. Transmission of leptospires to human occurs when directly contact urine of the carrier or contaminated water. It penetrates through wound or mucus membrane (37). After leptospires enter the body, it circulates and multiplies in bloodstream and then damages endothelial cells of blood vessels, resulting in ischemia in multi-organ such as necrosis of renal tubules, meningitis, hepatocellular and pulmonary damage (5, 38).

Leptospirosis is a worldwide distribution, particularly rural, town, and suburban, and also depends on population of rodents and ruminants (4, 20). Leptospirosis highly occurs during the rainy season. It has been reported that the trend of morbidity and mortality rate slightly decreased (26).

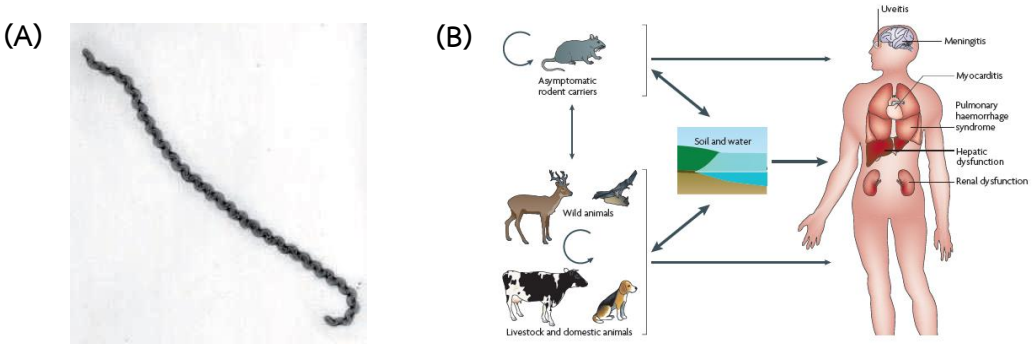


Figure 5 (A) Morphology of *Leptospira interrogans* and (B) life cycle of Leptospirosis infection (modified from (38)).

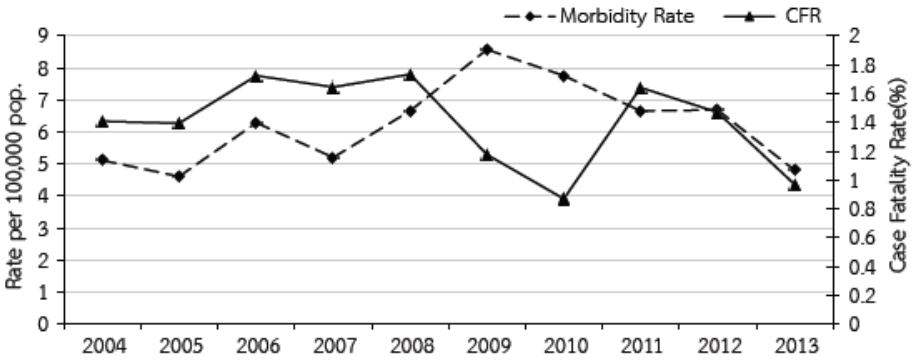


Figure 6 Reported cases of leptospirosis per 100,000 populations and case fatality rate by year Thailand, 2004 – 2013 (26).

Diagnosis of scrub typhus, leptospirosis, and murine typhus

Laboratory investigation is necessary for precise conformation because clinical feature presentation is non-specific. Recently, the laboratory investigations of scrub typhus, leptospirosis, and murine typhus are numerous including

1. Microscopy

Dark-field microscope investigation is used to directly observe leptospires in blood samples and culture. The threshold for leptospires detection is approximately 10^4 cells/ml of blood. The method is not diagnostic tool because a high risk of false positive and false negative from proteins and cell debris in blood (20, 37, 39).

2. Bacterial cell culture

Culture technique is used for isolation of *O. tsutsugamushi* and *R. typhi* because these are obligate intracellular bacteria. Orientiae and rickettsiae can be isolated from blood of patients, cultured in Vero cells, L929 cells, or inoculated into mice. Oriential and rickettsial cells are observed by Giemsa staining. Median time to positive detection is limited because propagation of these bacteria as early as 27 days. The method is inappropriate diagnostic tool, although it is a high specific method. Further, it requires biosafety level-3 to prevent risk of infection (40, 41). For Leptospires, they can be isolated from blood samples and cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and 5-fluorouracil is added to the medium to prevent contamination and observed by dark-field light microscope. The assay is not routine laboratory diagnosis because leptospires has a long doubling time and a high risk of infection (20, 42). Currently, the bacterial cell culture is a confirmation technique for scrub typhus, leptospirosis, and murine typhus.

3. Serological diagnostic tests

Nowadays, a serological test is gold standard for diagnosis of scrub typhus, leptospirosis, and murine typhus, which is antibody titer detection as indicator of acute or current exposure (41, 42). It has own limitation due to an insufficient antibody in the early phase for detection, even though the assay is more sensitive and faster than the bacterial cell culture (39, 43). There are several methods are used to detect pathogens causing scrub typhus, leptospirosis, and murine typhus such as

3.1 Microscopic agglutination test (MAT)

Microscopic agglutination test or MAT assay is a gold standard for leptospirosis diagnosis. The assay is a sensitive and specific because it can discriminate serovars of leptospires. A 4-fold increase antibody titer in paired sera or antibody titer ≥1:400 in single serum determine as positive results. However, the assay has own disadvantages including dark-field microscope and whole live leptospires requirement (37, 42). In addition it is time-consuming and low sensitive in first week of illness (39).

3.2 Indirect immunofluorescence assay (IFA)

Indirect immunofluorescence assay or IFA assay is a gold standard for oriential and rickettsial disease diagnosis. The method is antibodies against *O. tsutsugamushi* or *R. typhi* detection using fluorescent-labeled anti-human antibodies. Detection for positivity determines as ≥1:400 in a single serum or a 4-fold rise in the paired serum samples. Although the method is more sensitive than Weil-Felix test that is an old method, it requires fluorescence microscope and skilled technicians (19, 21, 44).

3.3 Indirect immunoperoxidase (IIP)

Indirect immunoperoxidase or IIP assay is a modified-IFA, which uses peroxidase-labeled anti-human antibody. The assay is a reference laboratory test as

same as IFA. The performance of the assay is easy, the microscope requirement is necessary for the reading (40, 45).

3.4 Weil-Felix test

Weil-Felix test is an agglutination assay, and an oldest method for scrub typhus and murine typhus diagnosis. The test is based on cross reaction of antibodies to *Proteus* sp. strains. Anti-Orientia antibodies cross react to *Proteus* OX-K, while anti-Rickettsia antibodies react to *Proteus* OX-19. Agglutination shows titer ≥1:160 in a single serum or a 4-fold rise in the paired sera. Although the test is low sensitive and specific, it is still uses for diagnosis in the resource-limited areas due to the test is either easy to perform or cheap (40, 41, 46).

3.5 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a detection of genus-specific IgM antibodies against orientiae, leptospires, and rickettsiae in a genus level. The conventional ELISA for three diseases uses the different antigen-coated microplate wells and measures the final concentration of antibodies by a Microplate Reader (39, 40). Though the assay is sensitive, specific, rapid, and easy to perform, its limitations including it uses whole antigen in large quantities, preparation of antigen is time-consuming and technician is risk to infections. Moreover, it needs confirm by other methods and skilled personals for readings (39, 41, 45).

3.6 Other serological tests

Recently, other serological methods were used as antibodies detection such as Rapid lateral flow assay, Passive Haemagglutination test, Indirect Haemagglutination assay, Dot immunoassay, Latex Agglutination and Immunohistochemistry test (20, 39, 45).

4. Molecular diagnostic tests

Molecular diagnostic test is a direct bacterial DNA detection using Polymerase chain reaction (PCR) method. The assay is the claimed to be sensitive and specific diagnostic test because it is used to detect bacterial DNA in blood specimens of patients after presentation of clinical symptoms or early phase of illness (39, 41, 45).

Recently, 56-kDa type-specific antigen (TSA) has been used as the most common target gene for *O. tsutsugamushi* detection. 56-kDa TSA is the most surface antigen proteins of *O. tsutsugamushi*, consisting of approximately 1,600 base pairs. It is only expresses in *Orientia* species and is not expressed in other bacteria. The role of 56-kDa TSA is unclear; it plays a role in the immunogen, adherent factor, and determinant of virulence for each strain (47, 48).

Previously studies have been reported the detection of *O. tsutsugamushi* using 56-kDa TSA amplification. The researches of Manosri and coworkers (2003) and Saisongkorh and colleagues (2004) assessed the nested PCR on detecting 56-kDa TSA of *O. tsutsugamushi* in blood samples compared to other methods. Their results showed that it could detect bacteria in the early phase of infection and after antibiotic administration (49, 50).

In 2006, Kim and others determined buffy coat samples of scrub typhus patients using nested PCR on detection of 56-kDa TSA gene compared to IFA assay. Their results reported nested PCR were positive for buffy coat samples (51). Lee and others performed eschar PCR assay, 56-kDa antigen fragment was amplified by nested PCR in both eschar and buffy coat samples (52).

In 2014, Janardhanan and coworkers used primers targeting 56-kDa TSA gene in nested PCR to amplify scrub typhus patients' eschar samples compared to conventional PCR. This study presented both nested PCR and conventional PCR could detect oriential DNA in eschar samples as same as previously researches (53).

For rickettsial diseases diagnosis, 17-kDa antigen has been used as the target gene in identification of *Rickettsia* spp. 17-kDa antigen is a genus-common surface protein antigen of *Rickettsia* spp. The nucleotide sequences comprise around 530 base pairs; it is conserved among *Rickettsia* species (54). 17-kDa antigen is associated in the survival of *Rickettsia* species. Functions of 17-kDa antigen may play a protective and a scaffolding role in all rickettsiae (55).

There are many previous studies reported about 17-kDa antigen was used as target gene on rickettsiae detection. In 1990, Webb and colleagues detected the pathogens of murine typhus in infected fleas using the primer pair is derived from 17-kDa antigen sequences. Their study reported 17-kDa antigen was amplified in individual infected fleas and was not amplified in control flea (uninfected) (56).

In 1994, Schriefer and others detected *R. typhi* in suspected patients' blood samples using PCR that amplify a gene encoding 17-kDa antigen. Their study reported 17-kDa antigen gene was amplified in blood samples of murine typhus patients using rickettsia specific primers (57).

In 2013, Watthanaworawit and colleagues and Kato and others evaluated real-time PCR for scrub typhus and rickettsial diseases. Their results showed that rickettsia assay targeting 17-kDa antigen of *Rickettsi*a species could amplify 17-kDa antigen gene of *Rickettsia* spp. in all of whole blood, buffy coat and tissue samples (43, 58).

In 2015, Chao and coworkers developed recombinase polymerase amplification method using lateral flow test and real-time fluorescent detection targeting 47-kDa antigen gene of *O. tsutsugamushi* or 17-kDa antigen of *R. typhi.* Their results showed that the method could detect *O. tsutsugamushi* or *R. typhi*. The limit of detection of the recombinase polymerase amplification method for detecting *R. typhi* was 2,000 copies/ml *R. typhi* spiked-bloods (59).

LipL32 lipoprotein has been used for leptospirosis diagnosis. LipL32 lipoprotein is a major outer membrane protein, consists of approximately 820 base pairs. It expresses in pathogenic *Leptospira* species and is not expressed in non-pathogenic *Leptospira* sp. The nucleotide sequences are highly conserved among pathogenic leptospires. The roles of lipL32 are associated in inflammation and virulence (38, 60). In 2007, Cheema and coworkers studied the efficacy of primers based on outer membrane protein (OMP) genes such as *lipL21* and *lipL32* were tested in serum and tissue samples from cattle and infected guinea pig using PCR. Their study showed primers derived from *lipL21* and *lipL32* genes were the same sensitivity as conventionally primers G1/G2 (61).

In 2013, Kucerova and colleagues examined real-time PCR method for lipL32 gene detection in biological materials of suspected leptospirosis patients. Their results showed lipL32 primers could amplify lipL32 gene of pathogenic leptospires in several biological materials (blood, urine, and liqour) (62).

In 2015, Chen and others developed loop-mediated isothermal amplification (LAMP) for leptospira detection using primers targeting lipL32 and lipL41 gene. Their study showed primers targeting lipL32 gene could be used in LAMP assay as same as other PCR techniques (63). The study of Najian and coworkers developed reported a label-based multiplex loop-mediated isothermal amplification (LAMP) with a set of lipL32 primers. Their study reported a label-based multiplex-LAMP lateral flow dipstick detected the pathogenic leptospires (64).

Previously studies suggested that the three target genes could be used for detection of pathogens causing scrub typhus, leptospirosis, and murine typhus.

Multiplex PCR and other infectious diseases

The multiplex PCR, which is one of PCR techniques, is simultaneously amplification with several specific primers in the single tube (22). It is depended on discrimination the different PCR product sizes by gel electrophoresis and visualized by ethidium bromide staining. Although it uses the many specific primers in the reaction, its principle is similar to single PCR assay. The assay used as routine laboratory is high sensitivity and specificity, detectable multiple pathogens, time-saving, and reliable data (24, 65).

Currently, it has been applied in various studies, especially the differentiation and identification of pathogenic pathogens including bacteria, viruses, fungi, and parasite (23). Rapid and appropriate method for pathogens detection is able the proper treatment. It has been reported that identification by using multiplex PCR in the field of infectious diseases.

In 2013, Foroahni and colleagues developed a novel diagnostic test for *Salmonella typhi*, *Bacillus anthracis* and *Yesinia pestis* detection based on singleplex and multiplex PCR. Their study reported that the primers, which used in multiplex PCR, were specific to each of bacteria and also it could detect the different bacteria species in the single tube. Limit of detection was 1-10 copies of the genome or 1-10 colony forming units (CFU) of each of bacteria (66). And Brata and coworkers explained the efficiency of multiplex PCR with a new primer sets for *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia pseudomallei* and *Brucella* sp. detection. Their study showed that the primers used in this study did not cross-react to closely related microorganisms and the lower limit of detection were 50 CFU/25µl in *B. anthracis*, *Y. peritis* and *Brucella* sp. whereas lower limit of detection of *B. pseudomallei* was 150 CFU/25µl (67).

In 2014, Wei and others developed a multiplex PCR method on *Vibrio* sp. detection. Their study presented that the multiplex PCR are detectable *Vibrio* sp., limit of detection was 10 and 10^5 CFU of each *Vibrio* species, and the results of multiplex PCR are comparable with physiological and biochemical methods (68). The research of Arabestani and colleagues developed multiplex PCR method on the common bacteria of bacteremia detection. Their results presented that the sensitivity and specificity of multiplex PCR were 91.58% compared to blood culture as gold standard diagnostic test. And also 6.34% of cases were found to be positive but negative in blood culture (69). In addition, Dhib and colleagues developed the multiplex PCR amplification based on chitin synthase 1 and internal transcribed spacer genes. Their data showed that the sensitivity of multiplex PCR was higher than mycological examinations and it is specific to dermatophytic strains (70).

In 2015, Sánchez and coworkers developed serogroup-specific multiplex PCR method for detecting serogroups of *Escherichia coli* in humans. Their study showed that 21 primers used in three multiplex PCR were specific to the serogroups of *E. coli;* the results suggested that the multiplex PCR are available serogroups of *E. coli* identification (71). And Bier and others developed and validated the multiplex PCR on virulent markers of *Vibrio vulnificus* detection. Their study showed the performance of multiplex PCR is high sensitivity, specificity, and reliable results with four of virulence markers (72).

Table 1 List of representative infectious diseases diagnosis using multiplex PCR (73).

Infectious agent	Pathogens targeted	Clinical manifestation(s) and/or specimen
Virus	HIV-1, HIV-2, HTLV-1, and HTLV-2 HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs	Blood Meningitis, encephalitis, or meningoencephalitis; CSF
Bacterium	*Haemophilus influenzae, Streptococcus pneumonia, Mycoplasma catarrhalis,* and *Alloiococcus otitidis* *Campylobacter jejuni* and *C. coli* *Actinomyces actinomycetemcomitans, Porphyromonas intermedia,* and *P. gingivalis* *Neisseria gonorrhoeae, Chlamydia trachomatis, Ureaplasma urealyticum,* and *Mycoplasma genitalium*	Upper respiratory tract Human campylobacteriosis Periodontal infection Genital infections
Parasite	*Giardia lamblia* and *Cryptosporidium parvum* *Leishmania* spp.	Diarrheal disease; water Leishmaniasis
Combination	HSV, *H. ducreyi*, and *Treponema pallidum* HPVs, HSV, and *C. trachomatis* Adenovirus, HSV, and *C. trachomatis* EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, and *C. pneumoniae*	Genital ulcer disease Genital swabs Keratoconjunctivitis Acute respiratory tract infections

CHAPTER III

MATERIALS AND METHODS

Microorganisms

In this study used *O. tsutsugamushi* included Karp, Kato and Gilliam strains and *R. typhi,* were obtained from Armed Forces Research Institute, Thailand. *L. interrogans* consisted of Pyrogenes, Pomona and Bratislava serovars and *L. biflexa* Patoc serovar, were collected from Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand. These bacterial strains used as positive control.

Other unrelated pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella* spp., dengue viruses (DENV1-DENV4), *Plasmodium falciparum,* and *P. vivax* were acquired from Department of Microbiology and Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Thailand. These pathogens used in the specificity testing.

Clinical samples

Eighty-three EDTA-whole blood samples were collected from Chulalongkorn hospital, Armed Forces Research Institute, Loei hospital, Takuapa hospital, and Chokchai hospital, Thailand; were double-blind experiment. In this study, inclusion criteria were patients' age were ≥ 18 years, presented with high fever (≥38°C) during 3-5 days, non-specific symptoms (headache, muscular pain, anorexia, eschar, or rash) and a negative result of virus test. Blood samples from healthy donors used as negative control. The present study was approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Thailand (IRB no. 534/57).

Genomic DNA Extraction

Bacterial DNA was extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen, USA). For orienatial and rickettsial DNA were isolated from infected cell cultures. Infected cell were harvested by trypsinization. Subsequently, proteinase K, RNase A and PureLink™ Genomic Lysis/Binding Buffer were added, mixed by vortexing and incubated at 55°C for 10 minutes to stimulate protein digestion. Leptospiral DNA and other bacterial DNA were isolated from pure cultures. Bacterial cell were harvested by centrifugation, resuspended the pellets in PureLink™ Genomic Digestion Buffer and incubated at 55°C for 30 min. Then, protein and RNA was digested by adding proteinase K, RNase A and PureLink™ Genomic Lysis/Binding Buffer to the lysate. Binding and washing DNA were carried out according to PureLink's manufacturer instruction. A purified genomic DNA was eluted in 50 µl PureLink™ Genomic Elution Buffer. DNA concentration was measured by UV absorbance at 260/280 nm using NanoDrop2000 (Thermo scientific, USA).

Primer design

The target genes consisted of *56-kDa TSA*, *lipL32,* and *17-kDa antigen* genes, were selected for this study. 56-kDa TSA (56K TSA_F and 56K TSA_R) primers were designed and provided from Miss Nareethorn Udomthongsuk, which amplified a 166-bp fragment from 56-kDa TSA gene of *O. tsutsugamushi* (position 1873-1896 and 2014-2038 of nucleotide sequence accession no. M33004) (74). LipL32 (lipL32_45F and lipL32_287R) primers were slightly modified from the study of González and provided from Assistant Professor Kanitha Patarakul, M.D., Ph.D. laboratory that amplified a 243-bp fragment in *lipL32* gene of pathogenic *Leptospira* sp. (position 45-64 and 268-278 of nucleotide sequence accession no. AY442332) (75). 17-kDa (17kDa _F and 17kDa_R) primers followed the research of Webb and others, which amplified

a 434-bp fragment in *17-kDa antigen* gene of *Rickettsia* sp. (position 86-105 and 500-519 of nucleotide sequence accession no. M28481) (56). The specificity of primers was bioinformatically aligned with databases in National Center for Biotechnology Information (NCBI) using BioEdit program (Ibis Biosciences, USA).

Table 2 Primer nucleotide sequences used in this study

Bacteria species	Primer name	Sequence (5'→3')	Length (bp)	Tm (°C)	Product size (bp)	References
Orientiae	56-kDa_1873F	GGC-CAA-GTT-AAA-CTC-TAT-GCT-GAC	24	61.0	166	Udomthongsuk, 2011.
	56-kDa_2038R	CAG-CAT-TAA-TTG-CTA-CAC-CAA-GTG-C	25	61.3		
Pathogenic leptospires	lipL32_45F	AAG-CAT-TAC-CGC-TTG-TGG-TG	20	57.3	243	González *et.al,* 2013.
	lipL32_287R	CGA-ACT-CCC-ATT-TCA-GCG-AT	20	57.3		
Rickettsiae	17-kDa_86F	GCT-CTT-GCA-GCT-TCT-ATG-TT	20	55.2	434	Webb *et.al,* 1990
	17-kDa_519R	CAT-TGT-TCG-TCA-GGT-TGG-CG	20	59.3		

Optimization of the initial PCR condition

In this experiment, we tested individual primer pair in single PCR amplification. The single PCR was performed in 20 µl per reaction with individual primer pairs. A reagent mixture included 1x Gotaq® Flexi buffer (Promega, USA), 1.5mM $MgCl_2$ (Promega, USA), 0.2mM dNTPs (Promega, USA), 0.2µM individual forward primer, 0.2µM individual reverse primer, 0.5U/µl Platinum™ *Taq* DNA Polymerase (Invitrogen, Brazil), and 5ng/µl template DNA. Amplification of each primer pair was performed in PCR thermal cycler (Applied Biosystems, USA). A PCR cycling condition for the single PCR were initial denaturation at 94°C for 5 min, followed by 35 cycles of three steps; denaturation at 94°C for 30 sec, annealing at 57°C for 1 min, extension at 72°C for 1 min, and the final extension at 72°C for 10 min.

Optimization of the multiplex PCR condition

The three causative agents of scrub typhus, leptospirosis, and murine typhus were tested in the multiplex PCR. The multiplex PCR with the three target-specific primers was optimized reaction to improve the efficiency of amplification for each product. The final condition for multiplex PCR was conducted on a total of 20 µl per reaction consisted of 1x Gotaq® Flexi buffer (Promega, USA), 2.5mM $MgCl_2$ (Promega, USA), 0.2mM dNTPs (Promega, USA), 0.2µM/each forward primer, 0.2µM/each reverse primer, 0.5 U/µl Platinum™ *Taq* DNA Polymerase (Invitrogen, Brazil), and 5ng/µl template DNA. Amplification of the multiple PCR was performed in the thermal cycler (Applied Biosystems, USA). The PCR cyclic condition for the multiplex PCR were initial denaturation at 94°C for 5 min, followed by 35 cycles of three steps; denaturation at 94°C for 30 sec, annealing at 61°C for 1 min, extension at 68°C for 1 min, and the final extension at 68°C for 10 min.

Specificity of the multiplex PCR assay

In this experiment, we evaluated specificity and efficiency of the designed multiplex PCR assay. The three different primer pairs were tested with other unrelated pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella* spp., dengue viruses (DENV1-DENV4), *Plasmodium falciparum,* and *P. vivax* and performed in the masking condition that was contaminated other pathogens including *S. aureus* and dengue virus in the desired target bacteria.

Sensitivity of the multiplex PCR assay

The sensitivity of the designed multiplex PCR assay was determined using a mixture of oriential, leptospiral, and rickettsial DNA in different concentration ranging from 0.5 fg/µl to 5 ng/µl. For a spiked-blood sample, 1 ng/µl of mixed oriential, leptospiral, and rickettsial DNA was added in 200 µl healthy huamn blood and then was extracted DNA from blood followed by PureLink's manufacturer instruction. Then, a 10 fold-sereial dilution of mixed target bacteria-spiked blood was tested by the developed multiplex PCR assay.

Performance of the multiplex PCR assay

Eighty-three whole blood samples of acute undifferentiated febrile illness patients were isolated by using PureLink™ Genomic DNA Mini Kit (Invitrogen, USA). DNA isolation was carried out according to PureLink's manufacturer instruction. Subsequently, Genomic DNA was tested in the designed multiplex PCR in order to validate the performance of the designed multiplex PCR assay compared to the serological methods (either IFA or MAT test). Diagnostic accuracy of the multiplex PCR was measured in term of sensitivity, specificity, positive predictive value, and

negative predictive value, compared to the serological methods (either IFA or MAT tests).

Gel electrophoresis

Each product was loaded onto 2.0% agarose gel (Invitrogen, USA) in 0.5X Tris-borate with EDTA buffer, separated by Horizontal electrophoresis (i-MyRun, USA) for 30 min at 100 volts, stained in 0.5 μg/ml ethidium bromide (Sigma, USA) for 5 min and re-stained in deionized water for 10 min. Amplicons were visualized by UV transilluminator (Bio-Rad, USA) and size of amplicons were compared with 100 bp DNA ladder (Bio-Helix, Taiwan).

CHAPTER IV

RESULTS AND DISCUSSION

Primer design

Three different primers consist of 56-kDa TSA, lipL32 and 17-kDa primers, which were bioinformatically aligned with the database in NCBI using BioEdit program. The results presented that the three primer pairs can bind conserved nucleotide sequences of each gene and did not cross-hybridize between bacteria species. (Figure 7)

A)

```
lipL32

Forward primer: 5'-AAGCATTACCGCTTGTGGTG-3'
      JN886738   CGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCG
      HM026175   CGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCG
      AY442332   CGTTGCACTCTTTGCAAGCATTACCGCTTGTGGTGCTTTCG
                 ****************************************
Reverse primer: 5'-CGAACTCCCATTTCAGCGAT-3'
      JN886738   CTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCA
      HM026175   CTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCCA
      AY442332   CTGCCGTAATCGCTGAAATGGGAGTTCGTATGATTTCCCAA
                 **************************************** *
```

B)

```
17K genus-common antigen

Forward primer: 5'-GCTCTTGCAGCTTCTATGTT-3'
      M28479     TTATAGCTCTTGCAACTTCTATGTTACAAGCCTGTAACGGTC
      M28481     TTTTAGCTCTTGCAGCTTCTATGTTACAAGCATGTAATGGTC
      M28482     TTATAGCTCTTGCAGCTTCTATGTTACAAGCTTGTAATGGTC
                 ** ********** ********** ***** ****
Reverse primer: 5'-CATTGTTCGTCAGGTTGGCG-3'
      M28479     AATGCATGCCGCCAACCTGACGGACAATGGCAAGTTGTGAAT
      M28481     AATGCATGCCGCCAACCTGACGGACAATGGCAAGTTGTGAAT
      M28482     AATGCATGCCGTCAACCTGATGGGCAATGGCAAGTTGTGAAT
                 ********** ******** ** ******************
```

C)

```
56K type-specific antigen

Forward primer: 5'-GGCCAAGTTAAACTCTATGCTGAC-3'
      M33004     TATGATTGTCGGCCAAGTTAAACTCTATGCTGACGTAATGAT
      AY283180   TATGATTGTCGGCCAAGTTAAACTCTATGCTGACGTAATGAT
                 ****************************************
Reverse primer: 5'-CAGCATTAATTGCTACACCAAGTGC-3'
      M33004     TCAGGAGCACTTGGTGTAGCAATTAATGCTGCTGAAGGTGTG
      AY283180   TCAGGAGCACTTGGTGTAGCAATTAATGCTGCTGAAGGTGTG
                 ****************************************
```

Figure 7 Primer nucleotide sequences alignment with; (A) lipL32 genes of pathogenic *Leptospira* sp., (B) 17-kDa antigen genes of *Rickettsia* sp., and (C) 56-kDa TSA genes of each strain of *Oreintia* sp.

Initial condition optimization of PCR reaction

In this experiment, to find out the initial PCR condition for individual primers using single PCR in the same reaction, we performed amplification with PCR condition that previously described (see materials and methods). The results showed that the different PCR product sizes of three bacteria in 2.0% agarose gel (Figure 8). Oriential DNA was amplified with 56-kDa TSA primer, which was a 166-bp PCR band, and 56-kDa TSA primer pair was detectable in all strains of *O. tsutsugamushi* (Karp, Kato, and Gilliam strains). *LipL32* genes were amplified with lipL32 primers that showed a 243-bp fragment of pathogenic *L. interrogans* (Bratislava, Pomona and Pyrogenes serovars) in 2.0% agarose gel, whereas non-pathogenic *L. biflexa* serovar Patoc were not amplified by lipL32 primers. 17-kDa primers could amplify 434-bp fragment of a gene encoding 17-kDa antigen of *R. typhi*.

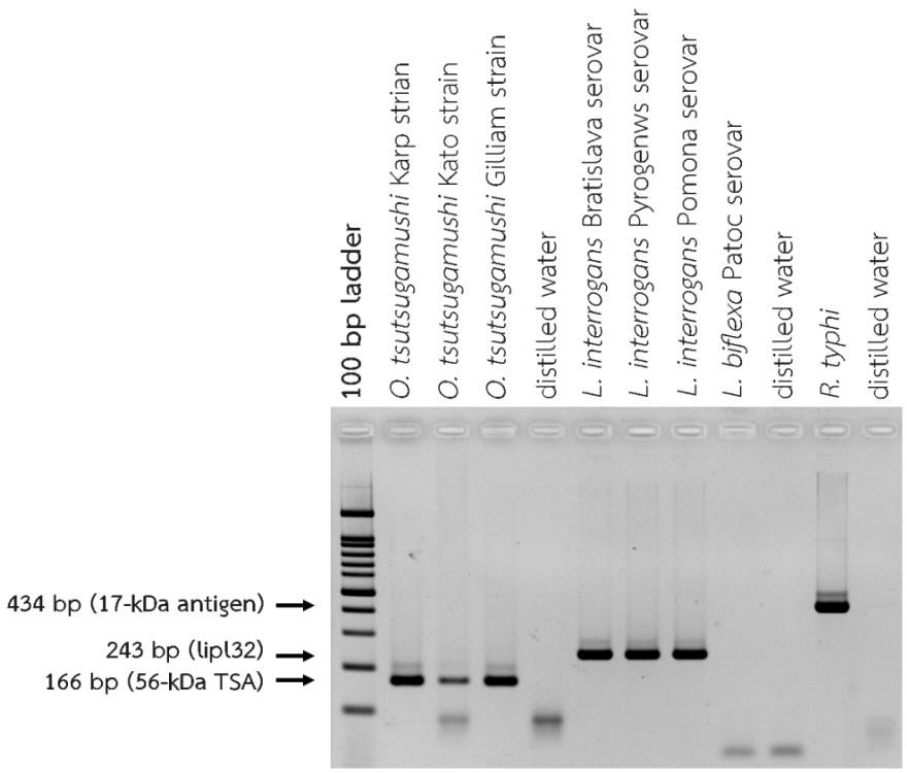


Figure 8 Amplification of individual primers in single PCR in 2.0% agarose gel. Amplicons in the region 160 bp showed oriential DNA was amplified by 56-kDa TSA primers. PCR product in the region 240 bp showed leptospiral DNA was amplified with lipL32 primers and product in the region 430 bp showed rickettsial DNA was amplified with 17-kDa primers compared to 100 bp DNA ladder.

Optimization of multiplex PCR condition

An optimization of the multiplex PCR is very important because a combination of several primers in the multiplex PCR reaction leads to unexpected product formation, self-priming, or uneven of desired product (76). Primer-template ratio, extension temperature, Mg^{2+} concentration, or annealing temperature is one of the parameters, which affect the multiplex PCR reaction (22). Therefore, some factors of the multiplex must be changed for the appropriate reaction to simultaneously identify the three bacterial pathogens. In this study, we optimized the parameters such as extension temperature, Mg^{2+} concentration, and annealing temperature for reduces non-specific interaction.

First, we aimed to find out the optimal extension temperature for the multiplex PCR reaction, we compared the extension temperature between 68°C and 72°C to amplify the maximum yield PCR product. The result presented that PCR bands at extension temperature 68°C was more intensity than PCR bands at extension temperature 72°C in the multiplex PCR reaction with three different primers (Figure 9A).

Second, to optimize the proper Mg^{2+} concentration for the multiplex PCR, we varied $MgCl_2$ concentration in order to increase the specificity of primer-template binding. The result showed that the optimal Mg^{2+} concentration for the multiplex PCR reaction was 2.5mM (Figure 9B).

Finally, we adjusted annealing temperature using a gradient PCR at 55-61°C. The result showed that the annealing temperature at 61°C were optimum for the three target genes amplification (Figure 9C).

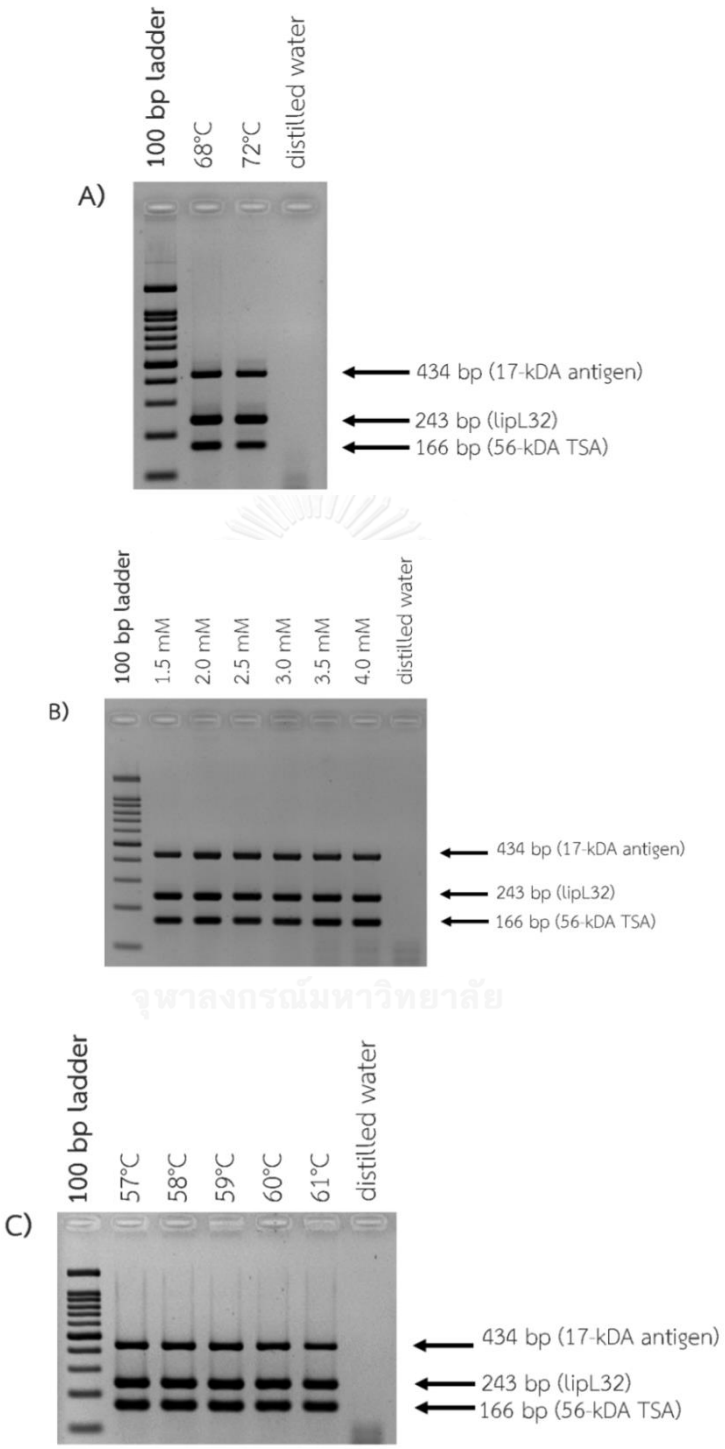


Figure 9 Amplification of the appropriate multiplex PCR condition. (A) the optimization of extension temperature, (B) the adjusting of Mg^{2+} concentrations, and (C) the optimization of annealing temperature using a gradient PCR.

Specificity of the multiplex PCR assay

In this experiment, to evaluate the specificity of the designed multiplex PCR assay, the three different primer pairs were tested with unrelated-pathogens including *E. faecalis*, *S. aureus*, *Salmonella* spp., *K. pneumonia*, *E. coli*, dengue viruses, *P. falciparum*, and *P. vivax*. The results exhibited that three primer pairs are detectable the three targeted bacterial pathogens. Furthermore, the three primer pairs did not detect the specific targets in all tested-pathogens. (Figure 10A)

Also, we assessed the efficiency of the multiplex PCR in a masking condition. The result presented that each primer pair was detectable a unique PCR product of each specific target in the masking condition. (Figure 10B)

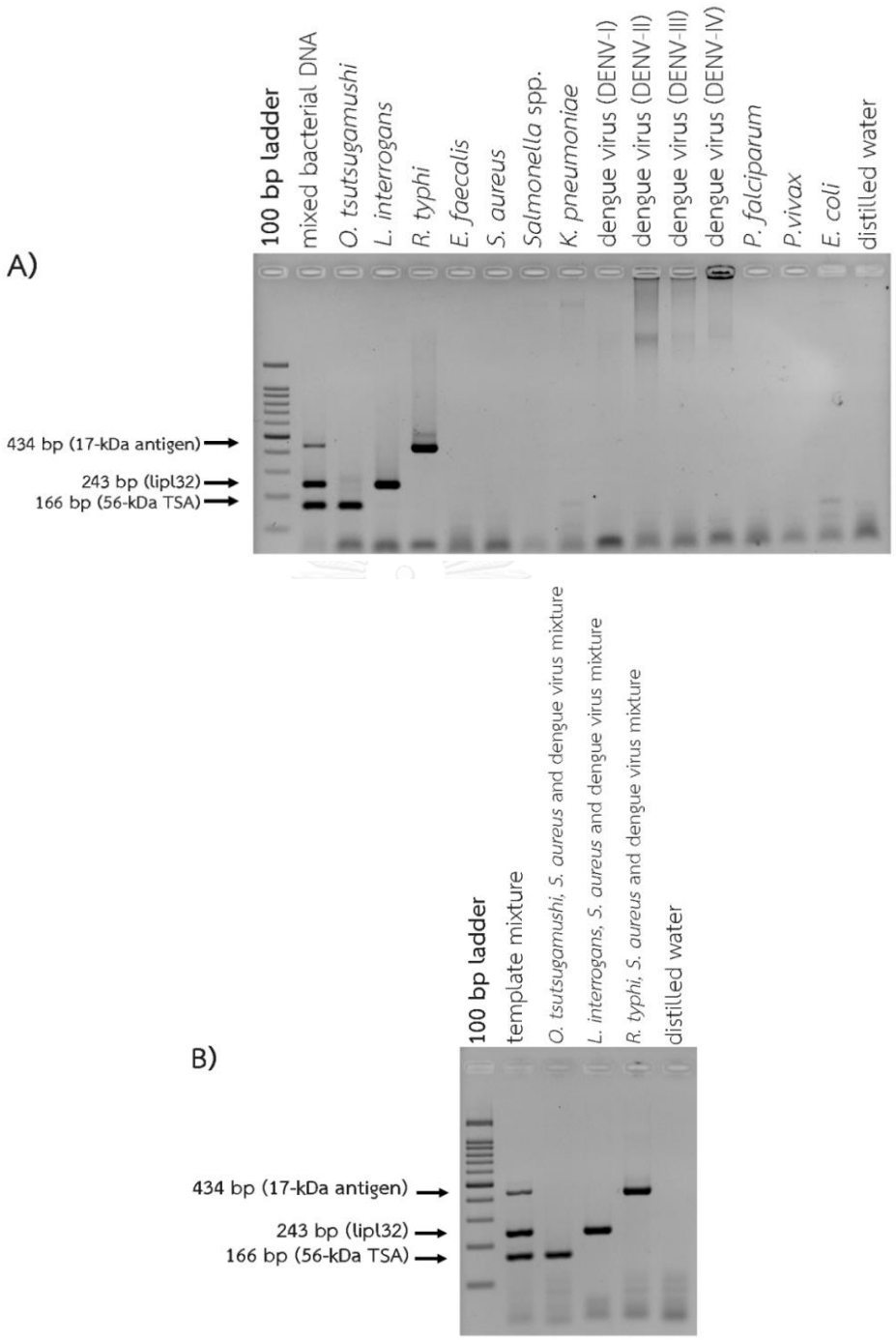


Figure 10 Specificity of the multiplex PCR reaction. (A) The three primer pairs were tested with other unrelated-pathogens and (B) the efficiency of the multiplex PCR assay in the masking condition.

Sensitivity of the multiplex PCR assay

In this study, the sensitivity of the developed multiplex PCR assay was evaluated detection limit of the multiplex PCR in term of the genomic DNA mixture containing *O. tsutsugamushi*, *L. interrogans* and *R. typhi* in the different concentrations. The result showed that the lowest detection limit was 0.5pg/µl or 230 copies for oriental DNA, 0.5pg/µl or 106 copies for leptospiral DNA, and 5pg/µl or 4,160 copies for rickettsial DNA (Figure 11A).

Furthermore, we determined the detection limit of the developed multiplex PCR with bacterial DNA-spiked blood samples in the different concentrations. The result showed that detection limit was 0.5ng/µl in all three bacteria (Figure 11B).

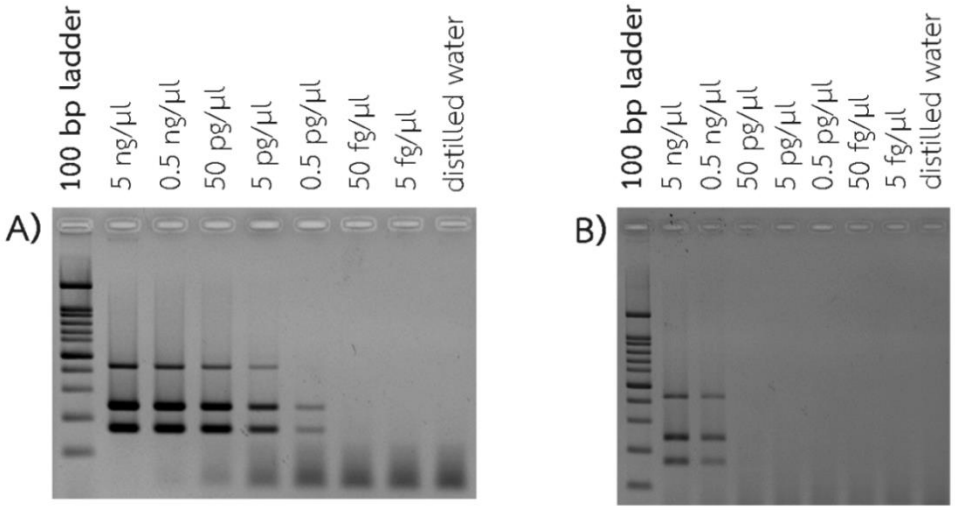


Figure 11 Sensitivity of the multiplex PCR assay. (A) Detection limit of the multiplex PCR in term of genomic DNA mixture and (B) detection limit of the multiplex PCR tested with spiked blood samples.

Performance of the multiplex PCR assay

To validate the performance of the designed multiplex PCR assay, we validated the performance of the designed multiplex PCR assay with 83 whole blood samples of acute undifferentiated fever patients. The results exhibited that a total of 83 samples, 39 samples (47%) showed multiplex PCR-positive including scrub typhus 22 samples (26.5%), leptospirosis 11 samples (13.25%), murine typhus 5 samples (6.02%), and co-infection of leptospirosis and scrub typhus 1 sample (1.2%), whereas either IFA or MAT assay could detect 20 samples (24.09%). (Table 3)

Comparison of multiplex PCR results to IFA or MAT results as the gold standard of three diseases (scrub typhus, leptospirosis, and murine typhus). The results presented the sensitivity and specificity of the multiplex PCR were 100% and 70%. Positive predictive value and negative predictive value were 51% and 100%. Twenty samples were both of serological methods and multiplex PCR positive, whereas 19 samples were positive in multiplex PCR but negative in IFA or MAT assay. Furthermore, 1 of 19 samples that were multiplex PCR-positive showed the co-infections of scrub typhus and leptospirosis (sample no. 82). (Figure 12)

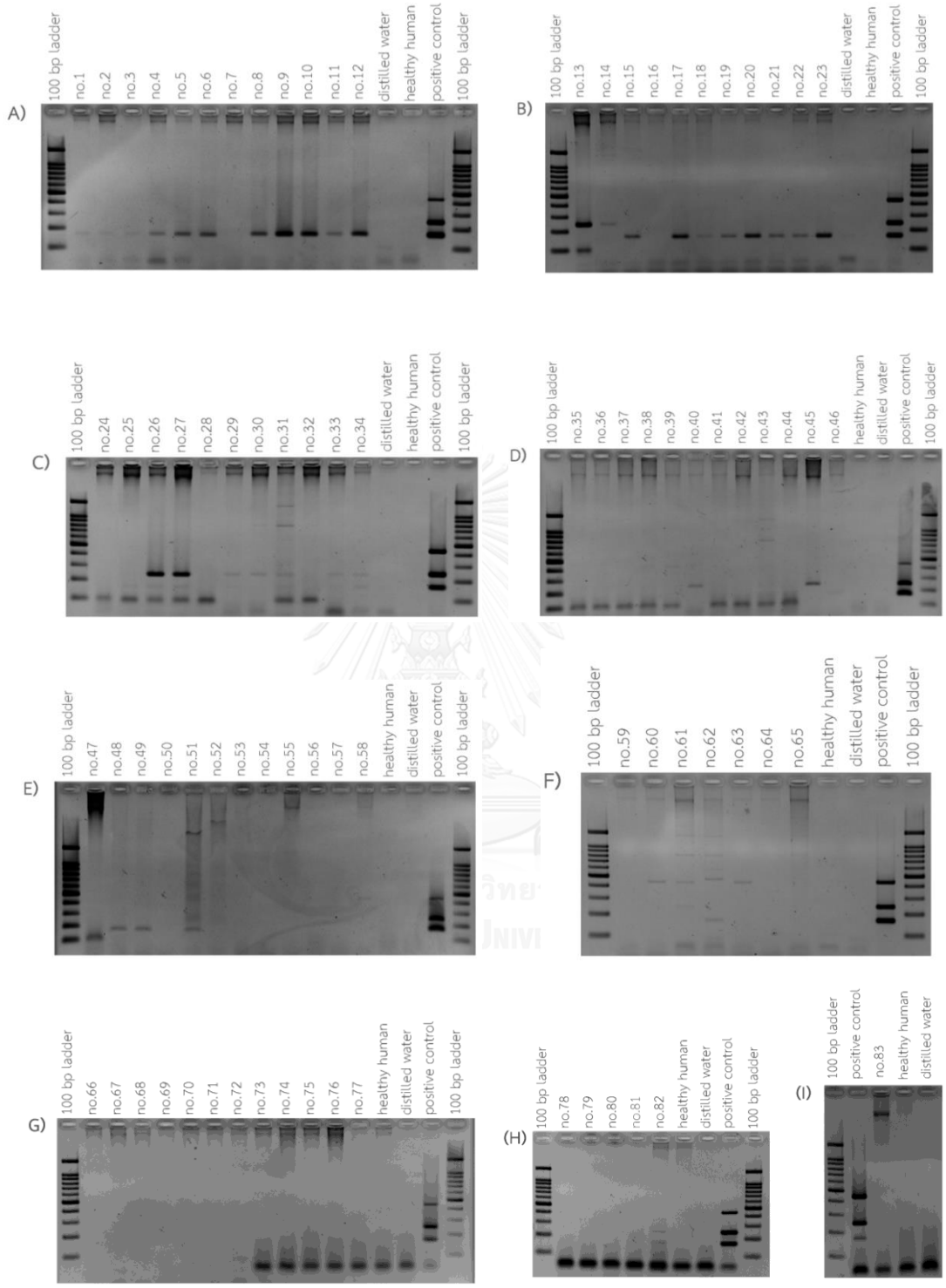


Figure 12 The performance of the designed multiplex PCR assay. This method was tested with 83 blood samples of acute undifferentiated fever patients.

Table 3 Comparison between the multiplex PCR results and either IFA or MAT results.

Methods/Results			Serological test (either IFA or MAT assay)		Total
			Positive	Negative	
Multiplex PCR	Positive	Scrub typhus	13	9	39
		leptospirosis	6	5	
		Murine typhus	1	4	
		Co-infection (leptospirosis and scrub typhus)	0	1	
	Negative		0	44	44
		Total	20	63	83

Discussions

To our knowledge, this work is the first study showing the improvement and reliable diagnostic tool for identifying the pathogenic agents causing scrub typhus, leptospirosis, and murine typhus. Our results showed positive detectable oriential, leptospiral, and rickettsial DNA and non-specific product disappeared. Previous studies reported that the detection of orientiae and rickettsiae is difficult because they are obligate intracellular bacteria, buffy coat samples that are isolated from whole blood are the sample of choice for *O. tsutsugamushi* and *Rickettsia* spp. In addition, orientiae and rickettsiae concentration is barely low in blood samples (3-5 bacterial cells/µl), so nested PCR has been utilized to identify *O. tsutsugamushi* and *Rickettsia* sp. in blood samples (41, 77). For leptospirosis, amount of leptospires in bloodstream is nominal due to the short period of leptospiremia, serum and urine samples are the sample of choice for detecting pathogenic *Leptospira* spp. (20, 39, 75) This study presented that the developed multiplex PCR could be used to discriminate three bacteria from whole blood samples of acute undifferentiated fever patients; detection limit of the assay was 0.5pg/µl or 230 copies for oriental DNA, 0.5pg/µl or 106 copies for leptospiral DNA, and 5pg/µl or 4,160 copies for rickettsial DNA.

The diagnosis of these diseases traditionally based on antibody detection; indirect immunofluorescence assay is a gold standard for detecting *O. tsutsugamushi* and *R. typhi*, and microscopic agglutination test is a gold standard for identifying leptospires. However, it requires paired sera and takes several days for confirmation (39, 43). The delayed diagnosis and treatment of patients result in severity of disease, complication, and mortality. Therefore, development the rapid method is necessary in order to improve identification of pathogens causing scrub typhus, leptospirosis, and murine typhus. In this study, we developed the multiplex PCR for distinguishing three bacteria. First, we compared extension temperature 68°C and 72°C, the

extension temperature 68°C was chosen because the higher extension temperature decreased the yield of some product (22). Then, we varied Mg^{2+} concentration range from 1.5-4.0mM , we chose Mg^{2+} concentration 2.5mM due to Mg^{2+} concentration affects specificity and efficiency of the reaction and also Mg^{2+} concentration depends on *Taq* DNA polymerase, template DNA, and dNTPs concentration. It is not appropriate lead to the yield of PCR product and the specificity of template-primer binding (65). Finally, annealing temperature was optimized using a gradient PCR. In this experiment, we chose the possible annealing temperature at 61°C to enhance the correctly primer-template binding and avoid decreasing sensitivity of the multiplex PCR assay (76). Our data showed maximal amplicons intensity and lowest non-specific background. The multiplex PCR were able to amplify the different band sizes in 2.0% agarose gel. Our study is concordance with previously studies that primers encoding 56-kDa antigen, lipL32, and 17-kDa antigen of each bacteria species for amplifying in the single PCR studies (50, 56, 75), and the research of Foroshani and colleagues performed the multiplex PCR with the unrelated bacteria species including *Salmonella typhi*, *Bacillus anthracis* and *Yersinia pertis*. Their result showed the different bacteria species could be detected by the multiplex PCR, and also the primer pairs in their study were specific to each bacteria species (66). This study suggests that the multiplex PCR is distinguished the different bacteria species and non-specific product disappears.

Detection limit is different in each target. These may explain from 1) the combination of many primers in the single tube affects effective reaction, 2) the multiplex PCR reaction prefers to amplify short band rather than to amplify long band (22, 68). As detection limit of bacterial-spiked blood is barely low may result from the presence of hemoglobin and other components in blood as PCR inhibitors and DNA concentration of bacteria, which were inserted into blood sample, was too

low (45, 78). The limit of detection of the assay is comparable with previous studies (79-82).

Currently, antigen and antibody detection have been used to diagnose scrub typhus, leptospirosis, and murine typhus. Antibody detection is a routine laboratory for detecting these diseases; it is inappropriate diagnostic tool for the early phase of disease detection because seroconversion usually arises 8-10 days, and may cross-react among species, whereas antigen detection, which is a direct microbial identification, is useful diagnostic tool on discriminate the pathogens in blood samples, these bacteria can be found in bloodstream in the first week of diseases (18, 19, 39, 41). In the present study, the developed multiplex PCR assay could be distinguish *O. tsutsugamushi*, *L. interrogans*, and *R. typhi* in whole blood specimens of acute undifferentiated fever patients. Our results demonstrated that the sensitivity, specificity, positive predictive value, and negative predictive value of the multiplex PCR were 100%, 70%, 51%, and 100%, respectively. Thirty-nine samples (47%) were found to be positive by multiplex PCR including scrub typhus 22 samples (26.5%), leptospirosis 11 samples (13.25%), and murine typhus 5 samples (6.02%), whereas either IFA or MAT assay showed 20 samples (24.09%), which were positive results. Furthermore, 19 (22.89%) samples were multiplex PCR-positive and either IFA or MAT-negative may result from the period of patients' blood collection and the phase of diseases. Our data are according to Ooteman and others' study showed that serum samples were found to be positive by PCR with negative by MAT assay (83). Our study suggests that the multiplex PCR is able to detect bacterial DNA in blood before increase of antibody against pathogens.

And also, one sample was dual positive of leptospirosis and scrub typhus by multiplex PCR with either IFA or MAT-negative. Previous studies have been reported that the occurrence of co-infection by *O. tsutsugamushi* and pathogenic *Leptospira*

sp. in Thai and Taiwanese patients with acute undifferentiated febrile illness (84, 85). Our results presents that multiplex PCR is a useful technique for detecting three disease or co-infection of each disease in suspected specimens.

The multiplex PCR can be completed within 4-5 hours; it is important to possible treatment of acute undifferentiated fever patients. Moreover, the multiplex PCR is less time-saving and faster than the conventional PCR and the serological tests, and also detectable multiple pathogens. This work suggests that the developed multiplex PCR can be applied in clinical diagnosis of scrub typhus, leptospirosis, and murine typhus patients. However, limitations of the assay were; 1) results of the multiplex PCR will have been confirmed by other laboratory investigations e.g. sequencing, and 2) 17-kDa antigen PCR product will has been identified *Rickettsia* species because 17-kDa antigen gene is a genus-common *Rickettsia* spp.

CHAPTER V

CONCLUSIONS

In conclusion, we developed the multiplex PCR on *O. tsutsugamushi*, *L. interrogans*, and *R. typhi* detection and evaluated the performance of the designed multiplex PCR in blood specimens of acute undifferentiated fever patients. Our study demonstrated that the developed multiplex PCR assay was detectable oriential, leptospiral, and rickettsial DNA. The detection limit of the assay was 0.5-5 pg/µl of bacterial DNA or 0.5ng/µl of spiked blood sample. Three different primer pairs were specific to the targeted bacteria; although these bacteria were contaminated with other pathogens. Evaluation of the performance of the developed multiplex PCR in blood specimens compared to either IFA or MAT methods. Our results showed the sensitivity and specificity were 100% and 70%, respectively. Therefore, the multiplex PCR is rapid, sensitive, and specific diagnostic laboratory test on the pathogens causing scrub typhus, leptospirosis, and murine typhus in whole blood samples of acute undifferentiated fever patients. Furthermore, the obtained knowledge from this study can be applied for developing the new technique on other bacteria identification.

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APPENDIX

LIST OF ABBREVIATIONS

AUF	Acute undifferentiated febrile illness
lipL32	LipL32 lippprotein
56-kDa TSA	56-kDa type-specific antigen
PCR	Polymerase chain reaction
IFA	Indirect immunofluorescence assay
MAT	Microscopic agglutination test
ELISA	Enzyme-linked immunosorbent assay
TBE	Tris-borate with EDTA
IgM	Immunoglobulin M
EMJH	Ellinghausen-McCullough-Johnson-Harris
DNA	Deoxyribonucleic acid

EQUIPMENT AND CHEMICALS

1. Autoclave (Hirayama, Japan)
2. Microcentrifuge (Hettich, Germany)
3. Vortex mixer (Scientific industries, USA)
4. Water bath (Memmert, Germany)
5. Microcentrifuge tube 0.2 mL (Coring, USA)
6. Microcentrifuge tube 1.5 mL (Corning, USA)
7. Cryoviral tube 2.0 mL (Corning, USA)
8. Centrifuge tube 15 mL (Corning, USA)
9. Centrifuge tube 50 mL (Corning, USA)
10. Filter Tip (Corning, USA)
11. Tip (Corning, USA)
12. Automatic dispenser micropipette (Transferpette)
13. Thermal Cycler (Applied Biosystems, USA)
14. NanoDrop 2000 (Thermo scientific)
15. MY-RUN Horizontal electrophoresis (Bio-Rad)
16. UV transilluminator (Bio-Rad)
17. Erlenmeyer flask 250 mL
18. Cylinder glass 100 mL
19. Barrier Film (Parafim)
20. PureLink™ Genomic DNA Mini Kit (Invitrogen, USA)
21. 5X Colorless GoTaq® Flexi Buffer (Promega, USA)
22. 5X Green GoTaq® Flexi Buffer (Promega, USA)
23. 25mM $MgCl_2$ (Promega, USA)

24. dNTP mix 10mM each (Promega, USA)
25. GoTaq® Flexi DNA Polymerase (Promega, USA)
26. Platinum® Taq polymerase (Invitrogen, Brazil)
27. DNase/RNase-Free distilled water (Invitrogen, USA)
28. Ethanol solution (Emsure, Germany)
29. 100 bp DNA ladder (Bio-Helix, Taiwan)
30. Ethidium bromide (Sigma Aldrich, USA)
31. 6X loading dye (Thermo Scientific, USA)
32. Agarose, molecular grade (Invitrogen, USA)

CHEMICAL PREPARATIONS

1. 10X Tris-borate with EDTA (TBE)

Boric acid	27.5	g
Tris-base	54	g
0.5M EDTA (pH 8.0)	20	mL

Add distilled water 300 mL, then mix to homogenous solution and add distilled water to 500 mL.

2. 0.5X TBE

Distilled water	950 ml
10X TBE	50 ml

Mix to homogenous solution.

3. 2.0% agarose gel

Agarose gel	2.0	g
0.5X TBE	100	ml

4. 10X Phosphate buffer saline (PBS)

KCl	0.2	g
KH_2PO_4	0.2	g
NaCl	8.0	g
Na_2HPO_4	1.15	g

Mix all of chemical components and add DI water to 1,000 mL, then adjust pH to 7.4 with HCl

5. 1X PBS

Distilled water	950 ml
10X PBS	50 ml

Mix to homogenous solution.

Formula for calculation of diagnostic accuracy

1) Sensitivity = true positive / (true positive + false negative)
2) Specificity = true negative / (true negative + false positive)
3) Positive predictive value = true positive / (true positive + false positive)
4) Negative predictive value = true negative / (true negative + false negative)

VITA

Nutchanart Sae-liang was born in Yala province, Thailand on 23rd July, 1990. She graduated with a Bachelor Degree of Science (Biology) with first-class honors from Faculty of Science, Srinakarinwirot University in 2012. She enrolled in Master Degree of Medical Science, Faculty of Medicine at Chulalongkorn University in 2013.