การประเมินความไวระหว่างเทคนิค PCR, quantitative PCR, FISH และ IHC สำหรับการตรวจ *Leishmania spp.* โดยศึกษาจากเซลล์เพาะเลี้ยง *Leishmania siamensis*

นางสาวสุมล จึงอุดมเจริญ

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EVALUATION OF THE SENSITIVITY AMONG CONVENTIONAL PCR, QUANTITATIVE PCR, FI SH AND IHC FOR THE DETECTION OF *LEISHMANIA SPP*. USING *LEISHMANIA SIAMENSIS* CELL LINE

Miss Sumon Jungudomjaroen

จุฬาลงกรณ์มหาวิทยาลัย
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สุมล จึงอุดมเจริญ : การประเมินความไวระหว่างเทคนิค PCR, quantitative PCR, FISH และ IHC สำหรับการตรวจ *Leishmania spp.* โดยศึกษาจากเซลล์เพาะเลี้ยง *Leishmania siamensis* (EVALUATION OF THE SENSITIVITY AMONG CONVENTIONAL PCR, QUANTITATIVE PCR, FISH AND IHC FOR THE DETECTION OF *LEISHMANIA SPP*. USING *LEISHMANIA SIAMENSIS* CELL LINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.เผด็จ สิริยะเสถียร, หน้า.

ลิชมาเนียสายพันธุ์ไทย (*Leishmania siamensis*) เป็นสายพันธุ์ที่พบในประเทศไทยไม่นานมานี้ ซึ่งผู้ป่วยจะมีอาการแสดงที่อวัยวะภายใน อาการแสดงที่ผิวหนัง หรือทั้งสองแบบร่วมกัน การตรวจวินิจฉัยโรคได้เร็วตั้งแต่ระยะแรกของโรคถือว่ามีความสำคัญมากในการรักษาซึ่งจะสามารถลดภาวะทุพลภาพและอัตราการตาย สำหรับการตรวจวินิจฉัยที่นิยมใช้เป็นการตรวจดูด้วยกล้องจุลทรรศน์โดยการย้อมสี H&E (Haematoxylin and Eosin) หรือ การย้อมโดยใช้สี Giemsa ซึ่งเทคนิคนี้ต้องอาศัยประสบการณ์มากและความสามารถในการตรวจเจอเชื้อได้น้อย ส่วนเทคนิค IHC และ FISH เป็นทางเลือกอีกทางหนึ่งซึ่งสามารถใช้ในการตรวจหาเชื้อลิชมาเนีย ซึ่งวิธีเหล่านี้ใช้กันเป็นประจำอยู่แล้วในห้องปฏิบัติการสำหรับการตรวจวินิจฉัยเชื้อก่อโรคอื่นๆ ส่วนการตรวจโดยเทคนิคทางอณูชีววิทยา (PCR และ qPCR) เป็นเทคนิคการตรวจระดับดีเอ็นเอ เป็นวิธีที่มีความไวในการตรวจปริมาณเชื้อลิชมาเนียที่มีจำนวนน้อยๆได้มีประสิทธิภาพสูงแต่ขณะเดียวกันก็มีค่าใช้จ่ายสูงมากกว่าวิธีมาตราฐาน การศึกษานี้ได้ทำการศึกษาเปรียบเทียบความสามารถในการตรวจเชื้อลิชมาเนียจากเซลล์เพาะเลี้ยง โดยทำการเจือจางจากความเข้มข้นจาก 10^7 ถึง 10^1 เพื่อใช้ทำการทดลองกับเทคนิค IHC ที่ตำแหน่ง GP63 เทคนิค FISH ที่ตำแหน่งยีน ITS1 เทคนิค PCR และqPCR ใช้ DNA ที่สกัดได้จากตัวอย่างในแต่ละความเข้มข้น ผลที่ได้จากการสังเกตของอาสาสมัครพบว่า ที่ความเข้มข้นต่ำ (10^1) พบว่าทั้งเทคนิค PCR และ qPCR สามารถสังเกตเห็นผลได้ที่ความเข้มข้นนี้ และ มีค่า median 4.00 ขณะที่เทคนิค IHC และ FISH มีค่า median 5.71 และ 5.87 ตามลำดับทั้ง IHC และ FISH มีความสามารถในการตรวจหาเชื้อได้ไม่มีความแตกต่างกันทางสถิติ $P<0.05$ แต่ ทั้ง IHC และ FISH มีความสามารถในการตรวจหาเชื้อได้น้อยกว่าเทคนิค PCR และ qPCR จากความรู้พื้นฐานนี้ ทั้ง 4 เทคนิคสามารถใช้ในการตรวจ *L. siamensis* ในระยะ promastigote ได้ และ เทคนิคเหล่านี้จะมีประโยชน์มากขึ้น โดยนำมาประยุกต์ใช้กับการตรวจในระยะ amastigote ในชิ้นเนื้อที่ได้จากคนไข้ โดยเฉพาะ IHC และFISH ที่มีค่าใช้จ่ายต่ำกว่า เทคนิคทาง molecular มาก ซึ่งเหมาะห้องปฏิบัติการขนาดเล็ก นอกจากนี้การใช้เทคนิค qPCR ที่พัฒนาขึ้นมาเพื่อทดสอบกับตัวอย่างผู้ป่วยพบว่าสามารถให้ผลบวกกับตัวอย่างทั้ง เลือด เม็ดเลือดขาว น้ำลาย และปัสสาวะ เทคนิคที่พัฒนาขึ้นมานี้สามรถนำไปประยุกต์ใช้สำหรับการตรวจวินิจฉัย การศึกษาการระบาดของโรค และการติดตามการรักษาได้ต่อไป

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สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต ....

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SUMON JUNGUDOMJAROEN: EVALUATION OF THE SENSITIVITY AMONG CONVENTIONAL PCR, QUANTITATIVE PCR, FISH AND IHC FOR THE DETECTION OF *LEISHMANIA SPP*. USING *LEISHMANIA SIAMENSIS* CELL LINE. ADVISOR: ASSOC. PROF. PADET SIRIYASATIEN, M.D., Ph.D., pp.

Leishmania siamensis is a novel Leishmania species described recently in Thailand. Patients infected with L. siamensis can be presented with cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) or combination of both CL and VL. Early diagnose is essential for therapeutic reasons and therefore decrease morbidity and mortality. Conventional diagnosis is usually based on microscopic examination by staining with haematoxylin and eosin or Giemsa. The technique requires expertise and shows low sensitivity. IHC and FISH are alternative techniques which used to detect Lesihmania parasites. These methods are known to use in routine laboratory for detection of other pathogens. Molecular techniques such as PCR and qPCR have been used to detect Leishmania DNA and have been shown high sensitivity for detection Leishmania spp. However, these techniques have higher cost than other conventional methods. The study is designed to compare the ability of IHC, FISH, PCR and qPCR for detection Leishmania parasites. The experiment sample was prepared from culture L. isamensis and was made to serial dilution of 10^{7}-10^{1}. IHC technique was used primary antibody anti-GP63, FISH technique was used probes designed label with fluorescent dye to hybridize the ITS1 gene region. PCR, and qPCR were used to detect the same extraction DNA from each dilution described previously. All result from 6 volunteers showed positive in low concentration (10^{1}) with both of PCR and qPCR and average of median are 4.00 can still observed in this concentration. While, IHC and FISH showed average of median are 5.71 and 5.87, respectively ($P<0.05$). However, they are lower sensitivity than molecular detections. The data obtained from this study can be used for detection promastigote of L. siamensis. These techniques can be applied for detection amastigotes form from clinical specimens. IHC and FISH is cheaper than molecular method that appropriates for a small laboratory. Moreover, qPCR developed from this study has been used to detect L.siamensis DNA in various clinical sample specimens. It showed positive in blood, buffy coat, saliva and urine. Techniques developed from this study therefore could be applied for epidemiological studies and follow up after treatment.

Field of Study: Biomedical Sciences Student's Signature ....
Academic Year: 2014 Advisor's Signature ....

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LIST OF ABBREVIATIONS

Ab	Antibody
AIDS	Acquired immune deficiency syndrome
bp	base pair
BSA	Bovine serum albumin
CL	Cutaneous leishmaniasis
cm	Centimeter
cm^3	Cubic centimeter
Cq	Quantification cycle
CSA	Crude soluble antigen
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DAT	Direct agglutination test
ddH_2O	Deionized distilled water
et al.	*et alii* (latin), and others
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
GP63	Glycoprotein 63
GPI	Glycosylphosphatidylinositol
hr	Hour
H&E	Hematoxylin and Eosin
IFAT	Indirect fluorescent antibody test

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LIST OF ABBREVIATIONS

IHC	Immunohistochemistry
ITS1	Inter transcribed spacer 1
kDNA	Kinetoplast deoxyribonucleic acid
KDa	Kilodalton
Max	Maximum
mg	Miligram
Min.	Minimum
min.	Minute
ML	Mucocutaneous leishmaniasis
ml	milliliter
mRNA	messenger ribonucleic acid
NTC	Negative control
PCR	Polymerase chain reaction
PKDL	Post-kala-azar dermal leishmaniasis
qPCR	Quantitative Polymerase chain reaction
rK39	Recombinant kinesin 39
RNA	Ribonucleic acid
rpm	Revolutions per minute
spp.	Species
ssrRNA	Small sub-unit ribosomal RNA
TBS	Tris buffer saline
TBST	Tris buffer saline add tween-20

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LIST OF ABBREVIATIONS

Taq	Thermus aquaticus
TAE	Tris acetate Ethylenediamine-tetra acetic acid
U	Unit
UDG	Uracil DNA glycosylase
VL	Visceral leishmaniasis
WHO	The World Health Organization
1°	Primary
2°	Secondary
$^\circ C$	Degree Celsius
µL	Microliter
µm	Micrometer
%	Percent

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CHAPTER I
INTRODUCTION

1. Background and rationale

Leishmaniasis is a neglected tropical disease affects one-sixth of the world's population (more than one billion people) which is reported by the World Health Organization (WHO) [1, 2]. It is a major public health problem in vast areas of the world, with a huge impact on the economy of developing countries. Leishmaniasis is endemically presented in 88 countries with a total of 12 million people infected and a mortality rate approximately 60.000 per year [3]. WHO reported that during of 2007 to 2011, visceral leishmaniasis and cutaneous leishmaniasis cases occur approximately 0.7 to 1.2 million and 0.2 to 0.4 cases, respectively in each year [4]. Leishmaniasis is an infectious disease caused by the protozoa, in genus *Leishmania*. Approximately, 20 *Leishmania* species can caused human Leishmaniasis [5, 6] Infected female sand fly is vector of Leishmaniasis. The life cycle of *Leishmania sp*. have 2 forms; the flagellate form call promastigote when it resides in insect. This form can infect to mammals through the bite of infected female sand fly. Another form is aflagellate called amastigote, the shape change to the oval shape after infected with macrophage of vertebrate hosts. There are 3 main types of clinical pattern of leishmaniasis: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL). The pattern of disease depends on the infection of different *Leishmani*a spp. VL type is the most severe form because patients usually death without treatment [5, 7] and it is an important opportunistic co-infected in AIDS patients. VL can cause of Post-kala-azar dermal leishmaniasis (PKDL) [8] and VL is usually caused by the *Leishmania donovani* species complex.

Bangladesh, Nepal, India, Brazil and Sudan are cover 90% endemic areas of VL [3, 9, 10]Thailand has never been reported as endemic area of the disease but leishmaniasis cases are found. Leishmaniasis case report in Thailand can be divided into 2 periods: imported leishmaniasis cases between 1960-1986[11, 12] and autochthonous leishmaniasis cases since 2006[13-16]. Reported autochthonous VL cases in Thailand cause by *L. infantum* [17] , *L. donovani* [18] and *L. siamensis* which was reported to be a novel specie[16].

Visceral leishmaniasis diagnosis is easily mistaken because other commonly occurring diseases such as typhoid, malaria, tuberculosis share its clinical features; many of these diseases can be presented along with VL (as co-infection). Laboratory diagnosis of leishmaniasis can be made by the following methods: (i) parasitological diagnosis (ii) serological diagnosis (iii) molecular diagnosis[10].

Parasitological diagnosis is the most commonly used for diagnosis VL. It is usually based on microscopic detection of amastigotes in clinical sample such as smears of tissue aspirates including liver, bone marrow, lymph node, spleen[10, 19]. The highest sensitivity in these specimens is the splenic smear (95%) that takes a small amount but definite risk of serious intra-abdominal hemorrhage [7] Direct examination such as bone marrow aspirates, microscopic examination of lymph node smears can give the false negative result because the low number of parasites [20]. Other method to demonstrate *leishmania* in clinical sample is *in vitro* culture. *Leishmania* found in culture is a promastigote form. However, this method has low sensitivity and time consuming compared to other methods [10, 21] Another methods for demonstrating the parasite such as immunohistochemistry (IHC) [22] Fluorescent *in situ* hybridization (FISH). FISH has been shown to be more sensitive

and begun to be established in the laboratory routine as auxiliary or alternative methods [23]. They can be used as supplementary tool to confirm the diagnosis based on haematoxylin and eosin stained sections, particularly in organs that do not have a high parasite load [24].

Serological diagnosis is based on the presence of specific humoral immune response [25] (detection of antigen-antibody reactions in the serum). An available of serological methods is used to diagnosis of VL in endemic regions. Several serological tests are used, such as direct agglutination test (DAT), the indirect fluorescent antibody test (IFAT), rK39 immunochromatographic and enzyme-linked immunosorbent (ELISA). All methods have high sensitivity but cannot discriminate between past and current infections [21, 26].

Molecular diagnosis is high sensitive than serology and microscopic methods. Polymerase chain reaction (PCR), which is a high sensitivity and high specificity method. PCR diagnosis is based on the in vitro amplification of specific-nucleotide sequences such as kinetoplast DNA (kDNA) minicircles, rRNA genes. Each kDNA of *Leishmania spp.* has a 120-200 base pair (bp) conserved region [27]. Leite R.S. et al., 2010, studied the sensitivity of PCR by using conjunctival swab (CS) samples the kDNA PCR-hybridization for VL diagnosis was evaluated an asymptomatic dogs that was found 90% by kDNA primer and 83.3% by internal transcribed spacer 1 (ITS1) primer. On the other hand, for blood sample, the positivity of kDNA PCR-hybridization was significantly lesser than the one obtained by the ITS1 PCR, indicating that sensitivity of PCR methods can vary according to the biological sample examined [28] However, the prognosis of PCR is still far from standardization in analysis of the amplification products because it is usually not suitable for

quantification of the template DNA. Advantage of quantitative real-time PCR (qPCR) is it able to monitor of the accumulation of PCR products during the amplification reaction [29] and is a useful method to detect and diagnose of microorganisms belonging to the same genus. Moreover, it can not only detect the quantification of specific DNA in various biological samples, but also identify the species or strains of several medically important pathogenic microorganisms to be differentiated by melting curve analysis of fluorescent PCR products [30] In conclusion, the advantages of qPCR are to reduce of risk contamination, reduce in assay time and improvement of sensitivity [31].

Early diagnosis of VL is crucial for controlling and early treatment of the disease would decrease morbidity and mortality. Routine laboratory diagnosis of leishmaniasis relies on microscopic demonstration of the organisms (amastigotes) in infected tissues [7]. However, this method has lower sensitivity than serological test such as direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA) and molecular test [10]

Although several techniques have been developed for diagnosis leishmaniasis, but leishmaniasis cause by *L. siamensis* have never been evaluated. The result in this study could determine sensitivity of IHC, FISH, PCR and quantitative PCR for detection *L. siamensis*

2. Research questions

-Which is the most sensitive technique (FISH, IHC, conventional PCR or quantitative PCR) for the detection of *Leishmania sp.* using cultured *L. siamensis* promastigote stage?

-Can FISH or IHC be used in *Leishmania* detection instead of using conventional or quantitative PCR technique?

3. Objective

-To develop protocol FISH, IHC, conventional PCR and qPCR for detection *L. siamensis* and determine their sensitivity.

4. Hypothesis

-Quantitative PCR is able to detect small amount of *L. siamensis* DNA.

-FISH and IHC able to detect cultured promastegotes of *L. siamensis*.

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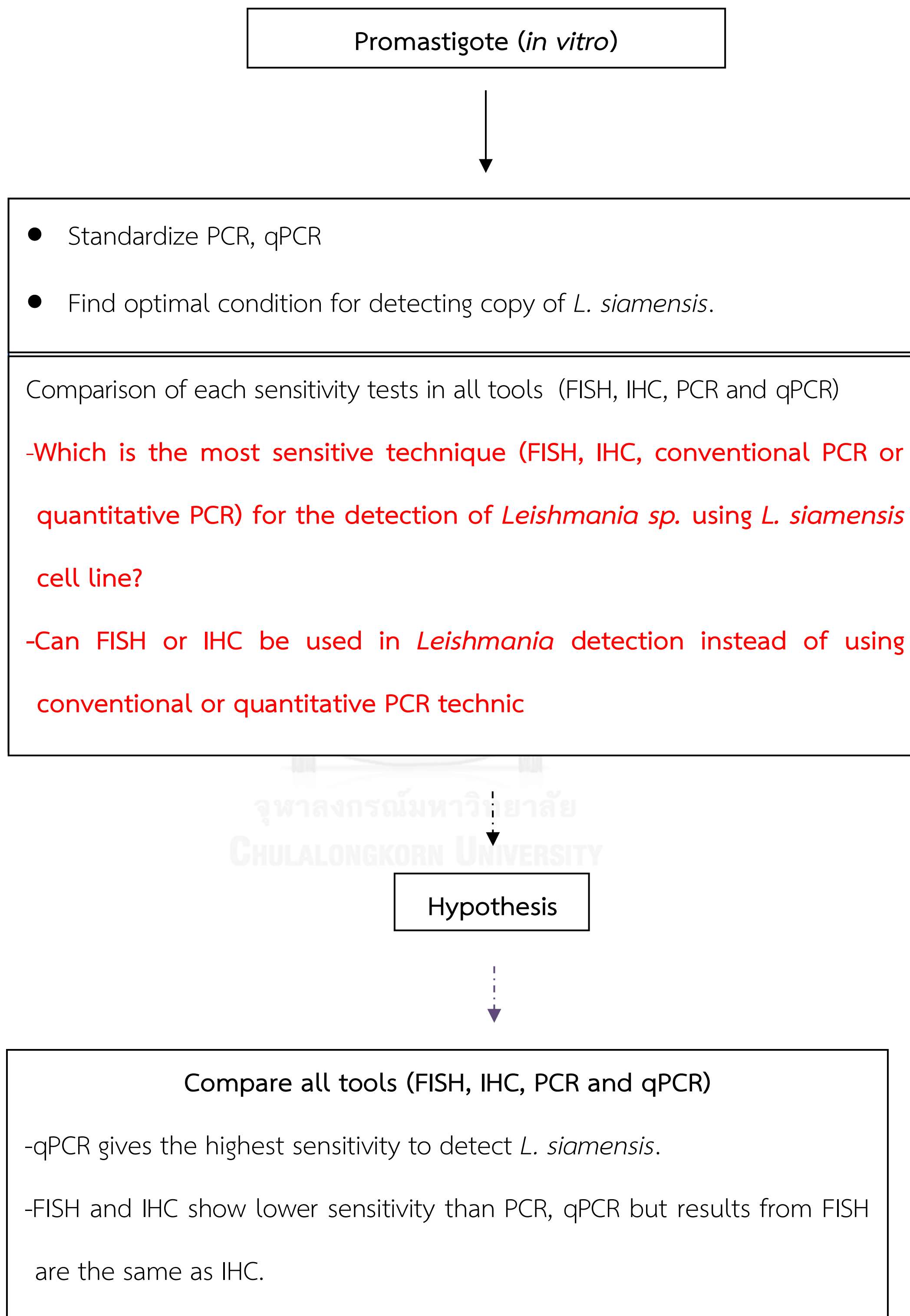
5. Conceptual framework



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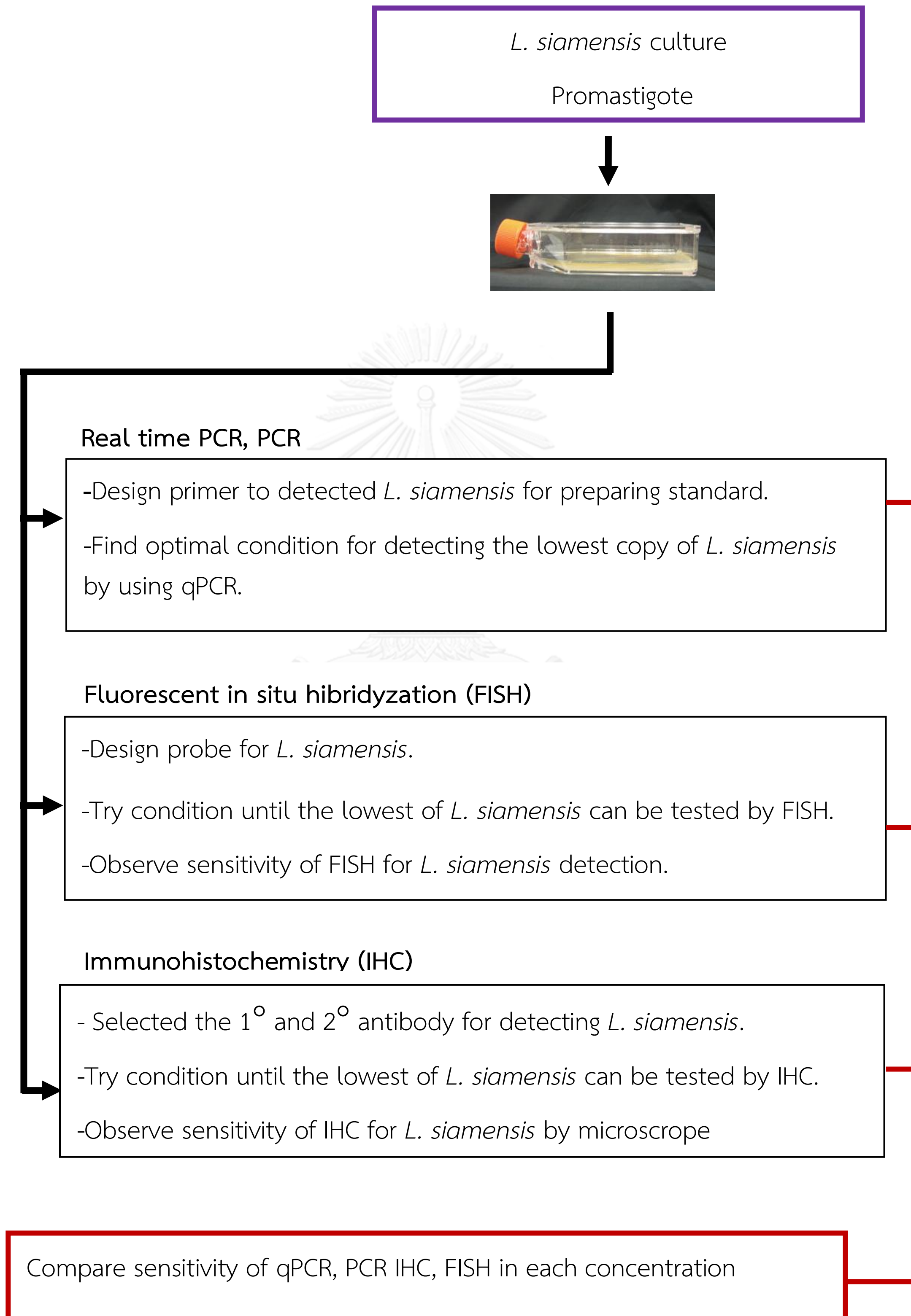
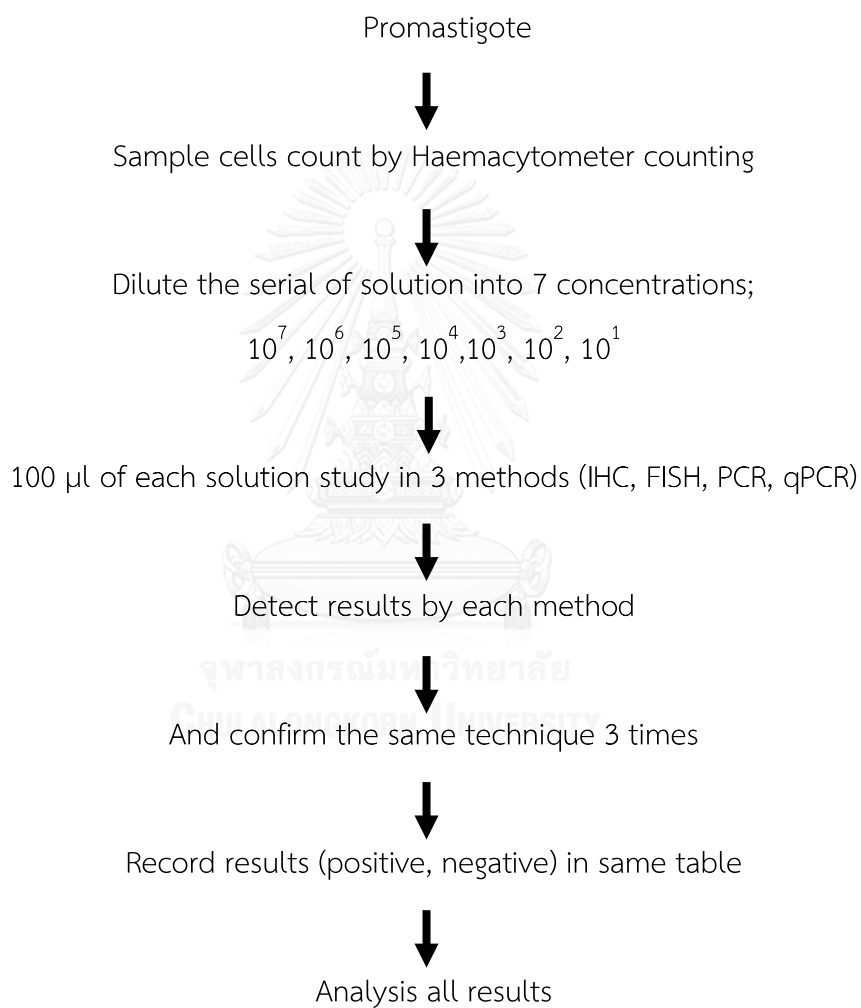
6. Experimental design



6. Experimental design (continue)



CHAPTER II
Review of related literatures

1. *Leishmania*

1.1 Biology

A group of disease known as leishmaniasis is caused by infection with protozoa of the genus Leishmania. Leishmaniasis is a zoonosis, involving domestic or wild animals as reservoir hosts, or an anthroponosis usually find only in humans as reservoirs [32]

The taxonomic position of Leishmania is as follow [33]

Kingdom:	Protista
Subkingdom:	Protozoa
Phylum:	Sarcomastigophora
Subphylum:	Mastigophora
Class:	Zoomastigophora
Order:	Kinetoplastida
Suborder:	Trypanomatina
Family:	Trypanosomatidae
Genera:	Leishmania spp.

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Literature reviews about a genetic of Leishmania spp. in the GenBank, can divide into 11 groups as showed in table 1.

Table 1: Leishmania spp. presented in the GenBank [34]

Leishmania species	Leishmania species
1) Leishmania aethiopica species complex (1) • *Leishmania aethiopica* *2) Leishmania aristidesi* *3) Leishmania deanei* *4) Leishmania donovani species complex (3)* • *Leishmania chagasi* • *Leishmania donovani* • *Leishmania infantum* *5) Leishmania hertigi* *6) Leishmania major species complex (2)* • *Leishmania major* • *Leishmania cf. major* *7) Leishmania mexicana species complex (4)* • *Leishmania amazonensis* • *Leishmania enriettii* • *Leishmania Mexicana* • *Leishmania pifanoi* *8) Leishmania tropica species complex (1)* • *Leishmania tropica* *9) Lizard Leishmania (5)* • *Leishmania adleri* • *Leishmania gymnodactyli* • *Leishmania hoogstraali* • *Leishmania tarentolae* • *Leishmania sp. NC29/Iran/2007* *10) Viannia subgenus group (10)* *10.1) Leishmania braziliensis species complex (4)* • *Leishmania braziliensis* • *Leishmania colombiensis* • *Leishmania equatorensis* • *Leishmania peruviana* *10.2) Leishmania garnhami* *10.3) Leishmania guyanensis species complex (3)* • *Leishmania guyanensis* • *Leishmania panamensis* • *Leishmania shawi* *10.4) Leishmania lainsoni species omplex (1)* • *Leishmania lainsoni*	*10.5) Leishmania naiffi species complex (1)* • *Leishmania naiffi* *11) Unclassified Leishmania (28)* • *Leishmania arabica* • *Leishmania gerbilli* • *Leishmania guliki* • *Leishmania herreri* • *Leishmania killicki* • *Leishmania turanica* • *Leishmania sp.* • *Leishmania sp. AM-2004* • *Leishmania sp. BK-2007* • *Leishmania sp. Ghana-2006* • *Leishmania sp. IMT208* • *Leishmania sp. IRN580* •*Leishmania sp. MHOM/IN/2003/NAV-122* • *Leishmania sp. MHOM/IN/2003/NAV-131* • *Leishmania sp. MHOM/IN/2003/NAV-132* • *Leishmania sp. MHOM/IN/2003/NAV-135* • *Leishmania sp. MHOM/MQ/92/MAR1* • *Leishmania sp. SA-2000* • *Leishmania sp. shifai* • *Leishmania sp. siamensis* • *Leishmania sp. SL/R/1* • *Leishmania sp. SL/R/2* • *Leishmania sp. SL/R/3* • *Leishmania sp. SL/R/4* • *Leishmania sp. SL/R/5* • *Leishmania sp. SL/R/6* • *Leishmania sp. SL/R/7* • *Leishmania sp. SL/R/8* • *Leishmania sp. SL/R/9* • *Leishmania sp. SL/R/10*

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1.2 Morphology

Leishmania spp. is a parasite that has 2 forms; promastigote (Figure 1) and amastigote (figure 2). Promastigote has a slender shape, a flagellated form that found in sand flies and culture medium. The size of this form is 10 to 15 µm in length. While amastigote form, which lives in the mammalian macrophage with an aflagellated form, has an oval or round shape, the size in this stage is 2 to 5 µm. They show a small rod and a big size of nucleus [35] The small rod shape of kinetoplast DNA (kDNA) is a single mitochondrial DNA that shape is shown a common disc. They are unusual arrangement of deoxyribonucleic acid. The fine structure of kDNA is a filamentous body containing thousands of catenated DNA molecules that divided into two classes: minicircle and maxicircles. The maxicircle role to edit of uracil residues into mRNA nucleotides, the minicircle encode guide RNAs (gRNA) for editing cytochrome oxidase subunit III mRNA [35-37]

Leishmania spp. can be alive and replicating intracellular of host because they have a surface coat such as GP 63. GP63 (glycoprotein 63) or leishmanolysin, which was a major surface antigen expressed on Leishmania promastigotes more than amastigotes, is the 63 KDa zinc-dependent metalloprotease. The molecules of GP63 are attached to the cell surface via glycosylphosphatidylinositol (GPI) [38, 39], its role is to protect Leishmania from mammalian hosts by avoiding the infection process (innate immune response) [39-42].

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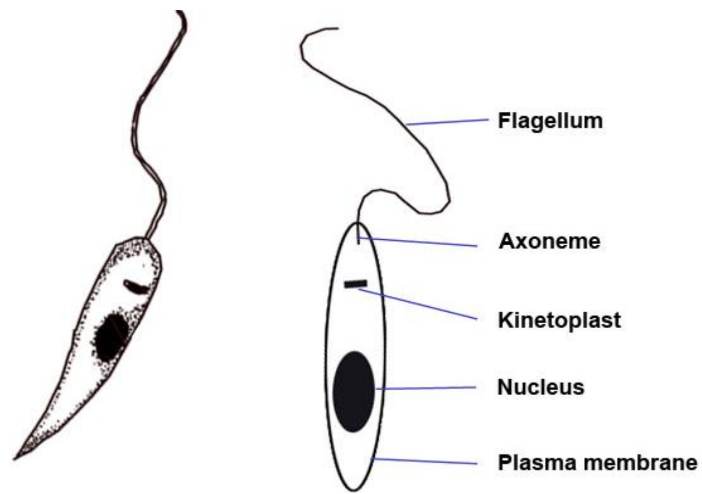


Figure 1: Morphology of promastigote stage of *Leishmania spp*. (modify picture) [35]

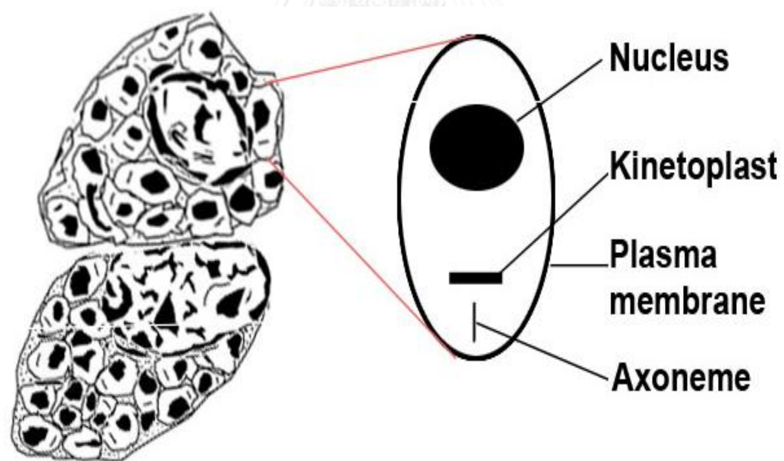


Figure 2: Morphology of amastigote stage. (modify picture) [35]

1.3 Life cycle

Leishmania spp. is transmitted by the bite of female sand flies that need blood for their eggs (*Phlebotomus* species in the Old World, *Lutzomyia* species in the New World). In sand flies stage, female sand flies become infected when they feed on the blood of an infected mammalian or animal host during blood meal. The parasites amastigote (non-flagellate) form within phagolysosomes of macrophages are ingested up by sand flies, which differentiate into promastigote (flagellate) in the sand flies' midgut. Changes of the conditions form mammalian host to the sand fly midgut (increase in pH, decrease in temperature) are activated the parasite in the vector from amastigote to promastigote form which contain motility organ, the flagella at the anterior end [32, 43]. This form develops and changes their structure from avirulent to virulent. While they migrate to the mouthpart of insect, the term of process calls metacyclogenesis [44]

On the other hand, in human stage (mammalian stage), the infectious sand flies not only take a blood meal but also inject promastigote form into human host by inserting the mouth part through the host's skin and therefore create a small wound. After that promastigote form enters the skin, they are quickly phagocytosed by macrophage in the inoculation site by the inflammatory reaction. Then promastigote changes their form into mammals (intracellular) aflagellated as amastigote forms [3] and/or free amastigotes into blood stream. They can survive and proliferate within the phagolysosome of macrophages. The life cycle of Leishmania is shown which in the figure 3

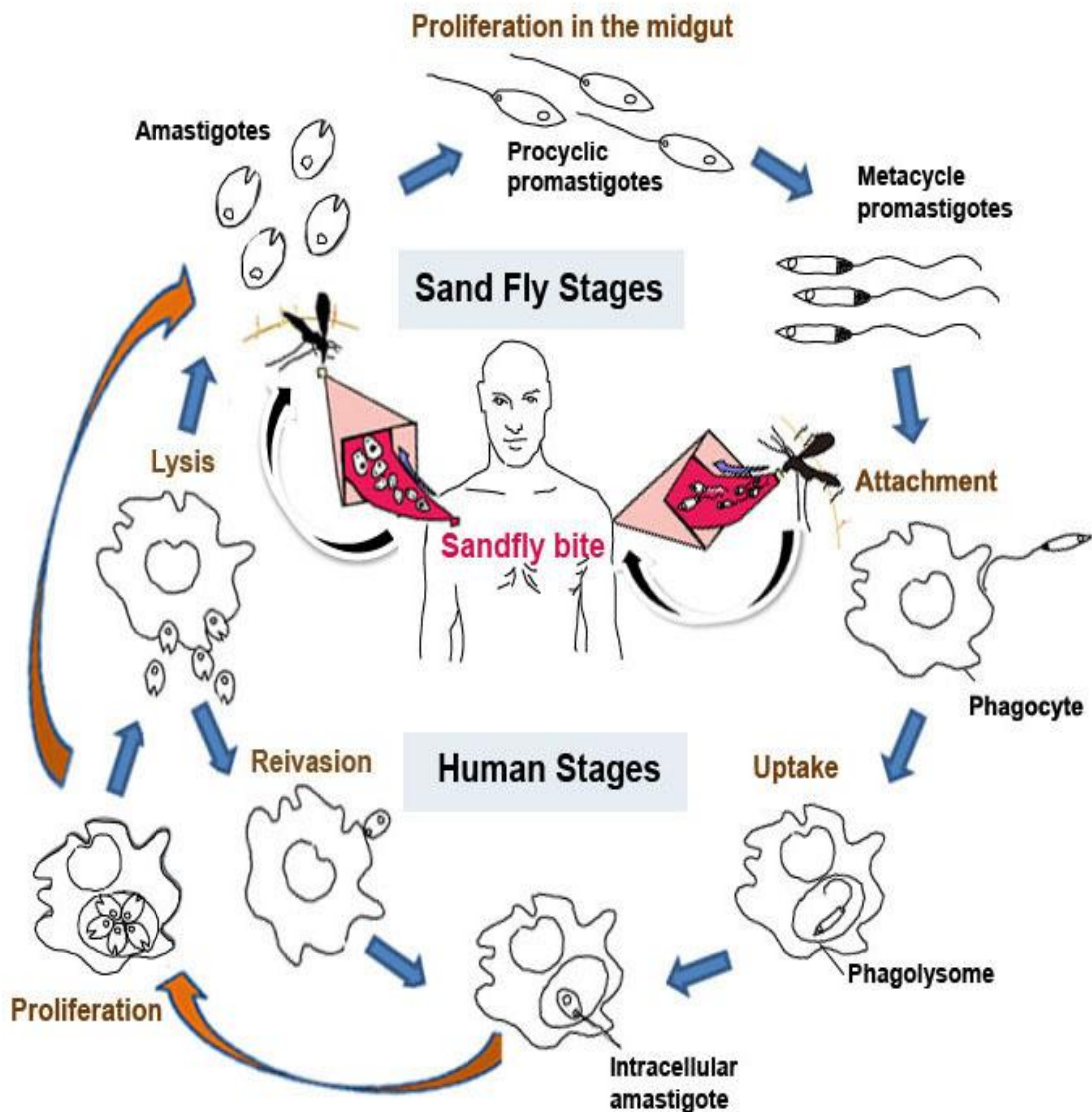


Figure 3: Life cycle of *Leishmania* (modified from)

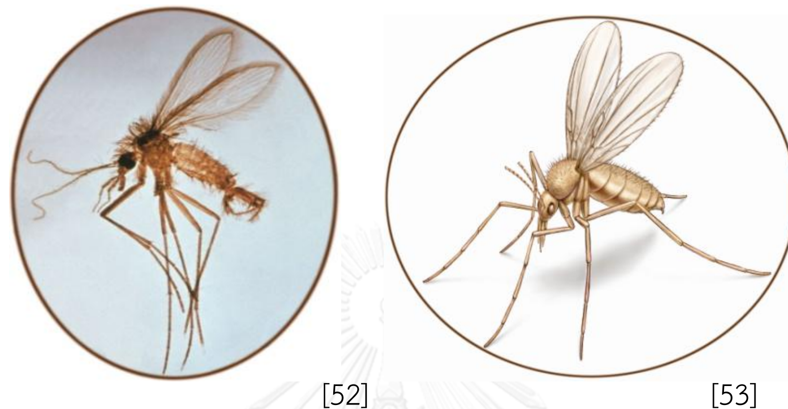
(National Institute of Allergy And Infectious Diseases; [45]

2. Vectors

The vectors of Leishmaniasis are the infected female sandflies (figure 4) that the distribution of disease depends on the widespread of vectors. There are over 800 species of sandflies but 30 sand fly species are vector of the disease; the main species of sand fly are *Phlebotomous* in (old world) Asia, Europe, Africa and *Lutzomyia* in (new world) Central and South America [46]. The size of body length is usually 1.5-2 mm [47] and wings length is less than 3 mm [9]. The variety of vector species can act some roles in preservation of the transmission cycle. The activity of sand fly forages in the nocturnal (6:00 PM - 6:00 AM) more than the diurnally (6:00 AM – 6:00 PM) in the ratio 2.6:1, respectively [48]

In Thailand, many investigators in different areas found and recorded 24 species of sand flies in different area; *Phebotomus asperulus*, *P. argentipes*, *P. hoepplii*, *P. burguesae*, *P. major major*, *P. stantoni*, *P. mascomai*, *P. philippinensis gouldi*, *P. teshi*, *Sergentomyia barraudi*, *S, indica*, *S. anodontis*, *S. bailyi*, *S. dentata*, *S. quatei*, *S. gemmea*, *S. silvatica*, *S, hodgsoni hodgsoni*, *S. mahadevani*, *S. perturbans*, *S. punjabensis*, *S. iyengari*, *Nemopalpus vietnamensis* and *Chinius barbazani*.[49]The predominant vector of *L. siamensis* is *Sergentomyia (Neophlebotomus) gemmea* [50]. The sand flies distribute in the north and the south; the north at Phitsanulok province in Naresuan Cave is not the leishmaniasis-affected area. The reporter showed 13 species of sand flies belonging to 4 genuses; 4 *Phlebotomus*, 1 *Nemopalpus*, 7 *Sergentomyia* and 1 *Chinius. N. vietnamensis* (49.15%) is abundance specie while *Sergentomyia gemmea* is lesser than 1% (0.05%). Moreover, the important information of *P. argentipe* species is the vector of *Leishmania sp. that* had been found in all day. Therefore, this observation shows the risk period in day

time of leishmaniasis [48]. Recently new knowledge of sand fly, that investigate the risk areas of leishmanias at southern of Thailand, showed 6 species of genus *Sergentomyia* and one of genus *Phlebotomus*.[51] *S. gemmea* is the predominant and potential vector of *L. siamensis* [50, 51]



[52] [53]

Figure 4: Sand flies of the *Phlebotomus specie*

3. Geographical distribution

Leishmaniasis broadly distribute into the Old World and the New World that associated with the subgenus of *Leishmania species.* The New World detects species of *L. Mexicana* complex, *L. deanei, L. hertigi,* and all species of subgenus Viannia, while other subgenus of Leishmania are found in the Old World, except *L. major* and *L. infantum* or *L. chagasi* are isolated in both areas [54] Furthermore, *L. siamensis* is a new species recently described from Thailand that is found in the Old World [16] Wilson E. Mary, et al. shows table of major species of *Leishmania* in each geographic location in table 2.

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The endemic areas are tropics, subtropics, and southern Europe, in settings ranging from rain forests in the Americas to deserts in western Asia, and from rural to periurban areas [25]. This disease is one of the most significant of the neglected tropical disease that can estimates the infection results in 2 million new cases a year

furthermore leishmaniasis threaten 350 million people in 88 countries [55]. These main species and endemic areas of VL show in figure 5.

3.1 Epidermiology of leishmania in Thailand

In Thailand, 3 species of VL were reported; *L. donovani, L. infantum* and *L. siamensis* [56]. *L. siamensis* was filled in to unclassified *Leishmania sp.,* which is suspected a new species. The analysis method is used to identify the new species by amplification the ITS1 region of small subunit ribosomal RNA (ssrRNA) angene (GenBank accession no AF303938) and the amplified PCR products of the ITS1 region of the rRNA and DNA gene demonstrated. Moreover, Leelayoova S., et al. study the taxonomic diversity of *L. siamensis* differential from other *Leishmania sp.* By using multilocus characterization and phylogenetic tree [57].

VL is one form of leishmaniasis, which is the most severe form including a novel specie in Thailand. There are several reports of *L. siamensis* that can infect human [16] Report of Leishmaniasis in Thailand can be divided into 2 periods; i) during 1960 to 1986 in table 3, leishmaniasis causes reports were imported from Pakistanian, Indian, Bangladesh and the Middle East.[11, 12] ii) during 1996 to 2010 in table 4, leishmaniasis cases were autochthonous caused by *L. donovani, L. infantum* and *L. siamensis*. Number of autochthonous leishmaniasis caused by *L. siamensis*, has been dramatically increased autochthonouse VL not only is detected in the south but also the north in the figure 6, further the table case of autochtonous *Leishmania* in Thailand between 1996 and 2012 shows more detail in table 4.

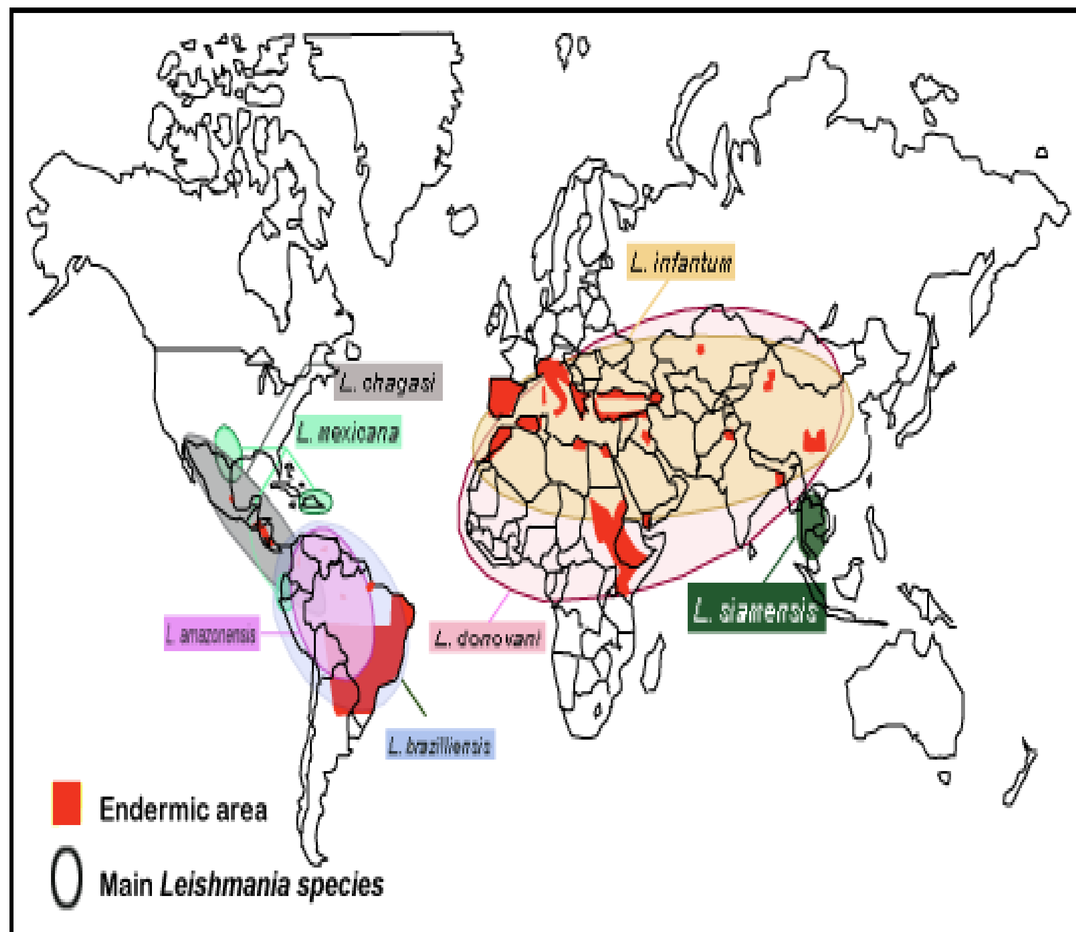

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Figure 5: Geographical distribution of main *leishmania species* and visceral leishmaniasis in the Old and New World (modify picture) [58]

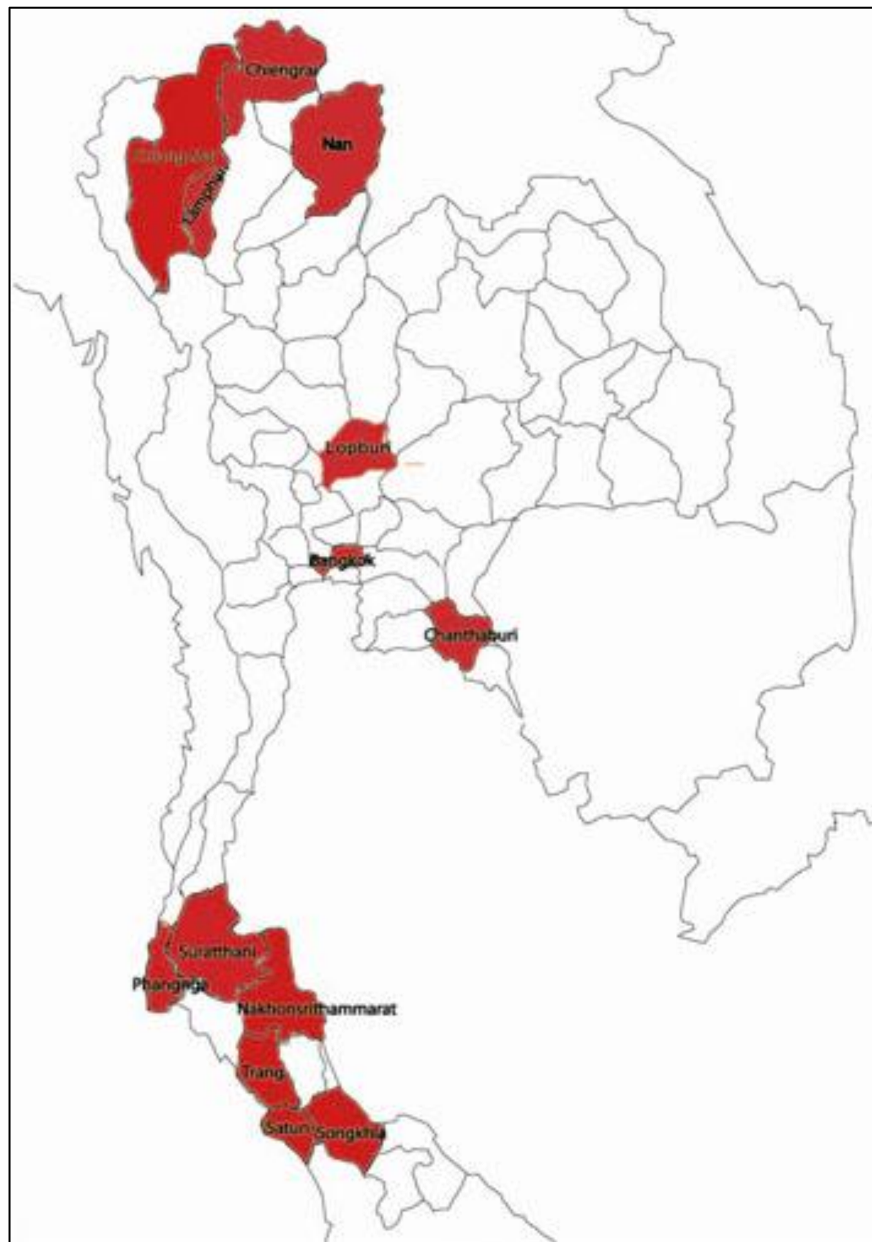

Figure 6: Thailand map**:** The red color showed the area of detection of autochthonous VL cases reported in Thailand during 1996-2014 [59]

Table 2 **:**Major species of *Leishmania* in each geographic location[60]

Species[a]	Major disease syndrome	Geographic location
L. dovononi	VL, PKDL[b]	India, North and Eastern China, Pakistan, Nepal
L. infantum[c]	VL	Mediterranean, Middle East, Balkans, Asia, northwest China, northern and sub-Saharan Africa
L. chagasi[c]	VL, CL (rare)	Latin America
L. archibaldi	VL, CL	Sudan, Kenya, Ethiopia
L. major	CL	Middle East, northwest China, northwest India, Pakistan, Africa
L. tropica	CL, VL	Mediterranean, Middle East, west Asiatic region, India
L. aethiopica	CL, diffuse CL	Ethiopia, Kenya, Yemen
L. mexicana	CL, rarely ML	Mexico, Central America, Texas
L. amazonensis	CL, diffuse CL, rarely VL	Amazon basin, Brazil
L. pifanoi	CL, diffuse CL	Venezuela
L. garnhami	CL	Venezuela
L. venezuelensis	CL	Venezuela
L. braziliensis	CL, ML	Latin America
L. guyanensis	CL	Guyana, Surinam, Amazon basi
L. peruviana	CL	Peru, Argentina highlands
L. panamensis	CL	Panama, Coata Rica, Colombia

[a] The subspecies Leishmania Viannia are indicated on this table.
The subspecies designation is not indicated throughout the text.
[b] PKDL-post-kala-azar dermal leishmaniasis.
[c] Evidence is accumulating that L. infantum and L. chagasi are the same species.

Table 3: Previous reported VL cases in Thailand

Year	Country	Reference
1960	An imported Pakistanian case	[11, 12]
1977	An imported Indian case	
1984	An imported Bangladesh case	
1985-1986	5 Thai-worker cases, who come back from the Middle East	

Table 4: **T**he representative of autochtonous *Leishmania* case in Thailand

Province, Year	**Age(year), Sex**	**Occupation**	**Species, form of *Leishmania***	**Reference**
Suratthani, 1996	3, Female	Not applicable	No species identified	[61]
Nan, 2005	40, male	Construction worker in several	*L. donovani*, VL	[18]
Phangnga , 2006	55, male	Worker in rubber plantation	*L. siamensis* VL	[16]

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Table 4: The representative of autochtonous Leishmania case in Thailand (Continue)

Province, Year	Age(year), Sex	Occupation	Species, form of Leishmania	Reference
Bangkok, 2007	66, male	Lumber truck driver	L. infantum, VL	[17]
Nakom Sri Thammarat, 2007	44, Male	Rubber plantation	L. donovani, VL	[62]
Chiangrai, 2007	36, Male	Employee	L. siamensis, CL	[59]
Chantaburi, 2009	37, Male	Fisherman, a history of travel to North Indonesia	L. siamensis, VL	[15]
Songkhla, 2012	46, Male	Rubber planter	L. siamensis, VL overlapping with CL	[14]
Trang, 2012	30, Male	Pet store owner		
Lop Buri 2012	3 female	-	L. siamensis, CL	[59]
Satun 2013	5 female	-	L. siamensis, VL	
Lumpoon, 2013	52 Male	farmer	L. siamensis, VL	[35]
Chiang Mai 2014	39 Male	Unpublished data	L. siamensis, VL overlapping with CL	
Chiang Mai 2014	52 Male	Unpublished data	L. siamensis, VL overlapping with CL	

4. Disease pattern

There are 3 main forms of the leishmaniasis: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL). VL form causes by *L. infantum* or *L. chagasi* and *L. donovani* [63]. The clinical manifestations of the disease may be present severe symptom or no symptom, depends on the infecting of *Leishmania species* that shows in table 5 [19]The aggressive symptom is determined on the specific subtype and the host immune situation [3]

Table 5: *Leishmania sp*. responsible for CL, MCL, VL and PKDL[a] [64]

Pathology	*Leishmania* spp.	Country (region)
CL	*Leishmania major*	Old World (African continent, Middle East)
	Leishmania tropica	Old World (African continent, Middle East)
	Leishmania aethiopica	Old World (African continent, Middle East)
	Leishmania venezuelensis	New World (South American continent)
	Leishmania mexicana	New World (South American continent)
	Leishmania amazonensis	New World (South American continent)
	Leishmania braziliensis	New World (South American continent)
	Leishmania panamensis	New World (South American continent)
	Leishmania peruviana	New World (South American continent)
	Leishmania guyanensis	New World (South American continent)
	Leishmania donovani[b]	Mediterranean and Caspian sea region
	Leishmania infantum[b]	Mediterranean and Caspian sea region
MCL	*Leishmania braziliensis*	New World (South American continent)
	Leishmania panamensis	New World (South American continent)
	Leishmania guyanensis	New World (South American continent)
	Leishmania amazonensis	New World (South American continent)
	Leishmania mexicana	New World (South American continent)
VL	*Leishmania donovani*	Old World (African continent, Middle East, India)
	Leishmania chagasi	Old World (African continent, Middle East, Mediterranean basin)
	Leishmania infantum	Old World (African continent, Middle East, Mediterranean basin)
	Leishmania archibaldi	Old World (African continent, Middle East, Mediterranean basin)
	Leishmania tropica[c]	Old World (Middle East)
	Leishmania amazonensis[c]	New World (South American continent)
PKDL	*Leishmania donovani*	Old World (African continent, Middle East, India)

[a]Abbreviations: CL, cutaneous leishmaniasis; MCL, mucucutaneous leishmaniasis; VL, visceral leishmaniasis; PKDL, Post-kala-azar dermal leishmaniasis.
[b]In rare instances, *L. infantum* and *L. donovani* have caused CL.
[c]In rare instances, *L. tropica* and *L. amazonensis* have caused VL.

4.1 Cutaneous leishmaniasis (CL)

The Leishmania species is commonly caused of CL such as *L. mexicana, L. major, L. tropica.* CL symptoms are usually absent the lesions; begin with an area of small redness and papule up to 2 cm in size at the inoculation site (figure 7). After that it enlarges to a nodule, crusts and eventually ulcerates with exudates formation

or dry with a crusted scab (figure 7). The lesions, which are usually found on exposed parts of the body such as arms, face and legs, may develop anywhere from a few weeks to months. Moreover the lesion is often chronic and unresponsive to antibiotics or steroids [19, 65, 66]

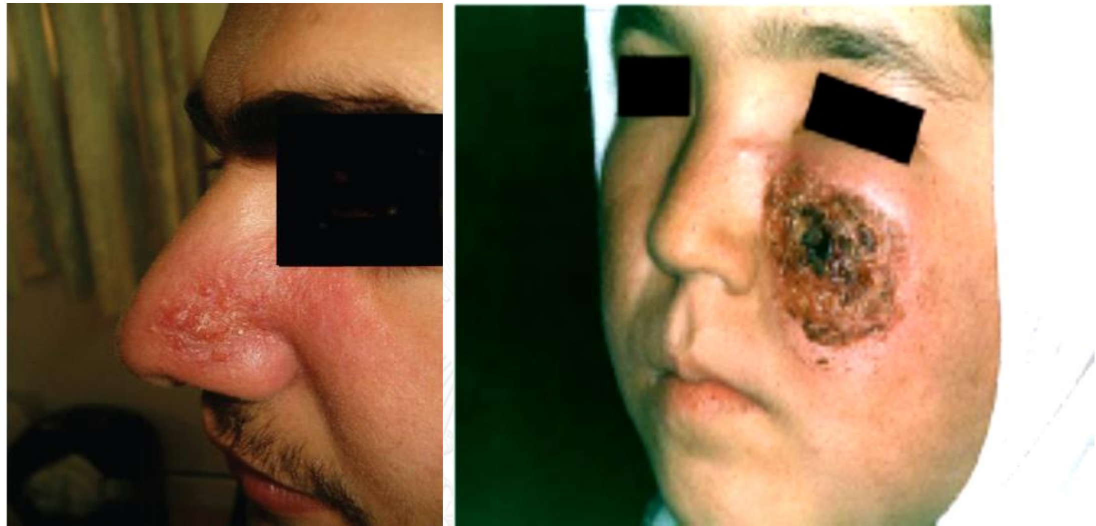

Figure 7: CL at crusting stage in a patient from Malt and Kabul [3, 19]

4.2 Mucocutaneous leishmaniasis (ML)

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Leishmania braziliensis is the majority cause of ML that is often developed after the primary lesion has healed. The initial symptoms are mild, with nasal stuffiness and inflammation, but ulceration and perforation of the septum may slowly ensue. These lesions characterize of the entire nasal mucosa which can lead to nasal obstruction and bleeding. The lesion may extend to soft palate, the face, larynx or pharynx such as the palates can also become deformed along with ulceration of the nasal septum and lips (figure 8). Other signs can be found including gingival edema, periodontitis and adenopathy. Secondary infection plays outstanding

role in the size and persistence of ulcers. This causes difficulty with eating and an increased risk of secondary infection which carries a significant mortality [19, 65-67]

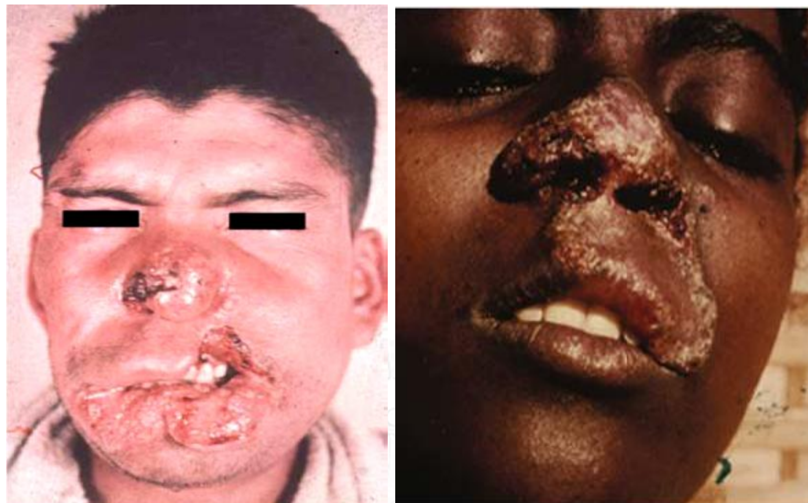

Figure 8: Mucocutaneous leishmaniasis [3];[64]

4.3 Visceral Leishmaniasis (VL)

VL also known as "kala azar or black fever", it is the most severe form with chronic disseminating disease usually presents with hepatosplenomegaly (figure 9). The disease is often fatal unless treated by chemotherapy. The fatal courses may be disturbed from coagulation. The clinical symptoms are characterized by undulating fever, loss of weight, hepatospenomegaly, lymphadenopathy, pancytopenia, anemia and thrombocytopenia [5, 6, 15]. This disease comprises a broad range of manifestations of infection. It may be asymptomatic; self-resolving or subclinical in many cases or presents with acute but usually runs a chronic course. Death usually occurs in case of severe secondary bacterial infections in advanced disease [6, 7]

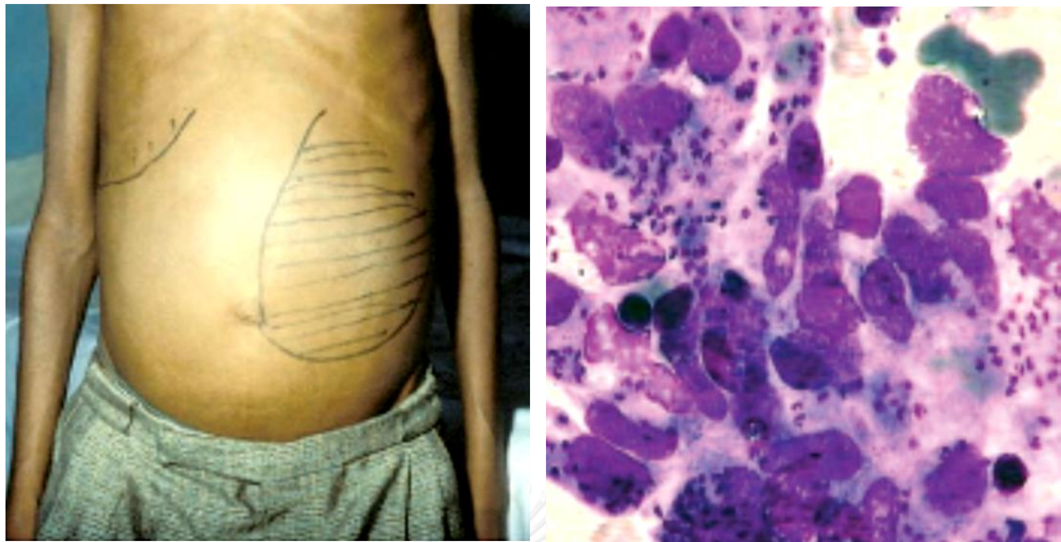

Figure 9: Visceral leishmaniasis (*L. donovani*) [9]

4.4 Post-kala azar dermal leishmaniasis (PKDL)

PKDL is suspected with patients live in endemic areas or use to be travelled to one because some PKDL patients have not the history of VL or kala-azar. Clinical diagnoses assess from the parasite or the clinical criteria or both [68]. The diagnosis of skin lesions are macular, maculo-papular or nodular rash (Figure 10). The rash usually starts around the mouth area to other parts of the body depending on severity. The interval development of PKDL is variable. The predominant distributed of PKDL occur in the Indian subcontinent and East Africa (1-3% of patients in India and 50% in Sudan) that is the endemic area VL. This is usually due to infection by the *L. donovani* complex that includes *L. donovani*, *L. infantum* and *L. chagasi*.[8, 69] The report of PKDL relapses after treatment for VL with miltefosine,[70] sodium stibogluconate, amphotericin B and paromomycin. [68].

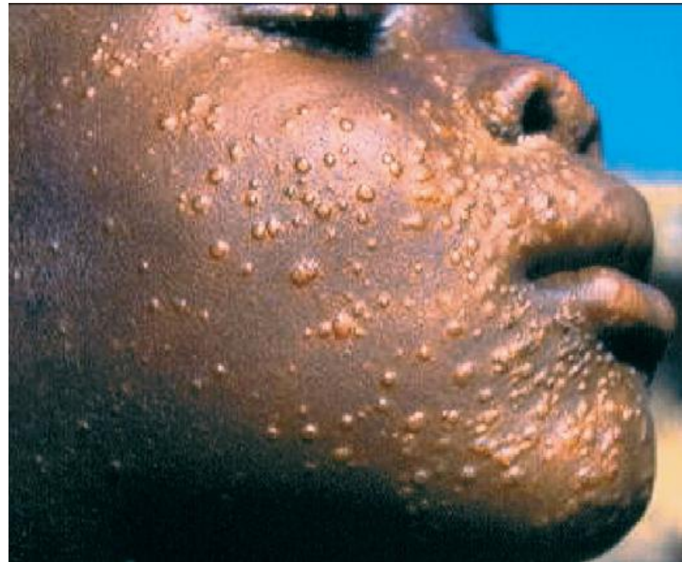

Figure 10: PKDL popular lesions [69]

4.5 Leishmania/HIV coinfection [71]

*Leishmania/*HIV co infection emerges as a serious new disease pattern and becomes dramatically increasingly frequent [65]. In 1999 co-infection of disease was reported that it was gradually increased worldwide. The symptoms occur from increase of immunosuppression and activate of virus replication [71]. The risk of developing VL by a factor is associated with HIV infection increases between 100 to 2,320 times in endemic areas, and greatly increases the probability of relapse and reduces the likelihood of therapeutic response [72]. At the same time, VL promotes clinical progression of HIV disease and the development of AIDS-defining conditions. Because the two diseases target similar immune cells, together they exert a synergistic damaging effect on the cellular immune response. Atypical presentations of leishmaniasis are reported in HIV patients, including vascularization of CL and cutaneous involvement in VL [2].

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4.6 Clinical pattern of *L. siamensis*

The pattern of disease can provide to 3 groups; VL, CL and VL overlapping with CL. The disease usually present relate to Human immunodeficiency Virus (HIV) infecton such as in the year 2014, the report of multiple cutaneous nodules related with an HIV infected patient [73]. Recently reported connection with systemic steroid that present multiple erythematouss, shiny infiltrative plaques and nodule on the body and face [74]. The next form VL, this form is the first detection and there are some report of VL overlap with CL [75] report the symptom in 2012. All pattern of diseases were conclusion by Suankratay C., 2014 in the table (appendix D) [59]. There are some report of *L. siamensis spp*., that is detected in CL form in other countries such as cows and a horse in Switzerland and Germany [76, 77]

5. Diagnosis

VL may be shared a symptom both cutaneous and VL, so the disease concerns an infected macrophages, which present in the blood stream of subcutaneous or intradermal and visceral organ [78]. Diagnosis methods use both the detective parasites and testing antibody based on traditional, serological and molecular diagnosis. Patient specimens collect for testing such as bone marrow, blood, tissue, saliva and urine [75].

5.1 Conventional diagnosis techniques: the common screening comprise of microscopy and cell tissue culture. General diagnostic of clinical parasitic infection uses microscope to be the gold standard for detection and identification morphology of parasite [79]. Light microscopy can be directly searched an amastigotes biopsy specimen such as tissue or isolation parasite in visceral organ collects from bone

marrow, splenic and blood aspiration. These parasite samples can be conventionally stained by Giemsa staining smear or histopathology hematoxylin - eosin staining. The Giemsa stain slide can help PCR to diagnose the suspect VL specimen, moreover, no problem in keeping for a long time (36 years) [80] The method of histopathology (72.34%) is high sensitivity than skin smear (54.33%).[81]. A remain aspirate and biopsy specimens culture a parasite cells in artificial culture agar, previous known as Schneider’s insect medium, M199, or Novy-MacNeal-Nicole medium (NNN) in order to get better the sensitivity of detection. Culture method is not imperative to be used in clinical detection (routine), because it has a high cost and a taking time. However, it is desired to apply for researching. In research field uses culture parasite (promastigote form) for studying in vitro screen drug, for inoculation in animal experimental, for confirmation the result when other problem techniques, for testing antigen-antibody. Moreover, the direct observations show low sensitivity except splenic aspirate is highly sensitivity (93.1-98.7%) [10], however, it causes of trauma to be fatal from hemorrhage. Singh R.K., et al, 2006 reports about splenic aspirate, is to be only the gold standard in diagnosis the VL patient [21, 67, 82] and Maia Z., et al. 2012 report that the diagnosis of amastigotes on hematological is a gold standard diagnosis [83]. The aspirate technic is used in endemic area; a large number of patients are not appropriated in using this method owing to takes time and skill in reading and preparing slide. Nevertheless, the experience of pathologist will be improved vary from an amount of diagnosis. The sensitivity of microscopic can increase by an advance staining such as immunofluorescence, immunoperoxidase, *in situ* hybridization [84], fluorescence in situ hybridization. However, these methods

give the low sensitivity than other method such as serological and molecular diagnosis[10]

5.2 Serological diagnosis: the diagnosis is based on the detection of antibody and parasites on the sample such as DAT (direct agglutination test), ELISA (enzyme linked immunosorbent assay) and recombinant kinesin 39 (rK39) [7] This type of method is not widespread in diagnosis of CL because it shows occurring cross reaction with other infection and low sensitivity [67]. It is commonly assayed of VL in the field. The problem of these methods cannot diagnose the VL patient who has a positive value for a long time after completely treatment [58] However, the endemic area of VL such as Ethiopia is appropriately diagnosed by using both DAT and rK39[85]

5.2.1 Direct agglutinate Test (DAT): This test is semi-quantitative test that is used to screen in endemic area in case of the required result only reads positive and negative [58, 86] This method can use a sample of blood or serum from the patient of VL. Start, the patient samples (blood, serum) are screened by doing the dilution. The plasma sample is diluted at 1:50 or 1:100 with β mercaptoethanol in 0.9% NaCl and adding control solution (negative, positive wells) [35] Only the blood test is started higher dilution at 1:1,600 so as to discriminate the confuse sedimentation positive from negative reaction. Then, following with single two-fold serial dilution to 1:3,200 [87] or 102,400 [35] for each specimen. Pipet 50 µL of DAT antigen solution put in the 96 V-shaped wells plate. After well mixing with dilution specimen, the micro-titer plate is sealed and incubated at room temperature (25°C) for 18 hr. or overnight [50]. The result of this test is observed from the present of agglutination that present from the reaction of parasite against with antibody. The

endpoint titer, which is the last well, show the agglutination same as the positive control [88] in figure 11A. The sensitivity and specificity of DAT is revealed in the table 6 that was 94.8% and 97.1% respectively [10]

5.2.2 *Enzyme-linked immunosorbent assay (ELISA):* This serodiagnosis is used to detect the quantity and present of all patient antibodies against a specific antigen so it is the high potential method to detect particular infectious diseases such as VL. The commonly used antigens are a crude soluble antigen (CSA) and rK39. First, CSA is prepared from a suspension of promastigotes by freezing and thawing. The antigen solution is the final product, be collected for coating microtiterplate of ELISA. There are the reported of sensitivity in range 80-100% [7, 21] and specificity 96% [10]. Second, rK39-related antigen is the micro form of ELISA (immunochromatographic strip test) so it is high sensitivity, specificity to be 93.9 and 95.3% respectively [10]. Ritmeijer K., et al., 2006 reports the suitability of rK39 rapidly screen and the convenience tests in diagnosis of VL [82]. Recombinant antigen (rK39) is comprised of 39 amino acids (117 base pairs) from the conserve kinesin region of *L. chagasi.* Moreover, the strip test is convenient equipment for diagnosis VL in the field [10, 58]. This strip is developed by fixing rK39 antigen on nitrocellulose paper. The patient blood or serum directly tested by smearing on the tip of the strip. It is several in dropped with phosphate buffer saline (PBS). They will move to react with rK39. The reaction product of antigen and antibody presents 2 pink bands of infected patient in case of no infected sample will appear only 1 pink band. In each reaction can read in 15 minutes [10] figure 11B. The limit of detection VL can cross reaction with other illness such as malasia, typhoid fever. The infected VL, which has already cured completely, cannot diagnosis of relapses because the antibody is still

presented for a long time [58]. However, these problems can dissolve with antigen diagnosis [21]

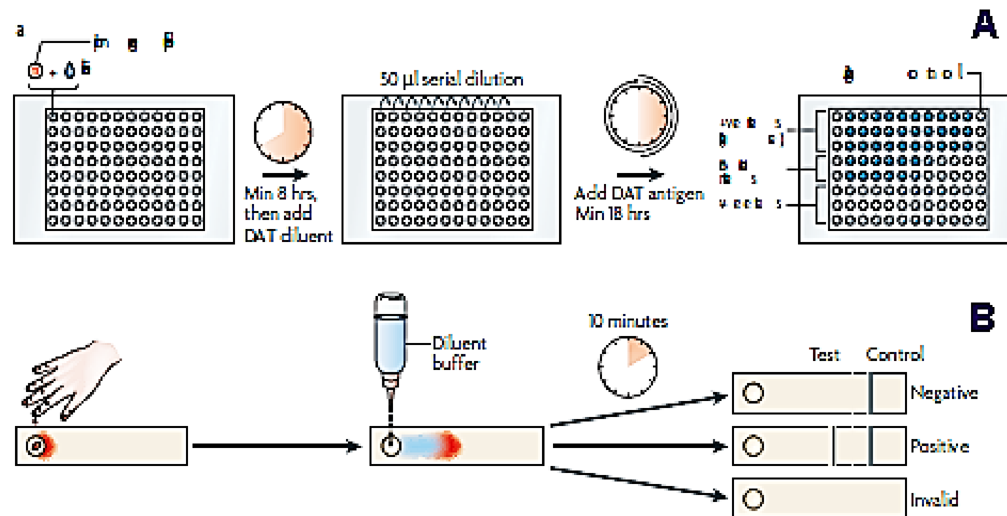

Figure 11: Serological tests for visceral leishmaniasis.[58]

A: The direct agglutination test,

B: The rK39 immunochromatographic test strip

5.3 Molecular diagnosis: many analytical of molecular techniques have been developed for diagnosis of leishmaniasis including PCR, qPCR. PCR is the important basic tool for research and clinical diagnosis of VL, that it is chose to identify the difference species of *leishmania* [89, 90] The different format of PCR can be classified in 3 wide range; Low, Mid and High pattern. The Low pattern is a simple PCR that comprise with a mini-set of tool such as LAMP (Loop-mediated isothermal amplification) technic. It is consisted the important direction of 2 steps, those are to amplify and assay the product of PCR. The Mid pattern is the popular tool such as conventional PCR, PCR-RFLP (PCR and restriction fragment length polymorphism) and

The final pattern (High pattern) is presented by real time PCR (qPCR) [91] .PCR tool is used to detect VL both immunocompromised and immunocompetent by using DNA. It can be rapidly identified species of parasite [37] and excellently dissolved the problem of serology by specificity and sensitivity [92]. Design primer of PCR assay amplifies region of ITS1 rRNA gene, Leishmania parasite is presented at 0.1 parasites / PCR reaction [90]. The diversity nucleic acid of both DNA and RNA are developed for targeted assay. The area of high copy number is usually found in conserved regions of DNA sequence that is designed to be primer. Leishmania 's primer is designed from DNA sequence of the genome such as ITS (internal transcribed spacer), gp63 locus[93], cysteine protease B, kDNA (kinetoplast minicircle DNA)[94], 18srRNA[7], (SSU)rRNA.[95, 96] The next step of PCR amplification is resolved by gel electrophoresis and observing result after staining with ethidium bromide. These techniques are operated in several laboratory [91]. Pandey K., et al, 2010, presents, 12% of all VL sample is not detected by microscope but it solves with PCR [97].

Real-time PCR (qPCR) is an analyzer amplification process of PCR tool that is stains with SYBR-green [91]. Table 7 and 8 shows some sample of qPCR primers that uses to detect and identify Leishmania sp. This method is commonly qPCR to study in quantify DNA during the amplification sequence target connects to fluorescent monitor. The fluorescent signal can be calculated the initiative sequence so as to check the end point of effect [98]. It is accurate and rapid tool to be gainfully diagnosed Leishmaniasis so early diagnosis is very important for anti-leishmania medication. This quality is applied to assay PKDL and VL patients However qPCR, that is compared with conventional PCR, is more expensive than semi-quantitative

PCR[99]. Mary C. et al., 2004 presents sensitivity 0.0125 parasites / ml by using qPCR to detect kinetoplast DNA of *L. infantum* [100] .

These technics are high sensitivity and specific method than traditional and serological diagnosis [67, 90]. *Leishmania* cell or the part of free DNA can release to fluid such as blood, secretion the molecular tool can detect a clinical sample not only whole cell but a piece of free circular. The development of molecular diagnosis with using nucleic acid amplification may apply to assay an asymptomatic patient from low load of parasite infection [79]. All methods (conventional, serological and molecular diagnosis) show the sensitivity and specificity in the table 6.

Table 6: Diagnostic assays for visceral leishmaniasis

Assay	Test time	Required skill level	Senstivity	Specificity	Comments
Parasitological diagnosis: Microscopic examination	Hours	Expert	Splenic aspirate 93.1-98.7%[9-11]; Bone marrow aspirate 52-85%[11]; Lymph node aspirate 52-58%[9]	100%	Restricted to endemic areas where clinicians are familiar with signs and symptoms, and for culture sophisticated laboratories are required.
Agglutination test (using urine)	Minutes	Medium	64-100%[62,63]	100%	Ideal method of diagnosing an infectious agent; antigen level correlates with parasite load.
Indirect Fluorescent Antibody Test	Hours	Expert	96%[15]	98%	Requires equipped laboratory setup.
Direct Agglutination Test	Hours	Medium	94.8% (95% CI, 92.7-96.4)[19]	97.1% (95% CI, 93.9-98.7)	Useful for epidemiology studies.
Immunochromatographic Strip Test	10 minutes	Low - Lab technician	93.9% (95% CI, 87.7-97.1)[19]	95.3% (95% CI, 88.8-98.1)	Used for screening by using rK39 strip, not useful in treated patient.
ELISA	Hours	Medium	100%[23]	96%	Cannot be used in field setting.
PCR	4-5 hrs	High	70-100%[36,41-43,61]	85-99%	Standardization is hindrance, lab to lab variation and contamination.

Table 7: Different target genes and primers using for detection of *Leishmania spp.* [35].

Target Gene	Primer Name	Nucleotide sequence (5' → 3')	References
ITS1	L5.8S LITSR	TGA TAC CAC TTA TCG CAC TT CTG GAT CAT TTT CCG ATG	[96]
kDNA	RV1 RV2 13A 13B	CTT TTC TGG TCC CGC GGG TAG G CCA CCT GGC CTA TTT TAC ACC A GTG GGG GAG GGG CGT TCT ATT TTA CAC CAA CCC CCA GTT	[94]
Mini-exon	Fme Rme	TAT TGG TAT GCG AAA CTT CCG ACA GAA ACT GAT ACT TAT ATA GCG	[101]
SSU-rRNA	S4 S12	GAT CCA GCT GCA GGT TCA CC GGT TGA TTC CGT CAA CGG AC	[95]
*hsp*70	HSP70sen HSP70ant	GAC GGT GCC TGC CTA CTT CAA CCG CCC ATG CTC TGG TAC ATC	[102]
cpb	Forward Reverse	CGT GAC GCC GGT GAA GAAT CGT GCA CTC GGC CGT CTT	[103]
rK39	rK39 sense rK39antisense	GAG CTC GCA ACC GAG TGG GAG GAC CTG GCT CGC CAG CTC CGC GGC GCG	[104]
rKRP42	rK39 sense 6His/*Eco*RI rK39 antisense	GAG CTC GCA ACC GAG TGG GAG GAC GTG ATG GTG ATG GTG ATG GAA TTG ATC CTG GCT CGC CAG CTC	[104]
7SL RNA	TRY 7SL.For1 M13 TRY 7SL.Rev 1M13	GTA AAA CGA CGG CCA GTG CTC TGT AAC CTT CGG GGG CT CAG GAA ACA GCT ATG ACG GCT GCT CCG TYN CCG GCC TGA CCC	[105]
Micro-satellite	AC01-forward AC01-reverse AC52-forward AC52-reverse AC16-forward AC16-reverse	GAG AGG CCA CCA GAC ACG TCA GCA CAC CCC CCT TCC TTC GCC TTC AAC ACC TTT AC CCA CCG CCG GCT TCA CTA C GCG GCA ATC GTC TGG CTA AA CTT CTT CTC ATG CTG CAC GGT CTC CTC CTT CCA TGG GCG GGC TTG TTT CGT TAC TTT TTA	[106]
cyt b	LEI-CYTB9 LEI-CYTB10 LEI-CYTB11 LEI-CYTB12	TTA TGG TGT AGG TTT TAG TYT AGG TT CCA TCC GAA CTC ATA AAA TAA TGT TTT GTT ATT GAA TWT GAG GWA GTG A TGC TAA AAA ACCACT CAT AAA TAT ACT	[107]
G6PD	*G6PD*-P1 *G6PD*-P2	ATH GAY CAY TAY YTN GGN AAR G TGK TTY TGC ATN ACK TCN CKD ATD ATN CC	[108]

Table 8: Primers used for *Leishmania* qPCR diagnosis and species identification[a] [109]

Type of DNA targeted, primer set designation	SYBR green primer sequence Forward	Sequence source[c]	Source or reference
Minicircle			
kDNA 1 minicircle	GGGTAGGGGCGTTCTGC	M94088	J.L.W.[c]
kDNA 2	AACTTTTCTGGTCCTCCGG GTAG	EU437406	Leish1 and -2 (20)
kDNA 3 minicircle	GGGTAGGGGCGTTCTGC	M94088	J.L.W.
kDNA 4 minicircle	GGGTGCAGAAATCCCGTTCA	Conserved kDNA[d]	J.L.W.
kDNA 5 minicircle	CTTTTCTGGTCCTCCGGGTAGG	*L. infantum* contig 1335	TaqMan qPCR (40)[e]
kDNA 7 minicircle	AATGGGTGCAGAAATCCCGTTC	Conserved kDNA[d]	J.L.W.
L. (L.) amazonensis kDNA 1	GGTCCCGGCCCAAACTTTTC	U19810	J.L.W.
L. (L.) amazonensis kDNA 2	GGTAGGGGCGTTCTGCGAAT	EU370875	J.L.W.
L. (L.) amazonensis kDNA 3	GGGTAGGGGCGTTCTGC	M94089	J.L.W.
L. (L.) amazonensis kDNA 4	TGAGTGCAGAAACCCCGTT CATA	EU370875	J.L.W.
L. (V.) braziliensis kDNA 1	AATTTCGCAGAACGCCCCTAC	U19807	J.L.W.
L. (V.) braziliensis kDNA 3	TGCTATAAAATCGTACCACCC GACA	AF231100	Adapted from PCR (56)[f]
L. (L.) infantum minicircle 1	TCCGCAGGAGACTTCGTATG	AF032997	Adapted from nested PCR (51)
L. (L.) major minicircle 1	ACGGGGTTTCTGCACCCATT	LM15_BIN_Contig406	J.L.W.
L. (L.) mexicana minicircle 1	AATGCGAGTGTTGCCCTTTTG	AY145437	J.L.W.
L. (L.) tropica minicircle 1	GGGGGTTGGTGTAAAATAGGG	AF308689	J.L.W.
L. (L.) donovani minicircle 1	GCGGTGGCTGGTTTTAGATG	FJ416603	J.L.W.
Maxicircle			
Cytochrome B 1	ATTTTAGTATGAGTGGTAGGTT TTGTT	AB095960	J.L.W.
L. (L.) amazonensis cytochrome B 1	GCGGAGAGGAAAGAAAAGG CTTA	AB095964	J.L.W.
L. (L.) tropica cytochrome B 1	CAGGTTGCTTACTACGTGTTTA TGGTG	AB095960	J.L.W.
L. (L.) tropica cytochrome B 2	TCAGGTTGCTTACTACGTGTTT ATGGTG	AB095960	J.L.W.
L. (L.) tropica cytochrome B 3	TGACACACATATTTTAGTGTGG GTGGTAGG	EF579904	J.L.W.
L. (L.) tropica cytochrome B 4	CACATATTTTAGTGTGGGTGGT AGGTTTTG	EF579904	J.L.W.
Maxicircle 1	GCTTGGTTGGATTATTTTT GCTG	DQ492252	J.L.W.
Genomic			
Alpha-tubulin 1	GAGGTGTTTGCCCGCATC	XM_001681731	J.L.W.
DNA polymerase 1	TGTCGCTTGCAGACCAGATG	AF009147	TaqMan qPCR (8)[e]
DNA polymerase 2	AGGAGGATGGCAAGCGGAAG	AF009136	J.L.W.
Miniexon 1	CGAAACTTCCGGAACCTGTCTT	AL389894	J.L.W.
Miniexon 2	GTGTGGTGGCGGGTGTATGT	LbrM02_V2.0710	J.L.W.
MAG 1 (MSP-associated gene 1)	AGAGCGTGCCTTGGATTGTG	AF058760	J.L.W.
MAG 2 (MSP-associated gene 2)	AGTTTTGGTTGGCGCTCCTG	AF058760	J.L.W.
SIDER repeat 1	CGACCCTGTCACCACCACAG	AM937229	J.L.W.
L. (V.) braziliensis DNA polymerase 1	TCGTTGAGGGAGGAGGTGTTTC	XM_001563712	J.L.W.
L. (V.) braziliensis DNA polymerase 2	ACGTCGCCAACTGCTTCACC	XM_001563712	J.L.W.
L. major MAG 1	GTCGTTGTCCGTGTCGCTGT	XM_001681328	J.L.W.
L. (L.) amazonensis DNA polymerase 1	GACGACGACGAGGAGGATGG	AF009136	J.L.W.
GPI	CCAGATGCCGACCAAAGC	AM117195	(66)
HSP70-1	GAAGGTGCAGTCCCTCGTGT	FN395029	J.L.W.
HSP70-4	TCGAGATCGACGCGTTGTT	FN395037	J.L.W.
SLACS	GGAGAAACTCACGGCACAGG	XM_001562078	J.L.W.
Leptomonas DNA			
Leptomonas miniexon 1	TGGAGCGGGTGCATTAACTC	S78663	J.L.W.
Leptomonas GAPDH 2	AGAAGCCGGATGTGCTTGTG	AF053738	J.L.W.
Human DNA			
Human TNF-α 1	GCCCTGTGAGGAGGACGAAC	NM_000594	J.L.W.
Human TNF-α 2	GCGCTCCCCAAGAAGACAGG	NM_000594	J.L.W.
Human GAPDH 1	GGGCTCTCCAGAACATCATCC	NG_007073	J.L.W.
Human GAPDH 2	CATCAAGAAGGTGGTGAA GCAG	NG_007073	J.L.W.
Human GAPDH 3	GCATGGCCTTCCGTGTCC	NG_007073	J.L.W.

[a] All sequences were developed for use in SYBR green and/or TaqMan assays.
[b] The TaqMan probe used for human DNA was 5'VIC/3'TAMRA. The TaqMan probe used for all other DNA was 5'FAM/3'TAMRA.
[c] Primers and probes were designed by one of us (J. L. Weirather [J.L.W.]) from sequences available on the World Wide Web. GenBank accession numbers are listed.
[d] The "conserved kDNA" sequences used multiple sources aligned with CLUSTALW.
[e] For TaqMan qPCR, the primers and probes are the exact ones used for TaqMan but not SYBR green assay reported in the literature cited.
[f] "Adapted from" indicates that the targeted gene in the literature cited was used by J. L. Weirather to redesign primers and probes.

CHAPTER III
MATERIAL AND METHODS

1. *L. siamensis* promastigote

L. siamensis used in this study was originated from a clinical leishmaniasis sample and was cultured in Schneider's insect medium (Sigma-Aldrich, USA). The media contained penicillin 100 U/ml, streptomycin 100 mg/ml and 20% fetal bovine serum (Sigma-Aldrich, USA). 5 – 10 ml of culture medium was added with some parasite in 25 cm^3 flask, incubated at room temperature (25 °C ± 2 °C) overnight. The next following day, *L. siamensis* was observed by inverted microscope (CKX41 Olympus, Japan). Serial dilutions of *Leishmania* were prepared from the stock cell. Solution of culture medium was washed with TBST buffer and fixed with 2% formalin minutes. First, stock *Leishmania* solution was pipetted 10 µl on haematocytometer (counting chamber) for counting cell by light microscope. Counting chamber was photo some pictures of 40x objective, photograph of *L. siamensis* were counted by image tool program (Figure 12). In order to know amount of promastigote, compare with other technique. After counting the cell, the variation of each dilution was prepared by starting 10^7 to 10^1. Each dilution pipetted 100 µl solutions on glass slide for FISH and IHC. Exception 100 µl solution for DNA extraction was used both PCR and qPCR. Then all specimens were collected in each technic (FISH, IHC, PCR, qPCR).

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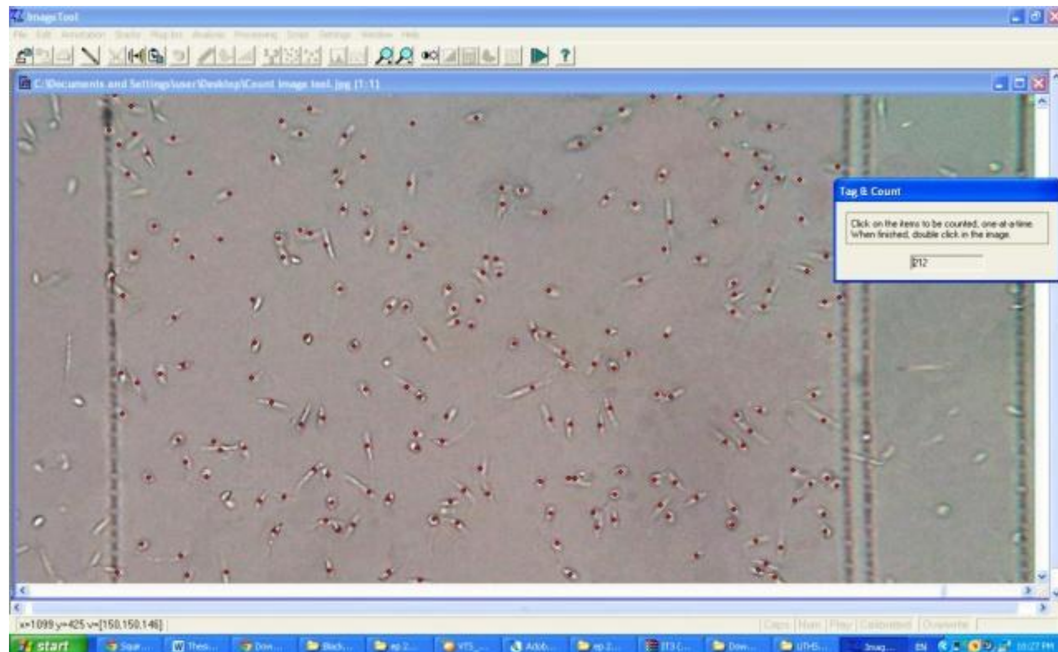

Figure 12: Image tool program use to count *L. siamensis* cell

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2. Reagents

Antibodies against GP63 (ab 65290), secondary antibody (ab 97245), DAB and substrate (ab 94665) (Abcam, Cambridge, UK). Others reagents were purchased as followed; Real time PCR method uses a set of Maxima@ SYBR Green with UDG pre-treatment (Thermo scientific, Thailand), Tissue DNA extraction (invisorb®spin tissue mini kit, STRATEC Molecular, Berlin, German), pGEM®-T Easy vector (Promega, USA), Plasmid extraction (NucleoSpin®Plasmid QuickPure, German, FISH kit (Dako, an agilent technologies company), PCR Kit (Fermentas, Pittsburgh, PA).

3. Equipments

-Culture Hood (Thermo scientific, USA)

-Pump (R-300, Boeco, Germany)

-pH meter (Denver instrument, ultrabasic, Becthai)

-Mini Centrifuge (Model 6K, Extragene)

-Centrifuge (Eppendorf Centrifuge 5417R, USA)

-Refrigerator 4 °C (LG, Thailand)

-Refigerator -20 °C (Whirlpool, Sanyo, Thailand)

-Freezer -80 °C (Thermo Scientific 995, Becthai, USA)

-Heat block (Labnet international, Inc, USA)

-Vortex (Fine vortex, Seoul)

-Autoclave (Hirayama Manufacturing corporation, Japan)

-Water baht (J.P.SELECTA, s.a, Barcelona, Spain)

-Hybridizer (Dako 92451, v 2, USA)

-Incubator (Memmert)

- Auto-pipette (Eppendorf) size 0.5-10, 2-20, 20-200 and 100-1000 µl

-Real time thermal cycle (Bio-Rad, Singapore)

-Microwave (LG, intellowave, Thailand)

-PAGE (Run Gel) (MiniProtein 3 cell, Bio-Rad, USA)

-Electrophoresis (Sub-Cell GT, Bio-Rad, USA)

-Spectrophotometer NanoDrop 2000c apparatus (Thermo Scientific Singapore)

-PCR machine thermal cycle (Eppendorf AG, Germany)

-PCR cabinet (Augustin ™, Thailand)

-Gel photo-documentation system (Gel Doc™ XR, Bio-Rad, USA)

-Fluorescent microscopic (BX 50. Olympus, Japan)

-Inverted microscope (CKX41 Olympus, Japan)

-Microscope (CH2, Olympus, Japan)

-Dry bath incubator (MD-02N-220, Cleaver Scientific, USA)

-Plastic box (25 x 18 cm)

-Tray slide, slide box, Plus microscope slide

-Centrifuge tube size 15, 50 ml

-Micro-tube size 1.5 ml and PCR tube size 0.2 µl

-Cell culture flask size 40 ml (Nunc, Denmark)

-Parafilm, pipette tip

4. DNA extraction

The solutions of cultured L. siamensis samples (100 µl) were brought to centrifuge in order to separate medium or fixative solution. After that all *L. siamensis* cells were used for the process of DNA extract by tissue DNA extraction (Invisorb®spin tissue mini kit, German).

Each dilution of sample 100 µL put in 1.5 ml micro-tube after that added 40 µl Proteinase K and 400 µl Lysis buffer G, mixed and incubated at 52 °C 2 hours, centrifuge for 2 minutes at 11,000 rpm. Pipette supernatant to a new 1.5 ml micro-tube, added 200 µl binding buffer A and vortex. Put Spin Filter into a 2.0 ml Receiver tube and transfer lysate onto the Spin Filter, incubate at room temperature for 1 minute., centrifuged, lysate onto the Spin Filter, incubate at room temperature for 1 minute. and centrifuged at 11,000 rpm for 2 minutes and discard filtrate. The dry lysate was washed 2 times with 550 µl Wash Buffer, centrifuge 11,000 rpm for 1 minute and

discard filtrate in each time, removed residual ethanol by centrifuge at maximum speed 4 minutes. The Spin Filter took to put into the 2.0 ml new receiver tube and added 50 µl Elution Buffer (warmed before use) then incubated at room temperature for 3 minutes and centrifuge at 11,000 rpm for 1 minute after that discard Spin filter [110]. The solution is measured optical density of DNA by spectrophotometer (NanoDrop 2000c machine). The DNA solution was stored in refrigerator until use.

5. DNA amplification by Polymerase Chain Reaction)

The specific DNA detection of *L. siamensis* was amplified by using these primers (Forward: 5-CGATATGCCTTTCCCACACAC-3 (21 bp) and Reverse: 5-CTGTATACGCGCGGCATTTG-3 (20 bp). These primers were designed from the sequences of ITS1 of 18S rRNA gene (Figure 13). The amplify PCR products consisted of 128 bp. The experiment used DNA extraction of *L. siamensis* as the positive control. Negative control did not add 2 µl of template but adding 2 µl of distill water. The total reaction mixture was 25 µl per reaction (in table 6). This set of primer was used to amplify by using the condition program that showed the detail in below.

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Figure 13: The sequence of *L. siamensis* compared with other species

6. PCR conditions

- Each PCR reaction composed of 25 µl of the final volume. Table 6 shows component of PCR mixture PCR condition is showed in Figure 14.

Initial denaturation at	95°C	3 minutes	1 cycle
PCR cycle			
-denaturation at 95 °C		30 minutes	35 cycle
-annealing at 60 °C		30 second	
-extension at 72 °C		1 minute	
Final extension at 72 °C		5 minutes	1 cycle
PCR products were store at 4 °C		5 minutes	

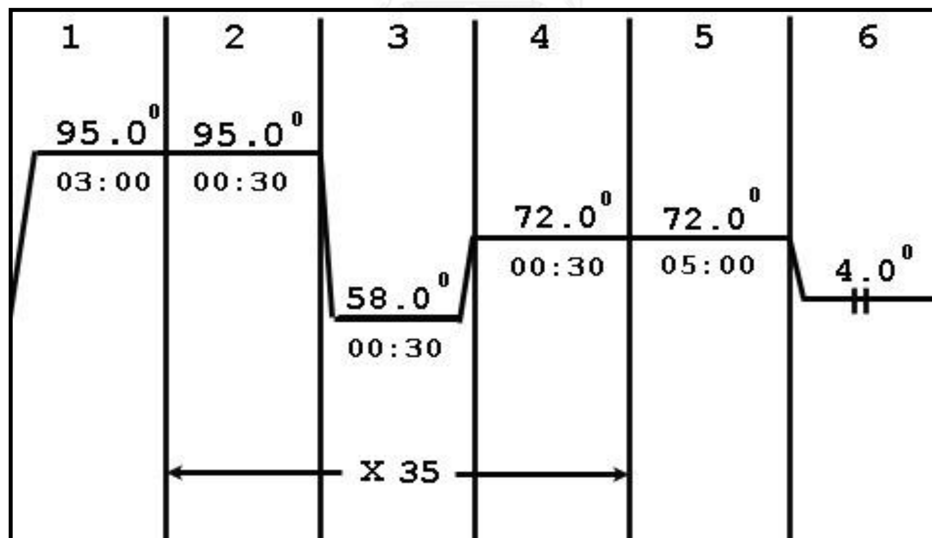

Figure 14: The optimal condition PCR for *L. siamensis*

Table 9: PCR components

Components	Volume (µl)
Deionized water	15.55
25 mM $MgCl_2$	1.25
2 mM dNTP	2.5
10 x PCR buffer	2.5
0.5 µM Forward Primer	0.5
0.5 µM Reverse Primer	0.5
5 U/µL *Taq* DNA polymerase	0.2
DNA template	2
Total reaction mixture	**25**

PCR reactions were consisted of positive control, negative control, all serial dilutions of *L. siamensis* DNA. After completed PCR cycles, all samples were mixed with bromthymol blue (loading dye) then gradually dropped in the hole of 1.5 % (wt/vol) agarose gel for electrophoresis. These products were visualized by staining with ethidium bromide and observe under UV light. These samples were compared with control and the standard marker.

7. DNA Cloning and sequencing:

DNA cloning: The PCR amplicons were ligated into pGEM®-T Easy vector (Promega, USA). Ligated plasmid was transformed into *E. coli* DH5α competent cells in order to amplify the large amount of plasmid. The transform colonies were

screened by LB-ampicillin plate contain βX – Gal IPTG. Ligated plasmid DNA was then purified by using NucleoSpin®Plasmid QuickPure (USA).

8. Prepare competent DH5α E. coli

DH5α *E. coli* took from the stock cell which was kept at -80°C, then cultured on the LB plate at 37°C overnight. The next fallowing day, the single growth colony was selected and incubated into the 5 ml LB broth. It was incubated in a shaker at 37°C overnight. After that, 50 ml Medium A (appendix A) was divided into 2 tubes, each tube contained 20 ml of centrifuge tubes size 50 ml. Then pipetted 200 µL from 5 ml LB broth put in both tubes and incubated shaker 250 rpm at 37°C for 2.30 hours. Set 50 ml centrifuge tube in ice box for 10 minutes and the chamber of centrifuge at 4°C, 4000 rpm for 10 minutes. Discarded the supernatant and kept the pellet cell in ice box. Gently resuspended pellet with 20 µl of medium A and added 1,000 µl of medium B (appendix) up and down with pipette. Finally, aliquot 200 µl in each microtube for storage at -80°C, until used.

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9. Ligation

Amplified PCR amplicons were ligated into pGEM-T Easy Vector (Promega; USA) (Figure 15). The ligation reactions (Table 7) were incubated for 1 hour at room temperature.

Table 10: Reaction of DNA lygation for insert in pGEM® -T Easy Vector Systems

Components	Volume (µl)
Deionized water	4
5x ligation buffer	3
pGEM®- T Easy Vector (pTz57R/T, 0.17 pmol)	1.5
T4 DNA ligase	0.5
PCR products	1
Total reaction mixture	**10**

10. Transformation

The competent cell took from the stock cell (-20°C), incubated in an ice box 3 - 5 minutes. 100 µl of competent cell was put into the 5 µl of DNA ligation (gentle mix). The mixture reaction incubated in ice box at 30 minutes then moved fast to incubate in hot plate or water bath at 42 °C, 45 sec. This activity called “heat shock”, the surface membrane of *E. coli* opened the channel to permit foreign plasmid that was transfer into bacteria cell. After that this tube were carried back to ice box 2 minutes [111] and adjust competent solution to 250 µl with SOC (bacteria medium) at 42°C or room temperature. Then, this appendorf tube was amplified by shaking at 37°C, 200 rpm 1 hour. The next step, centrifuged at 3,000 rpm 2 minutes and discarded 100-200 µl upper part of solution. Collected remain residual to continue the next step.

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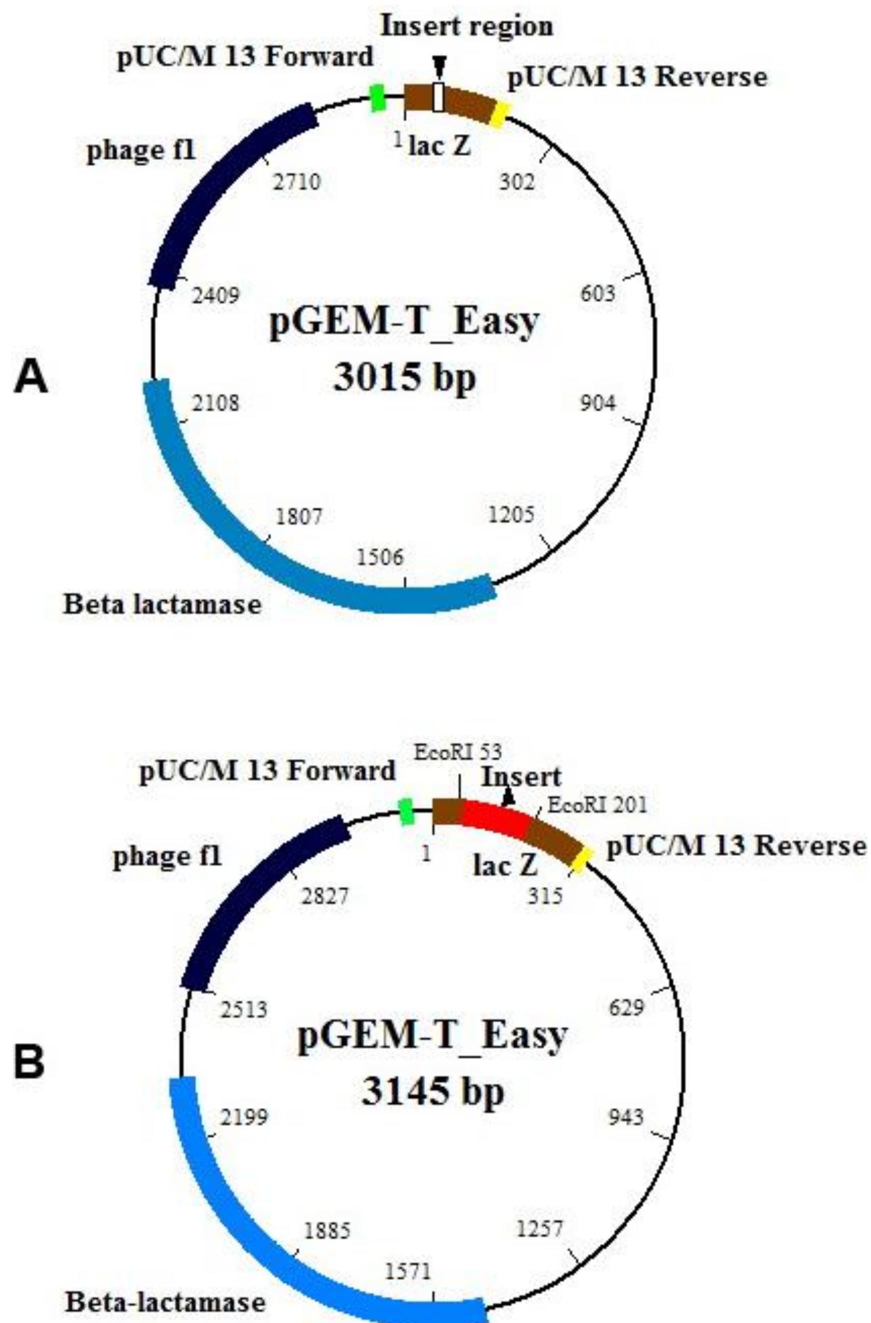

Figure 15: Figure A shows the original pGEM-T EASY, Figure B shows PCR product directly ligated into pGEM®-T Easy vector

11. Screening of transformed colonies

LB agar plate (LB + 50 mg/ml Amplicillin) was spread with 40 µl of 20 mg/ml X-Ggal + 40 µl of 24 mg/ml IPTG then this plates added competent cell (after shaking at 37°C, 200 rpm 1 hour.) 100 µl incubated at 37 $^{\circ}$C overnight. The next following day, checking blue-white colonies transformation showed in Figure 16. The successful of transformation presented in white colonies because these cells did not express the lacZ gene. However, they had the ampicilline resistance gene so they could be increased a quantity in LB broth with amplicillin.

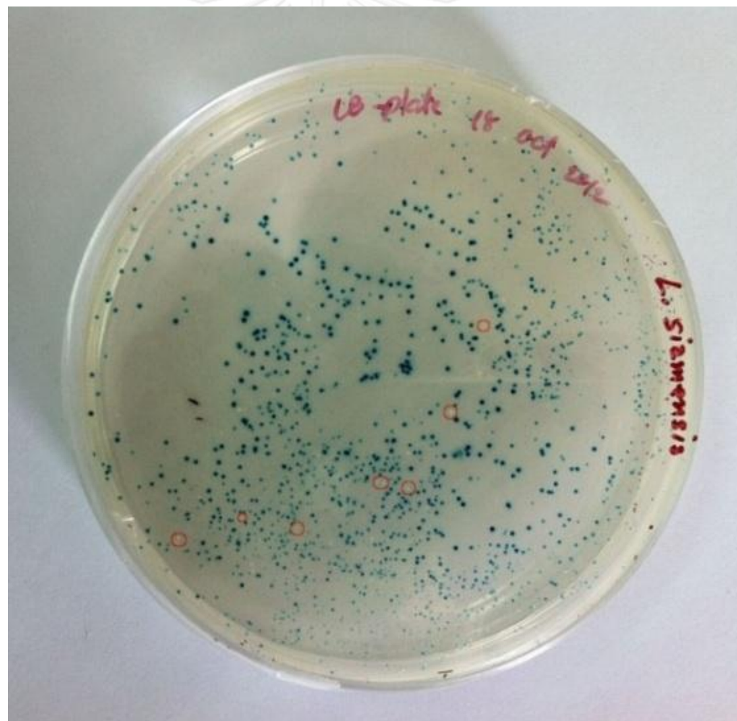

Figure 16: White colonies show expected plasmid contained inserted gene fragment transformed into *E. coli*

12. Colony PCR

Pipetted 4 ml LB broth in 15 ml centrifuge tube and added 4 µl of 50 mg/ml ampicillin. Moreover, PCR tube (0.2 µl) prepared for check inserted DNA by normal PCR cycle, except mixture reaction of Table 8. After that a single white colony was picked up by sterile toothpick so as to stir in PCR condition in Table 8. The sterile toothpick put in the 15 ml centrifuge that it took to shake at 37°C, 200 rpm overnight. If the checking result shows positive reaction, the next following day transformation cell will be extracted for plasmid purification.

Table 11: Condition for testing DNA insertion (colony PCR)

Components	Volume (µl)
ddH_2O	6.5
25 mM $MgCl_2$	0.8
2 mM dNTP	1
10 x PCR buffer	1
0.5 µM Primer forward	0.3
0.5 µM Primer reverse	0.3
5 U/µL *Taq* DNA polymerase	0.1
Total reaction mixture	**10**

13. Prepared Plasmid DNA extraction by Spin Plasmid Mini kit, Germany

Pipetted 1.5 ml *E. coli* LB culture (PCR positive) into microtube, centrifuge at 11,000 g 30 s. Then keep a residual by remove supernatant as much as possible. Added 250 µl Buffer **A1** dissolve completely the clump of residual such as vortex,

pipette up and down. Then add 250 µl Buffer A2 mix gently by inverting 6-8 times, incubate at room temperature. After that add 300 µl mixed thoroughly by inverting 6-8 times (Do not vortex). Centrifuge 11,000 g 5 minutes at room temperature. Prepared a Plasmid Filter into a 2.0 ml collection tube then decant lysate onto the Spin Filter. The lysate on the Spin Filter was centrifuged at 11,000 x g for 1 minute and discard filtrate. The dry lysate was added 600 µl Buffer A4, centrifuge 11,000 x g for 1 minute and discard filtrate of collection tube. After that, it back to the plasmid column and centrifuge again for 2 minutes at 11,000 x g. Discard the collection tube. The plasmid Filter took to put into the 1.5 ml new receiver tube and added 50 µl Buffer AE. (incubate 70°C before use) then incubated at room temperature for 1 minute and centrifuge at 11,000 x g for 1 minute after that discard Spin filter [112].

The solution is measured for optical density of plasmid DNA by spectrophotometer (NanoDrop 2000c machine). The DNA solution was stored in -20 °C refrigerators until used.

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14. The sequence of *L. siamensis:*

The plasmid sequence was determined by sending to First BASE Laboratories Sdn Bhs, Malaysia pass through Ward Medic Ltd., Part, Thailand. The sequence of *L. siamensis*, which was checked by using the NCBI BLAST programs, was aligned by using CLUSTAL X (Cluster algorithm).

15. Quantitative Real-Time PCR

All qPCRs were performed using the BIO-RAD, CFX96™ Optic Module. The total volume of 20 µl/reaction consisted of 10 µl Master Mix SYBR green (Thermo

Scientific), 0.2 µl of Forward and Revearse primers, 0.25 µl of UDG (Thermo scientific), 7.35 µL of ddH_2O, and 2 µl of DNA template (Table 9). The amplification conditions comprised a two-step initial denaturation at 50 ^{o}C for 2 minutes and 95 ^{o}C for 10 minutes, respectively, followed by 40 cycles of 30 sec at 95 ^{o}C, 30 sec at 61 ^{o}C, 30 sec at 72 ^{o}C, and one final melting cycle consisting of 5 sec at 65 ^{o}C and 50 sec at 95°C (Figure 17). The changing of fluorescence was used to measure the concentration of plasmid, and the equation below was used to calculate the copy number of the plasmid. A standard curve of *L. siamensis* was set up and the copy number of the samples was determined (Whelan *et al*., 2003).

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23} \text{(copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (dp)} \times 660 \text{ (g/mol/dp)}}$$

Table 12: qPCR mixture

Component	**Volume (µl)**
Mixture	10
Primer Forward	0.2
Primer Reverse	0.2
UDG	0.25
ddH_2O	7.35
Template	2
Total reaction mixture	20

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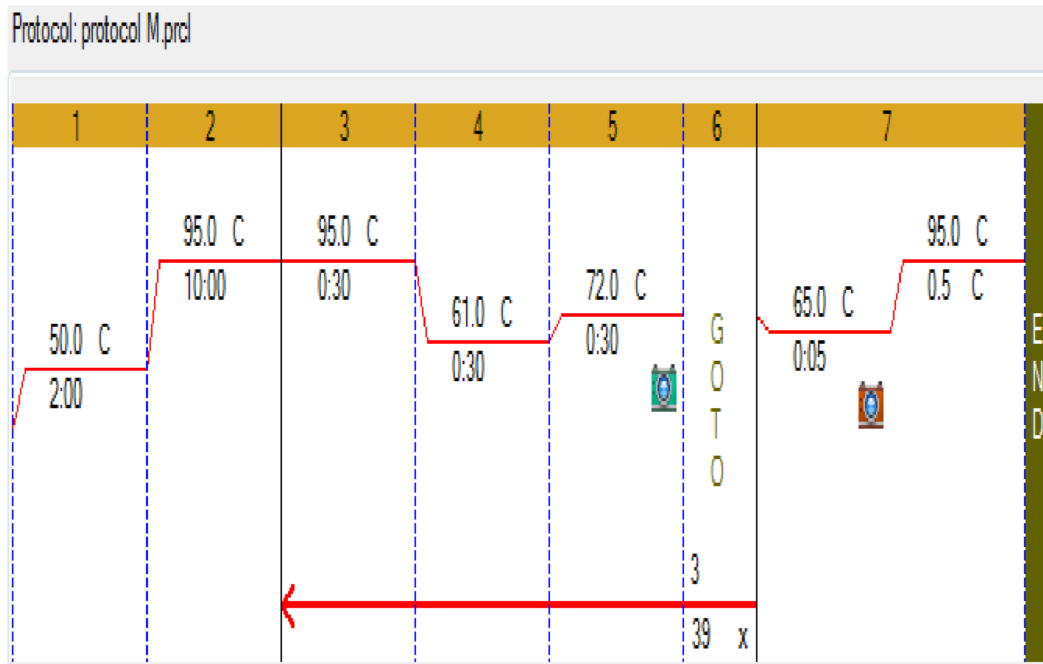

Figure 17: The qPCR condition for detect L .siamensis

16. Slide preparation for IHC and FISH

For fresh specimen (promastigote form) prepares from tissue culture which is centrifuged at X 4,000 rpm in order to separate parasite from the solution. After that the residual cell is washed with TBS (Tris buffered saline) 2 times. Then pipette solution and smear specimen on the slide plus (test negative and positive cell). These slides are fixed with acetone solution and incubate at -20 ^{o}C for 15 minutes. Allow slide to dry completely at room temperature.

17. Immunohistochemical (IHC) Method

The process of IHC consisted of 2 days

Day 1

Some preparation dry slides were provided for staining with IHC. PAP pen (a special marking pen) uses to draw outside around a specimen before the step of dehydration. These slides prepare by fresh tissue so skipping the step of deparaffinization. Only dehydration by serial of alcohol; 95%, 85%, 70% each 2 changes of reagent respectively, for 3 minutes each. After that transfer all slides to wash in running tap water twice times for 10 minutes each. Then incubate the slide in 3% H_2O_2/DW with 3% H_2O_2 in methanol 20 minutes. at room temperature. After that the slide washes in TBS buffer 3 times for 5 minutes each. Block with 4% bovine serum albumin 100-400 μL per reaction in each section for 30 minutes at room temperature. After that wash 3 times with TBS solution and block with 10% Normal horse serum in TBST 20 minutes at room Temperature. For the next step, only remove blocking solution (do not necessarily wash with buffer solution). Add primary antibody (1°Ab) consisting of anti-GP63 antibody diluted with 10 % Normal Horse Serum in TBST to each section. Incubate overnight 4°C

Day 2

Remove antibody solution and wash sections in wash buffer 3 times for 5 minutes each. Then add the secondary antibody (2° Ab) , goat anti-rabbit IgG conjugate with horseradish peroxidase (HRP) (1:400) dilute in 10 % Normal Horse Serum in TBST, incubate 30 minutes at room temperature. Finally the specimen will be immersed in DAB substrate working solution. Prepared reagent from the set of kit then incubated for 10 minutes at room temperature. After that, washes cells in

dH_2O_2 2 times for 5 minutes each. All specimen slides were brought to dehydrate process again and mounted with medium (permount) to cover the section. All slides were observed and photographed with an Olympus (BX 50) light microscope.

Note: For the control sections, the step of process had the same step as the sample except 1° Ab was changed to be the diluent of 1° Ab (TBST containing 10% normal horse serum) for overnight at 4 ^{0}C in the same chamber.

18. Fluorescent *in situ* hibridyzation (FISH) Method

Prepare Probe

Primer design (forward and reverse) of *L. siamensis* promastigote forms was labeled with Fluorochrome-labeled probes, was fluorescein isothiocyanate (FITC) at 3’ end. It synthesized by Invitrogen.

Day 1

Providing dry slides of *L. siamensis* from the stock skipped deparaffinization to dehydrate by serial of alcohol; 95%, 85%, 70% each 2 changes of reagent respectively, for 3 minute each. After that transfer all slides to wash in running tap water twice times for 10 minutes each. Water bath can be used for Per-Treatment (separated double stand of DNA). Prepared staining jar containing Pre-Treatment Solution (from set kit: dilute 1:20 in dH_2O) places in water bath. Set the temperature of water bath and Pre-Treatment Solution (close the chamber) to 95 - 99°C.

Immerse all slides from step 1 into the preheated chamber and incubate at 95 - 99°C for 10 ($\pm$1) minutes. Remove the entire chamber with slides from the water bath, open the lid and allow the slides to cool in Pre-Treatment Solution at

room temperature for 15 minutes. Transfer the slide to a chamber of dilute Wash Buffer (from set kit: dilute 1:20 in dH_2O) and soak for 3 minutes 2 times at room temperature (20-25°C) (continue to step 3). Apply pepsin solution as follows (pepsin: diluent: distill water = 1:1:8). All solution is mixed together and equilibrates at 37 (±2)°C an incubator or on a heating block such as Dako Hybridizer at 37°C for 50 minutes in a humid chamber box (apply from a plastic box with humid tissue). Tap off Pepsin and soak sections in the diluted Wash Buffer for 3 minutes 2 times at room temperature (20-25°C). Continue to dehydration (70%, 85% and 95%, respectively) for 3 minutes each. Apply 10 µL of design probe to the center of the tissue section. Immediately place coverslip over design probe and allow it to spread evenly under the coverslip. Avoid air bubbles. Seal coverslip with Coverslip Sealant or apply using with nail varnish around the periphery of the coverslip. Allow the Coverslip Sealant to overlap the coverslip and the slide, thereby forming a seal around the coverslip. Make sure that the Coverslip Sealant covers the entire edge of the coverslip. Prepare Dako Hybridizer for a hybridization run. Make sure that Humidity Control Strips are saturated and optimal for use. Start the Hybridizer and choose a program that will denature at 95°C for 5 minutes and hybridize overnight (16-18 hr.) at 42°C (Dako 92451, v.2.0, USA). Place slides in the Hybridizer, make sure the lid is properly closed and start program.

Day 2

Fill two staining jars, e.g. Coplin jars with the diluted Stringent Wash Buffer. A (from set kit: dilute 1:20 in dH_2O). Place one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 65°C. Ensure that the

temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Using forceps take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time As soon as all coverslips have been removed transfer slides from the room temperature pre-wash jar to the 65°C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65°C for 3 minutes at room temperature (20-25°C). Change diluted Wash Buffer and soak section for another 3 minutes. Dehydrate tissue sections through a graded series of ethanol: in 70%, 85 and 95, respectively, for 3 minutes each. Apply 15 µL of fluorescence Mounting Medium containing DAPI to the target area of the slide and close a glass coverslip. -The slides were examined on a fluorescence microscope at 494 nm (Bx50 Olympus, Japan).

Note: Slide may be read after 15 minutes or within 7 days after mounting. However, fading occurs if slides are exposed to light or high temperatures. To minimize fading, store slides in the dark at 2-8°C

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19. Statistical Analysis

In this study, Kruskal-Wallis statistic will be used to analyze the data. Then Multiple comparison test will be used to test for differences among means when Kruskal-Wallis test indicates a significant. Statistical analysis will be done using SPSS version 11.5. For all the tests, the result will be expressed as mean ± Standard error (SE). The value of $p < 0.012$ (.05/4= 0.012) will be considered to indicate statistical significance.

CHAPTER IV
Results

The culture cell of *L. siamensis* in promastigote stage was used to determine by 4 techniques, including Fluorescent in situ hybridization (FISH) analyzed by using fluorescent microscopy, Immunohistochemistry (IHC) analyzed by using light microscopy, PCR-ITS1 observed on agarose gel electrophoresis and qPCR-ITS1 analyzed by amplification curve. All techniques were observed by 6 persons that did not know these conditions and record the result in paper form (in appendix c).

1.Immunohistochemistry (IHC)

The culture of *L. siamensis* promastigotes were prepared for IHC method and observed by light microscopic examination. For negative control was not detected the area of brown color (Figure 18A), while positive samples indicated that gp63 was the most abundant protein on the promastigote surface and flagella, showing brown color of DAB (Figure 18 B, C and D). The positive samples were easily to observe with brown color (arrow), especially the cluster showed dark brown color (star symbol) when compared with negative control. This technique can be easier to observe the several forms of *L. siamensis* promastigote. This study can perform and evaluate IHC successfully on promastigotes of *L. siamensis*.

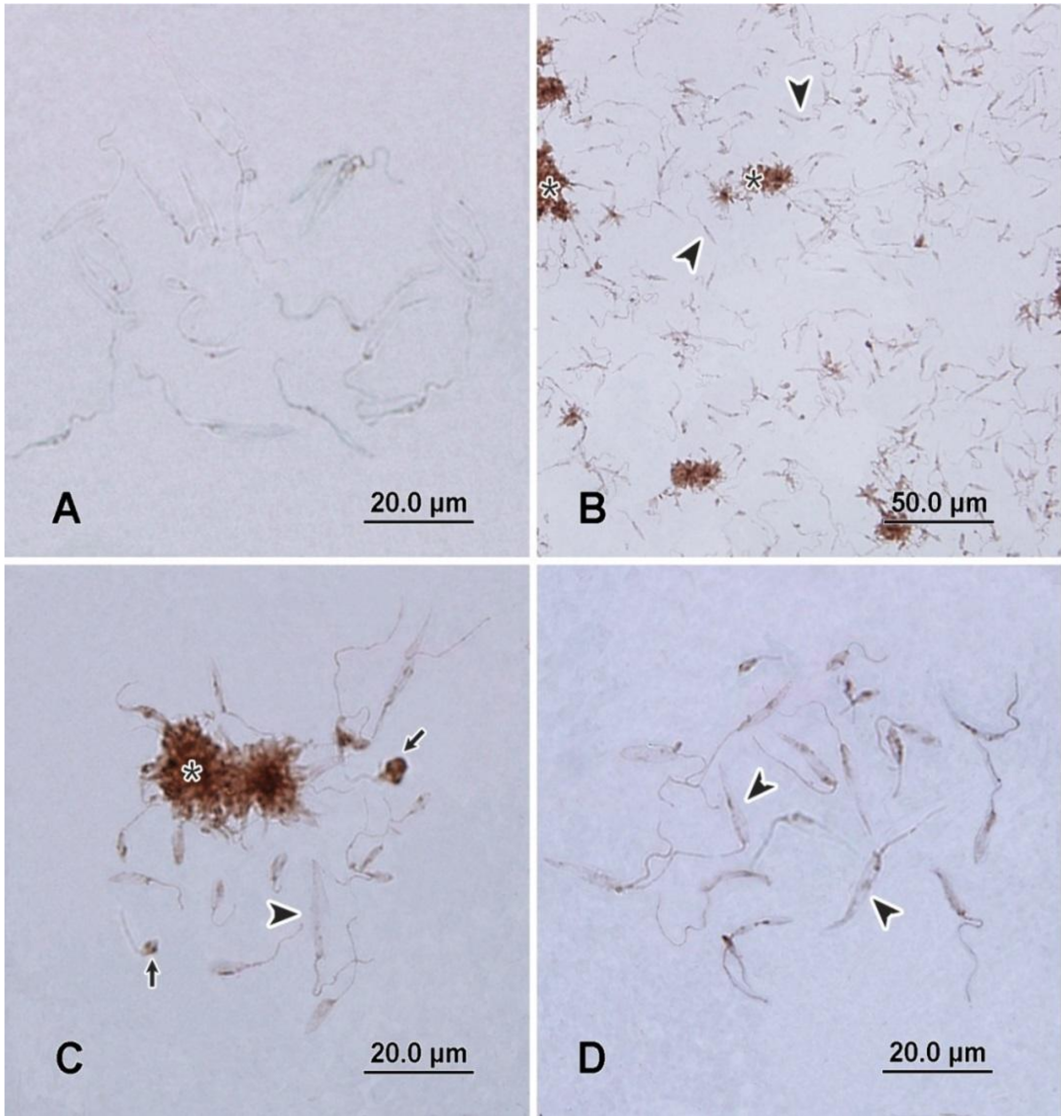
A
20.0 µm
B
50.0 µm
C
20.0 µm
D
20.0 µm

Figure 18: *L. siamensis* stained with IHC technic.

A: Control picture of *L. siamensis* were not present the brown color of DAB stain

B: The low magnification of objective lense (X40) showed a dark brown color in a cluster of *L. siamensis* (star) and general single cell presented a light brown color (arrow head).

C: The high magnification of clump parasite area showed strong brown color (star) that gathered with a short shape of parasite (arrow). Adding, single cell presented in this picture (arrow head).

D: The high magnification of positive cell stained with DAB, present a light brown color (arrow head).

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2. Fluorescent in situ hybridization (FISH)

L. siamensis of promastigotes were smeared on slides plus for FISH method. This study was to determine the location of the kinetoplast of promastigote. The oligonucleotide probes labeled with FITC (fluorescein isothiocyanate) were designed to anneal the ITS1 gene region. The negative controls were hybridized without probes (Figure 19 A, C, E) and the positive samples were hybridized with probes (Figure 19 B, D, F). In the negative control, *L. siamensis* promastigotes were not present the bright green spot of fluorescent at kinetoplast (Figure 19A), whereas the positive samples were presented with a small bright green spot in each individual parasite (Figure 19B; short arrow). All samples were stained with DAPI (4'.6-diamidino-2-phenylindole) for counterstaining that give the blue color (Figure 19C and D; arrow). The figure 19 E and F were merged from previous micrographs of both negative control and positive sample. However, the positive samples can observe a bright green spot on promastigotes (Figure 19F; arrow head) compared with the negative control, which cannot observe bright green spot (Figure 19E). The results of the study confirmed FISH-ITS1 as an accurate method for the diagnosis promastigotes of *L. siamensis*.

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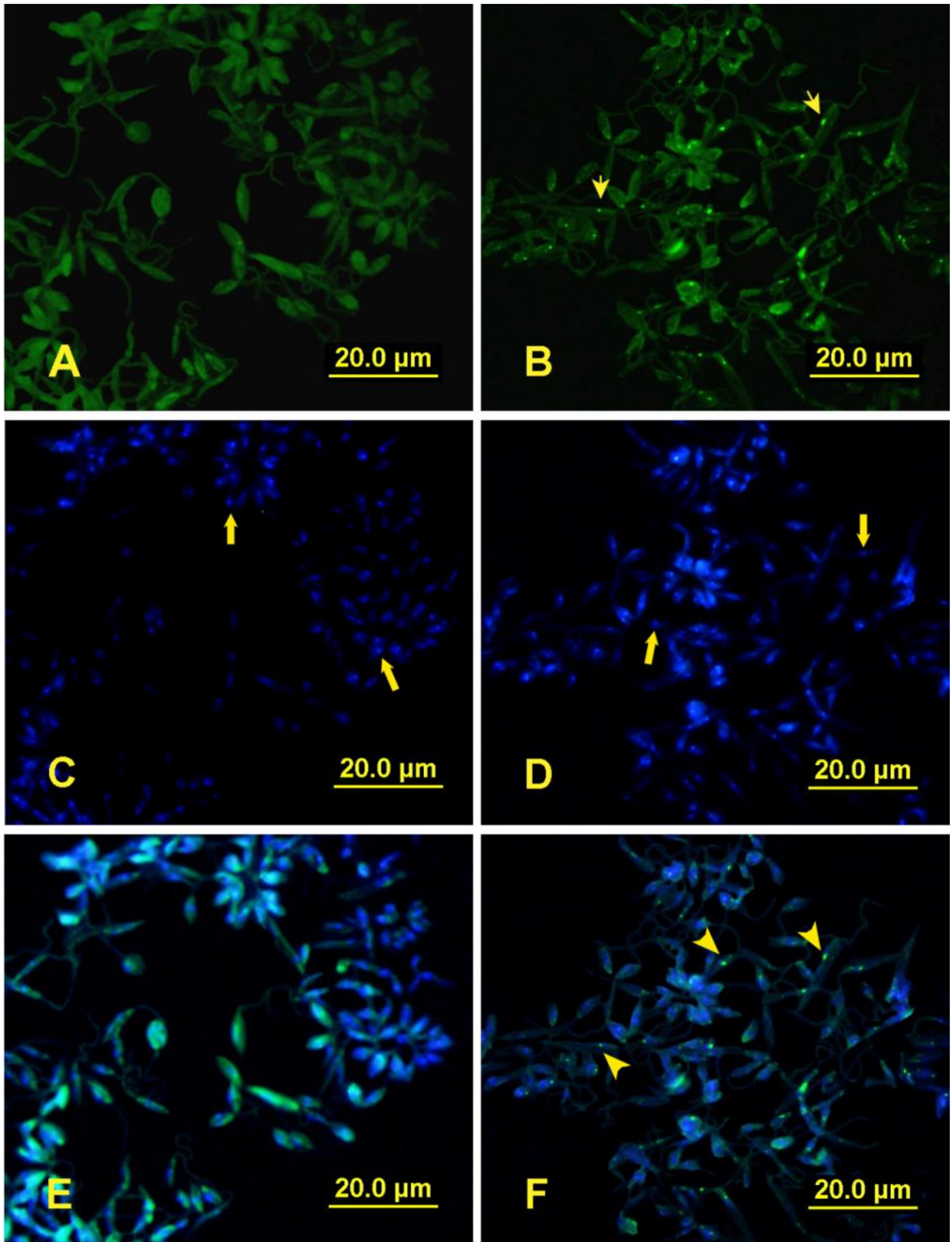

A
20.0 µm
B
20.0 µm
C
20.0 µm
D
20.0 µm
E
20.0 µm
F
20.0 µm

Figure 19: *L. siamensis* promastegotes stained with Fluorescent *in situ* hybridization

A: Negative control stained with fluorescent dye

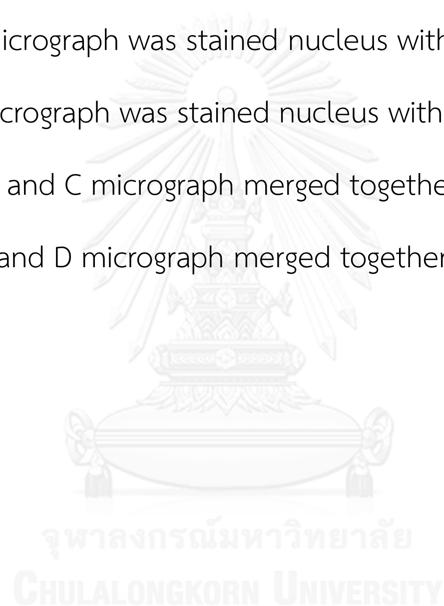
B: Positive sample micrograph showed a small bright green spot (short arrow)

C: Negative control micrograph was stained nucleus with blue color of DAPI

D: Positive sample micrograph was stained nucleus with blue color of DAPI

E: Negative control, A and C micrograph merged together

F: Positive sample, B and D micrograph merged together



3. Molecular detection

3.1 Polymerase Chain Reaction (PCR)

DNA of all concentration (10^7 to 10^1 cells/ml of promastigotes) of *L. siamensis* was prepared to detect by using PCR base on ITS1 rDNA gene (PCR-ITS1), which the primers were designed for *L. siamensis* only. These samples were prepared to be triplicate in each concentration. The product size of the PCR-ITS1 approximately 128 bp were demonstrated on 1.5% agarose gel. The PCR-ITS1 product band was gradually decreased from highest concentration (10^7cells/ml) to lowest concentration (10^1 cells/ml) (Figure 20). This study showed that the PCR amplifying of ITS1 region could detect *L. siamensis* with new designed primer.

For the positive from promastigotes was amplified PCR products were ligated into a pGEM-T Easy cloning vector as described previously. The sequences of the ITS1 region were compared to the reported sequences of *L. siamensis* (accession no JQ866907) and were found to be 100% identical (Figure 21). Plasmid DNA containing the ITS1 region of *L. siamensis* was used to set up a standard curve with concentrations ranging from 10^6 to 10^1.

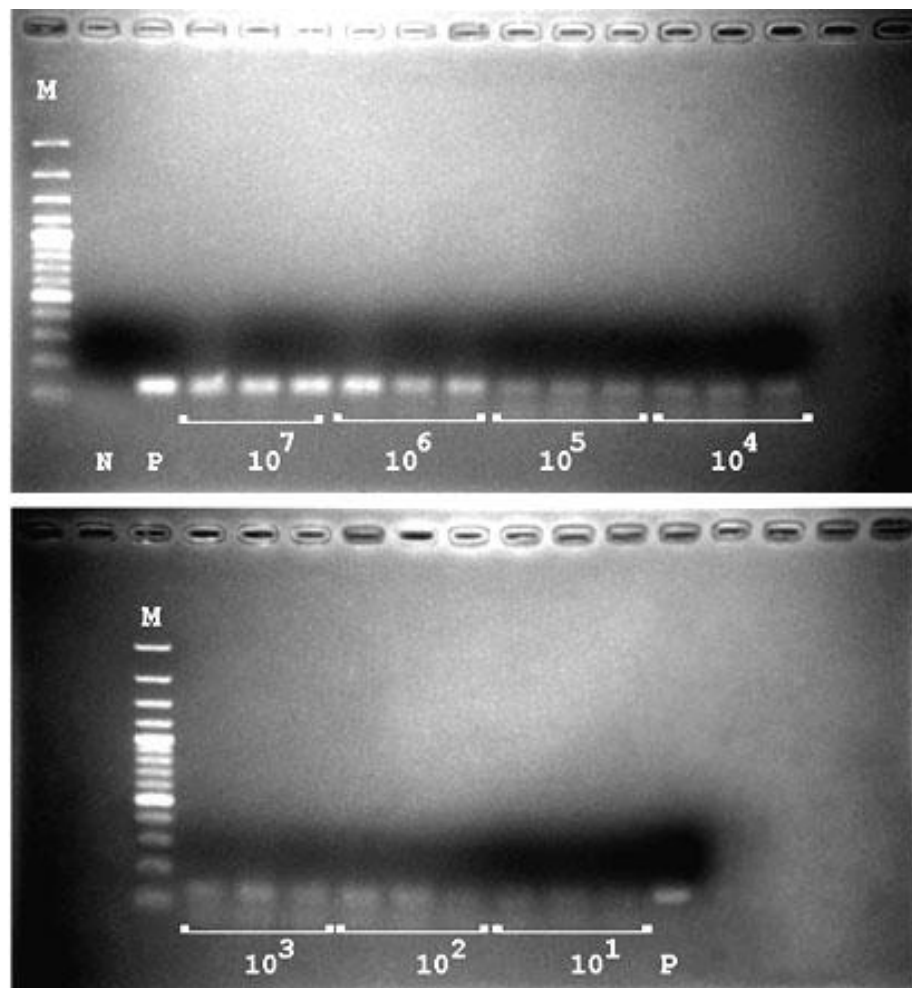


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Figure 20: The PCR-ITS1 amplified product of approximately 128 kb in length for *L. siamensis* promastigote. PCR was able to amplify extracted DNA from the serial dilution of 10^7 to 10^1. N: Negative control, P: Positive control, M: 100 bp, DNA standard marker

```
                      *        20         *        40         *        60         *        80
JQ866907   : TCCGCCCGAAAGTTCACCGATATTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT :  84
Design/L.  : ------------------------------------------------------------------------------------ :   -

                  *       100         *       120         *       140         *       160
JQ866907   : CATTTTCCGATGATTACACCAAAAAACATACAGGTAGAGAGTAGTAGAATACATCTACTCGGGGAGGCATGTTTTTTCCGATAT : 168
Design/L.  : ------------------------------------------------------------------------------CGATAT :   6
                                                                                           CGATAT

               *       180         *       200         *       220         *       240         *
JQ866907   : GCCTTTCCCACACACACAAACACAGCAATATATATGTATATATATACGTATATTGCTATACCCAAAAACCATACCGTAAAAAGC : 252
Design/L.  : GCCTTTCCCACACACACAAACACAGCAATATATATGTATATATATACGTATATTGCTATACCCAAAAACCATACCGTAAAAAGC :  90
             GCCTTTCCCACACACACAAACACAGCAATATATATGTATATATATACGTATATTGCTATACCCAAAAACCATACCGTAAAAAGC

                   260         *       280         *       300         *       320         *
JQ866907   : AAAAAGGCCGGTCGACGCCAAATGCCGCGCGTATACAGTGGAAAAGTCCGTTCGTTACGGCTCTTTCTCTCTCGCGGGTGTGTG : 336
Design/L.  : AAAAAGGCCGGTCGACGCCAAATGCCGCGCGTATACAG---------------------------------------------- : 128
             AAAAAGGCCGGTCGACGCCAAATGCCGCGCGTATACAG

               340         *       360         *
JQ866907   : TGTGGATAACGGCTCACATAACGTGTCGCGATGGATGACTTGG : 379
Design/L.  : ------------------------------------------- :   -
```

Figure 21: Comparison between the sequence amplified from cultures of *L. siamensis* and the sequence of the ITS1 region from *L. siamensis* previously reported in GenBank (accession no.JQ866907).

4. Real-time polymerase chain reaction (qPCR)

In this study developed a qPCR assay targeting ITS1 to detect and quantify *L. siamensis*. Ten-fold serial dilutions were performed concentration of promastigote cells from 10^7 to 10^1 cells/ml, DNA extraction and qPCR detection. The dynamic range of the ITS1-qPCR assay encompassed at least 10^1. The quantitative of parasite in this study is at least 50 parasites/µl by using qPCR (Figure 22). The Cq (quantification) value (n=3) were characterized by a mean ± SD (Table 16). The cycle was necessary to get a signal level of fluorescence threshold. The high concentration (10^7) was the Cq value 18.90, while the low dilution of *L. siamensis* was to be gradually increased a number of Cq. All average Cq is not more than NTC value, that were a positive result.

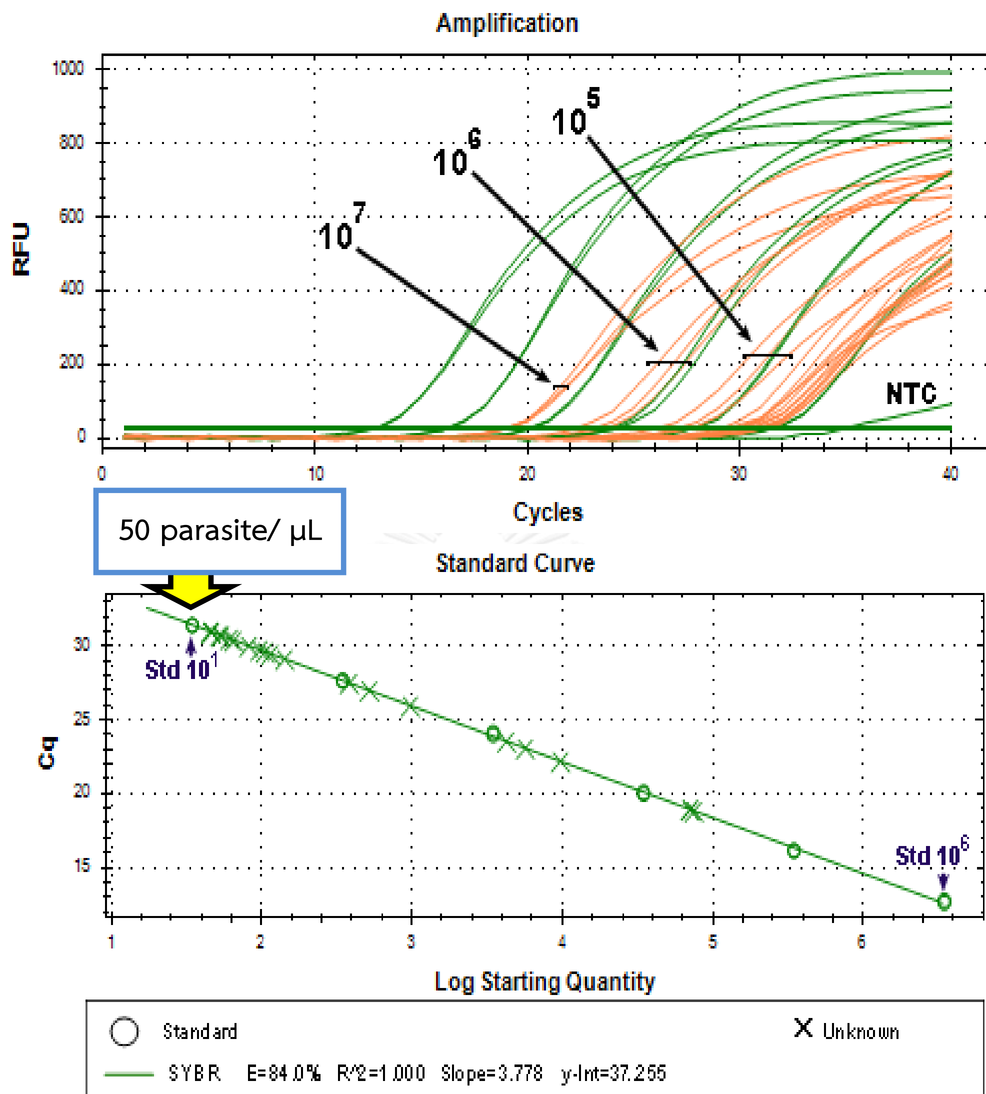

Figure 22: The result of qPCR technique. All sample presented in the red color, while the standard amplification showed in the green color. These samples rose up in the rage between the 10^6 to the 10^1 of standard (picture of amplification). In the standard curve showed the pattern of all sample (X) and standard (o) again. The yellow arrow showed the lower of parasite. R^2 = 1.000

Table 13: Show the Cq value

Sample conc.	Cq1	Cq2	Cq3	Average Cq	Note
10^7	18.93	18.83	18.93	18.90	NTC= 34.86
10^6	22.19	23.07	23.54	22.93	
10^5	26.99	27.49	25.96	26.81	
10^4	30.49	30.98	29.74	30.40	
10^3	30.02	29.47	29.12	29.54	
10^2	30.95	30.75	29.61	30,44	
10^1	30.97	30.70	30.39	30.69	

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5. Volunteer observes result

The result collected from some volunteers, who were graduate students in Faculty of Medicine, Chulalongkorn University. These specimens were observed by a light microscope (IHC and FISH), record in appendix C form, while PCR results observed the gel photograph and qPCR result observed the amplification curve. All volunteers observed the photograph by the naked eyes and those data recorded in the table 17.

Table 14: The observe result of 4 technique from all volunteers

Volunteer	Technic	107			106			105			104			103			102			101		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	IHC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
	FISH	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
	PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	qPCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	IHC	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–
	FISH	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	qPCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	IHC	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–
	FISH	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	qPCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	IHC	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	FISH	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	qPCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 14: The observe result of 4 technique from all volunteers (Cont)

Volunteer	Technic	10^7			10^6			10^5			10^4			10^3			10^2			10^1		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
5	IHC	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	FISH	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	qPCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	IHC	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
	FISH	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–
	PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	qPCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

-The final value of each technic from each person was selected for calculation the average value.

-IHC

Average = Person 1 + Person 2 + Person 3 +Person 4 + Person 5 + Person 6

= (3+3+3) + (4+4) + (4+4) + (5+5+5) + (5+5+5) + (4)

= 59/14

= **4.21**

-FISH

Average = Person 1 + Person 2 + Person 3 + Perso4 + Person 5 + Person 6

= (3) + (5+5+5)+ (5+5+5)+ (5+5+5) + (5+5) + (4)

= 62/13

= **4.76**

-PCR

Average = Person 1 + Person 2 + Person 3 +Perso4 + Person 5 + Person 6

= (1+1+1) + (1+1+1) + (1+1+1) + (1+1+1) + (1+1+1) + (1+1+1)

= 18/18

= **1**

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-qPCR

Average = Person 1 + Person 2 + Person 3 +Perso4 + Person 5 + Person 6

= (1+1+1) + (1+1+1) + (1+1+1) + (1+1+1) + (1+1+1) + (1+1+1)

= 18/18

= **1**

6.. Statistical Analysis

Analysis result from the statistic NPar Tests (nonparametic test), Kruskal-Wallis Test in order to check each pair of technic (IHC-FISH, IHC-PCR, IHC-qPCR, FISH-PCR, FISH-qPCR, PCR-qPCR) has different from others (Table 18).

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The result from each technic (IHC, FISH, PCR, qPCR) was compared by NPar Test, Kruskal-Wallis Test P< 0.05 (0.000) so at least, there was one pair differentiation.

The result from IHC and FISH was compared by NPar Test, Mann-Whitney Test (P > 0.05 (0.211). So both IHC and FISH had not the different result.

The result from IHC and PCR was compared by NPar Test, Mann-Whitney Test (P < 0.05 (0.002). So both IHC and PCR had the different result.

The result from IHC and qPCR, was compared by NPar Test, Mann-Whitney Test (P < 0.05 (0.002). So both IHC and qPCR had the different result.

The result from FISH and PCR was compared by NPar Test, Mann-Whitney Test (P < 0.05 (0.002). So both FISH and PCR had the different result.

The result from FISH and qPCR was compared by NPar Test, Mann-Whitney Test (P < 0.05 (0.002). So both FISH and qPCR had the different result.

The result from PCR, qPCR, was compared by NPar Test, Mann-Whitney Test (P > 0.05 (1.00). So both PCR and qPCR had not the different result.

Table 15: Show the median result of each method

Method	Median (min - max)
IHC	5.71 (5 - 6)
FISH	5.87 (5.31 - 6.13)
PCR	4 (4 - 4)
qPCR	4 (4 - 4)

-

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CHAPTER V
DISCUSSION

Leishmaniasis caused by a novel *Leishmania* species, *L. siamensis,* has recently been described in Thailand and Myanmar[13, 75]. Clinical presentations of *L. siamensis* infection include VL[15], and a combination of VL and CL[14]. The disease is usually found in immune-compromised patients, such as AIDS patients and those receiving systemic steroid therapy.[74] In Thailand, the disease is usually described in patients living in the southern region of the country[16]. Diagnosis of *L. siamensis* infection in local hospitals is based on microscopic examination. Cultivation of the parasites and PCR are only available in some university hospitals. Microscopic examination requires experienced personnel to discriminate between *Leishmania* and other pathogens [10, 65] such as *Histoplasma* and *Penicillium*. In many routine laboratories are frequently used the traditional microscopy for detection of *Leishmania* parasites by Giemsa's staining. Weigle and Heredia (1987)[113] revealed leishmaniasis cases were not detected by Giemsa or Hematoxylin-Eosin staining approximately 30% Sensitivity of microscopic examination[113] is lower than other techniques such as direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA) and molecular detection (PCR and qPCR) Although, serological tests are commonly used for diagnosis of some *Leishmania* species PCR for detecting the *Leishmania* DNA shows high sensitivity and specificity but it can be done only in some provincial and university hospitals in Thailand[21, 91]. PCR methods have been developed for the diagnosis of *L. siamensis* infection and have shown high sensitivity However, compared to qPCR, PCR is time consuming and unable to determine DNA copy number Several PCR and qPCR approaches have been published based on

different target genes in *Leishmania* parasites [35], such as the cysteine protease B (cpb) the cytochrome b (cyt b), The internal transcribed spacer 1 (ITS1) region of the small subunit ribosomal RNA (SSU-rRNA) gene, the glucose-6-phosphate dehydrogenase (G6PD) the heat shock protein 70 (hsp70) [102] , the spliced leader mini-exon, the SSU-rRNA gene, the triose-phosphate isomerase (tim) genes revealed that the ITS1-PCR method is a suitable target for PCR-based detection of *L. siamensis* infection in clinical specimens due to its high sensitivity and specificity [10].

This study developed the alternative method for efficient, accurate, simple, rapid and less-expensive for *L. siamensis* detection in routine laboratory, the advance of microscopy such as IHC and FISH are considered in addition to PCR and qPCR. Several reports showed IHC and FISH detection of *Leishmania* amastigotes in formalin-fixed, paraffin-embedded sections of canine and human tissues[114]. In case of smears or tissue section is usually complicate to identify leishmaniasis, especially small amount of parasites present in the lesion or in irregular site. The pathologist can use IHC or FISH for confirming their diagnosis or for obliteration other uncertain diagnoses. Both IHC and FISH are a practicable technique for increasing the specific binding methods. For immunostaining method, antibody GP63 is used in IHC method for detection at surface coat of *L. siamensis*. GP63 (glycoprotein 63)[39] or leishmanolysin, which was a major surface antigen expressed on *Leishmania* promastigotes more than amastigotes, is the 63 KDa zinc-dependent metalloprotease[93]. The molecules of GP63 are attached to the cell surface via glycosylphosphatidylinositol (GPI) its role is to protect *Leishmania* from mammalian hosts during its sojourn On the other hand, FISH-ITS1 was specific with *L. siamensis* and suitable for screening in *L. siamensis*. FISH is one method that is quickly

detected and diagnosed, therefore, it appropriates for screening of microorganisms grown in blood cultures, *Leishmania sp*. in tissues

In this study designed a new primer set for qPCR to specifically amplify the ITS1 region of *L. siamensis*. The primer set was used in PCR and was showed to amplify the desired region of the parasite's DNA, as confirmed by sequencing. The standard curve for qPCR was determined using various concentrations of *L. siamensis* DNA, ranging from 10^6 to 10^1 copies/µl, and the sensitivity of detection was 50 copies/µL or 50 parasites/µl. The qPCR and PCR techniques have been applied for the diagnosis of invasive and noninvasive forms of VL. Both qPCR and PCR [31] can solve the false-negative (small amount of parasite) that occurs from traditional and serological tests. A new qPCR method was recently used to detect the parasite genome in the blood of patients suffering from VL [115]. Saliva is a good source for *L. siamensis* DNA, and a report from Phumee et al. demonstrated that *L. siamensis* DNA was detected in saliva of leishmaniasis patients, including an asymptomatic patient Our study confirms that qPCR also can detect *L. siamensis* DNA in promastigotes.[116]

Conclusion

This is preliminary result for apply to integrate into routine testing in hospitals laboratory and field study. In the future, IHC and FISH will be useful tool for histopathological, epidemiological, and clinical studies. qPCR may be used instead of PCR in diagnostic routines because qPCR is very similar to PCR in its speed, quantitative, and amplification ability.

Appendice A

Chemicals for preparation of DNA cloning

1.1 Chemicals for preparation the media

1.1.1 LB agar total volume 100 ml

-Peptone 1.0 g.

-NaCl 1.0 g.

-Yeast extract 0.5 g.

-Agar 1.5 g.

- Dissolve in 100 ml of deionized water except agar. Adust the pH of the solution to 7.0 using sodium hydroxide (NaOH). Add agar and autoclave at 121°C for 20 min to sterilize the broth.

1.1.2 LB broth for incubate cell

- 4 ml LB broth adds 4 µl amplicillin by septic technic (ready for use)

1.1.3 SOC media total volume 1000 µl

-SOB 970 µl

-2M glucose 10 µl

-2M $MgSO_4$ $7H_2O$ 10 µl

-2 M $MgCl_2$ $6H_2O$ 10 µl

- All solution mix together, ready for use.

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1.1.4 Luria-Bertani broth (LB) total volume 100 ml.

-peptone 1.0 g.

-NaCl 1.0 g

-Yeast extract 0.5 g.

- Dissolve in 100 ml. of deionized water. Adjust the pH of the solution to 7.0 using sodium hydroxide (NaOH). Autoclave at 121°C for 20 min to sterilized the broth (The broth can be stored sealed at room temperature).

1.1.5 SOB solution total volume 100 ml.

-Yeast extract 0.5 g.

-Tryptone 2 g.

-1 mM NaCl 1 g.

-1 M KCl 0.25 ml

-1mM $MgCl_2$ 1 ml

-1mM $MgSO_4$ 1 ml

- Dissolve in 100 ml. of deionized water. Adjust the pH of the solution to 7.0 using sodium hydroxide (NaOH) or hydrochloric (HCl). Autoclave at 121°C for 20 min to sterilized.

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1.2 Chemicals for preparation the competent cell

1.2.1 TB solution total volume 100 ml

-PIPES 0.3 g.

-CaCl2 0.7 g.

-KCl 1.86 g.

- Dissolve in deionized water and adjust to pH 6.7 with NaOH or HCl and then add 1.09 g. of MnCl2. Adjust to final volume 100 ml. Sterilize by filtration with 0.2 μm filter and store at 4°C.

1.2.2 Dimethyl sulfoxide (DMSO) store at -20°C

1.3 Chemical for cloning

1.3.1 -50 μg/ml of ampicillin

- Dissolve 0.5 mg of ampicillin in deionized water. Adjust to final volume 10 ml. Sterilize by filtration with 0.2 μm filter and store at -20°C. (Add 50 μm/ml of ampicillin into LB broth)

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1.3.2 Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal solution) 20 mg/ml.

- Dissolve 20 mg of bromo-4-chloro-3indolyl-β -D-galactoside in 1 ml of dimethylformamide (DMF). Store at 20°C.

1.3.3 Isopropyl thio-β-D-galactoside (IPTG solution) concentration 24 mg/ml.

- Dissolve 120 mg of isopropyl thio-β-D-galactoside in 5 ml deionized water. Sterilize by filtration with 0.2 μm filter and store at -20°C.

1.4 DNA plasmid extraction kit (QIA Spin Miniprep Kit); QIAGEN@

-Lysis solution

-Wash buffer

-Elution buffer

-Fast plasmid spin column assembly

1.5 Chemicals for plasmid DNA extraction (agarose gel electrophoresis)

1.5.1 10X TAE buffer: volume 1 liter

-Tris-base	48.44	g
-$CH_3COONa_3H_2O$	16.4	g
-Na_2EDTA	7.44	g
-Glacial acetic acid	17	ml

- Dissolve in deionized water and adjust to pH 7.7 with glacial acetic acid. Adjust to final volume 1 liter and autoclave at 121°C for 20 min.

1.5.2 100 bp of DNA standard marker: Invitrogen®

1.5.3 Loading buffer solution

-Bromophenol blue	0.01	g.
-Tris-HCl (pH 6.8)	1.25	ml
-Glycerol	5	ml

- Dissolve bromophenol blue and tris-HCl and then adjust to final volume 5 ml with deionized water. Add 5 ml of glycerol. Store at 4°C.

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1.6 Chemicals for Polymerase Chain Reaction (PCR): Invitrogen®

1.6.1 The Chemicals for PCR consists:

-10X PCR buffer

-2 mM dNTP

-25 mM MgCl2

-Taq DNA polymerase

1.7 Buffer preparation

Buffers were prepared as concentrated stock solutions and stored at room temperature. Prior to use, stock running buffers were diluted to a 1 x working concentration with ddH_2O

1.7.1 10X TAE buffer (for gel electrophoresis)

Tris-base	48.4 g
0.5 M EDTA, pH 8.0	20 ml
Glacial acetic acid	11.42 ml

-Make up volume with water. Total volume 1,000 ml Adjust to pH 7.6-7.8

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Chemical for preparation immunohistochemistry

2.1 Tris buffered saline (TBS), stock 10X pH 7.6

-Trizma base (C4H11NO3)	24.2 g.
-Sodium chloride (NaCl)	80 g.

- Prepare Trizma base and Sodium chloride then dissolve in 1 L deionized water and adjust pH to 7.6 with concentrated HCl

2.2 Tris buffered saline (TBS), working 1X pH 7.6

-TBS stock 10X 100 ml

-Deionized water 900 ml

- Dissolve 100 ml of 10X TBS stock in deionized water 900 ml

2.3 Tris buffered saline, working 1X pH 7.6 with 0.1 % tween 20 (TBST)

-TBS 1X 100 ml

-Tween-20 1 ml

- Prepare 100 ml of 1X TBS add 1 ml tween-20 and mix

2.4 Set kit from Abcam compose:

-Mouse monoclonal to Leishmania Major Surface Protease (ab65290)

-Goat polyclonal Secondary Antibody to Mouse IgG2a-heavy chain (HRP) (ab97245)

-DAB substrate – (ab94665)

2.5 -3% normal horse serum

-Normal horse serum 3 ml

-TBST, working pH 7.6 97 ml

- Mix together, ready for use.

2.6 -3% Hydrogen peroxide

-30% Hydrogen peroxidase 10 ml

-Deionized water 90 ml

- Mix together, ready for use.

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Reagent for preparation fluorescent in situ hybridyzatio

3.1 Set kit from Dako compose

-Pre-Treatment Solution (20X)

-Pepsin

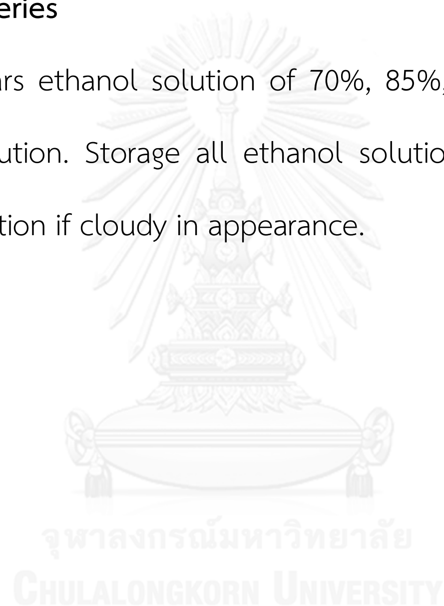
-Stringent Wash Buffer (20X)

-Wash Buffer (20X)

-Fluorescence Mouhting Medium

3.2 Ethanol series

- Prepare 2 jars ethanol solution of 70%, 85%, 95% respectively from 95% ethanol solution. Storage all ethanol solution at room temperature and discard solution if cloudy in appearance.



Appendice B

Protocol for preparation Scheider's insect medium (Sigma-alorich)

Supplements can be added prior to filtration or introduced aseptically to sterile medium. The nature of the supplement may affect storage conditions and shelf life of the medium.

Measure out 80% of final required volume of water. Water temperature should be 15-20°C.

1. While gently stirring the water, add the powdered medium. Stir until dispersed. Material will not go in solution completely. Do **not** heat water.
2. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2
3. To the solution in step 3, add 0.4 g. sodium bicarbonate or 5.3 ml of sodium bicarbonate solutio (7.5% W/V) for each liter of final volume of medium being prepared. Stir until dissolved.
4. While stirring, adjust the pH to at least 9.2 ± 0.2 with sodium hydroxide. Stir for a minimum of 10 minutes. Solution may become turbid.
5. While stirring, adjust the pH to 6.7 ± 0.2 with HCL, solution will clear.
6. Prepare a calcium chloride solution by dissolving 0.6 g of anhyfrous calcium chloride (C5670) in 50 ml of tissue culture grade water for EACH liter or final volume of medium being prepared. Slowly add the calcium chloride solution dropwise to the medium with rapid mixing to avoid precipitate formation.

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7. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1 N HCl or 1 N NaOH is recommended.
8. Add additional water to bring the solution to final volume.
9. An osmotic pressur of 360 mOsm ± 5% is suitable for the growth of cells derived from Drosophila melanogaster. If desired the osmotic pressure can be increased 10 mOsm by adding potassium chloride (0.4 g salt OR 2 ml of a 20% (w/v) solution) OR sodium chloride (0.3 g of salt OR 2 ml of a 15% (w/v) solution) for EACH liter of final volume of medium being prepared. The osmotic pressur can be decreased 10 mOsm by adding 27.8 ml of water for EACH liter of final volume of medium being prepared. Stir until dissolved.
10. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns or less.
11. Aseptically dispense medium into sterile container.

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Protocol for Immunohistochemical stain

Slide preparation

1) For tissue specimen (amastigote form) is formalin fixed, embedded in paraffin blocks and cut by microtome (5 μm) lay on adhesive superfrost plus slide.

2) For fresh specimen (promastigote form) prepares from tissue culture which is centrifuged at X 4,000 rpm in order to separate parasite from the solution. After that the residual cell is washed with TBS (Tris buffered saline) 2 times. Then pipette solution and smear specimen on the slide plus (test negative and positive cell). These slides are fixed with aceton solution and incubate at -20 °C for 15 minutes.

Allow slide of 1), 2) to dry completely at room temperature.

Deparaffinization and dehydration

Only slide of 1) is brought to deparaffinize slide with xylene 2 times, 5 min each then dehydration by serial of alcohol; 95%, 85%, 70% each 2 changes of reagent respectively, for 3 minute each. Transfer to wash slide twice in distilled water in running tap water for 10 minutes concentration prepares triplicate solution and washes in each solution 3 minutes. After that wash sections twice in distilled water for 5 minutes each.

Antigen retrieval

Slide of part 1) is prepared to unmask the antigenic epitope by putting in jar of 10 mM citrate buffer pH 6, is heated with microwave at hi-Power 3 minutes then adjust to power 30%, 10 minutes, bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 and maintain at a sub-boiling Temperature for 10 minutes. Washes section in 300 ml TBS 2 times for 5 min each (room temperature)

Block endogenous peroxidase activity

PAP pen (a special marking pen) uses to draw around a specimen of slide 1), 2) then incubate the slide in 3% H_2O_2 / DW 10 minutes at room temperature. After that the slide washes in TBS buffer 3 times for 5 min each.

Block non-specific background

Block with 4%bovine serum albumin 100-400 µL per reaction in each section for 30 minutes at room temperature. After that wash 3 times with TBS solution and block with 10% Normal horse serum in TBST 20 minutes at room Temperature. For the next step, only remove blocking solution (do not necessarily wash with buffer solution).

Primary antibody (1° Ab)

Add 1°Ab diluted with 10 % Normal Horse Serum in TBST to each section. Incubate overnight 4°C or 1-2 hr. room temperature.

Secondary antibody (2° Ab)

Remove antibody solution and wash sections in wash buffer 3 times for 5 min each. Then add 2° Ab conjugate with horseradish peroxidase (HRP) (1:400) dilute in 10 % Normal Horse Serum in TBST, incubate 30 minutes at room temperature.

Color development

Prepared reagent from the set of kit consists of DAB chromogen (3,3' diaminobenzidine) 30 µL = 1 drop and DAB diluent (1.5 ml = 50 drops), then incubate 10 minutes at room temperature. After that, washes these cell in dH_2O_2 2 times for 5 minutes each

- 1) tissue section takes to counter stain.

-Both 1) and 2) are dehydrated and mounted with medium (permount) to cover the section and observe the section by the light microscope

Protocol for Fluorescent in situ hybridyzation stain (FISH)

Day 1

-Step 1: Preparation slide

Preparation specimen slide of FISH method is the same step as IHC protocol, after all slide dry completely at room temperature. If the slide is tissue section from paraffin block, it has to pass the process of deparaffinization and dehydration.

-Step 2: Pre-Treatment

Water bath can be used for Per-Treatment (separated double stand of DNA). Prepared staining jar containing Pre-Treatment Solution (from set kit: dilute 1:20 in dH_2O) places in water bath. Set the temperature of water bath and Pre-Treatment Solution (close the chamber) to 95 - 99°C.

Immerse all slides from step 1 into the preheated chamber and incubate at 95 - 99°C for 10 ($\pm$1) minutes

Remove the entire chamber with slides from the water bath, open the lid and allow the slides to cool in Pre-Treatment Solution at room temperature for 15 minutes.

Transfer the slide to a chamber of dilute Wash Buffer (from set kit: dilute 1:20 in dH_2O) and soak for 3 minutes 2 times at room temperature (20-25°C) (continue to step 3).

-Step 3: Pepsin, (method A or B)

A) Place specimen with Pepsin (ready for use) in an incubator or on a heating block such as Dako Hybridizer at 37°C for 2-6 minutes. An incubation time of 3 minutes will be adequate for most specimens,.

B) Prepare pepsin solution as follows

For a chamber (jar) can contain 6 slides: prepare pepsin solution 60 ml

-Add 48 ml dH_2O or deionized at 20-25 ^{o}C in jar

-Add 6 ml of cold (2-8 ^{o}C) pepsin diluents (10X) from set kit

-Add 6 ml of cold (2-8°C) pepsin in the same set kit

All solution is mixed together and equilibrates at 37 ($\pm$2)^{o}C in water bath.

Tap off Pepsin and soak sections in the diluted Wash Buffer for 3 minutes 2 times at room temperature (20-25 ^{o}C). Continue to dehydration. Dehydrate tissue sections through a graded series of ethanol: 70%, 85% and 95%, respectively, for 3 minutes each

.

Note: Both of method 1 and 2, the optimal incubation time may depend on tissue fixation, should be determined by the user. So in the first time, the user ought to try the condition in order to find the appropriate time

-Step 4: Design Probe (minicircle kDNA lable with FITC)

- The following step should be performed in a fume hood.

-Apply 10 µL of design probe to the center of the tissue section. Immediately place a 22 mm x 22 mm glass coverslip over design probe and allow it to spread evenly under the coverslip. Avoid air bubbles. If air bubbles are observed, gently tap them away from the tissue using forceps.

-Seal coverslip with Coverslip Sealant or apply using with nail varnish around the periphery of the coverslip. Allow the Coverslip Sealant to overlap the coverslip and the slide, thereby forming a seal around the coverslip. Make sure that the Coverslip Sealant covers the entire edge of the coverslip.

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-Prepare Dako Hybridizer for a hybridization run. Make sure that Humidity Control Strips are saturated and optimal for use. Start the Hybridizer and choose a program that will denature at 82 (±2)°C for 5 minutes and hybridize overnight (14-20 hr.) at 45°C (please refer to Dako Hybridizer Instruction Manual for details). Place slides in the Hybridizer, make sure the lid is properly closed and start program.

- Instrumentation that allows for conditions similar to the ones described above maybe used for denaturation and hybridization.

-Pace slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 82 (±2)°C. Denature for 5 minutes ensuring that the temperature of the block does not drop below 80°C at any time.

-Place slide in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate overnight (14-20 hours) at 45 (±2)°C. Please note that a hybridization temperature of 37°C is not suitable for use with the probes contained within this kit.

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Day 2

Step 4: Stringent Wash

-Fill two staining jars, e.g. Coplin jars with the diluted Stringent Wash Buffer. A (from set kit: dilute 1:20 in dH_2O). A mininun volume of 100 ml or 15 ml per slide in each jar is recommended.

Place one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 65°C. Ensure that the temperature has stabilized.

Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 65°C; this will not affect performance.

-Using forceps or gloves take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time

-As soon as all coverslips have been removed transfer slides from the room temperature pre-wash jar to the 65°C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65°C for 3 minutes at room temperature (20-25°C)

-Change diluted Wash Buffer and soak section for another 3 minutes. Dehydrate tissue sections through a graded series of ethanol: in 70%, 85 and 95, respectively, for 3 minutes each.

-**Step 5: Mounting**

-Apply 15 µL of fluorescence Mounting Medium containing DAPI to the target area of the slide and apply a glass coverslip.

Note: Slide may be read after 15 minutes or within 7 days after mounting. However, fading occurs if slides are exposed to light or high temperatures. To minimize fading, store slides in the dark at 2-8°C

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Appendix C

Recording form

IHC | **FISH**

Symbol	Positive				Negative				Symbol	Positive				Negative			
I	1	2	3	4	1	2	3	4	F	1	2	3	4	1	2	3	4
M									M								
β									β								
□									□								
[symbol]									[symbol]								
Ω									Ω								
Δ									Δ								
E									E								

IHC | **FISH**

Symbol	Positive				Negative				Symbol	Positive				Negative			
I	1	2	3	4	1	2	3	4	F	1	2	3	4	1	2	3	4
◎									◎								
✳									✳								
☆									☆								
[symbol]									[symbol]								
[symbol]									[symbol]								
[symbol]									[symbol]								
∝									∝								

Appendix D

Summary of 12 authothonous leishmaniasis in Thailand

Table. Summary of 12 case reports of autochthonous leishmaniasis in Thailand.

Year, province	Age (years), sex	Occupation	Underlying disease	Clinical features; duration	Form of leishmaniasis, species of *Leishmania*	Investigations: Sandfly vectors	Investigations: Animal reservoirs (DAT)	Treatment	Outcome
1996, Surat Thani[3]	3, female	No	No	Fever, hepatosplenomegaly, anemia thrombocytopenia; 2 months	VL, no species identified	No study	No study	Pentamidine isethionate for 15 doses	Remission
2005, Nan[4]	40, male	Construction worker in several provinces	Amphetamine and opium addiction	Fever, hepatosplenomegaly, pancytopenia, mediastinal mass; 31 months	VL, no species identified	No potential vectors	Positive in 3 cows and 1 cat	2 courses of ABd for 30 days	Remission
2006, Phangnga[5]	55, male	Rubber planter	No	Fever, hepatosplenomegaly, pancytopenia; 3 years	VL, *L. siamensis*	No potential vectors	Positive in 9 cats	ABd (100 mg) mixed with 1 mg lipid for 14 days	Relapse 2 months after treatment
2007, Bangkok[6]	66, male	Lumber truck driver	Diabetes, hypertension	Fever, weight loss, hepatosplenomegaly pancytopenia; 6 months	VL, *L. infantum*	Inability to obtain vectors due to raining	Negative in 9 dogs, 1 cat, 3 rats	ABd every other day for 30 days	Remission
2007, Nakhon Si Thammarat	44, male	Rubber planter	Diabetes	NA; 6 months	VL, *L. siamensis*	*P. argentipes* and other 4 non-potential vectors	Positive in 1 cow and 1 cat	NA	NA

NA: not applicable, ABd: amphotericin B deoxycholate, VL: visceral leishmaniasis, DAT: direct agglutination test for *Leishmania* antibody, HCV: hepatitis C virus

[59]

Table. Summary of 12 case reports of autochthonous leishmaniasis in Thailand (continued).

Year, province	Age (years), sex	Occupation	Underlying disease	Clinical features; duration	Form of leishmaniasis, species of *Leishmania*	Investigations		Treatment	Outcome
						Sandfly vectors	Animal reservoirs (DAT)		
2007, Chiang Rai	36, male	Employee	AIDS	Skin papules, nodules; 1 year	CL, *L. siamensis*	*P. argentipes* and other 3 non-potential vectors	Negative	NA	Remission
2009, Chantha-buri[7]	37, male	Fisherman, history of travel to North Indonesia	AIDS, chronic HCV infection	Fever, nephritonephrotic syndrome, hepatosplenomegaly, anemia, thrombo-cytopenia; 8 weeks	VL, *L. siamensis*	No potential vectors	Negative	ABd every other day for 14 days, and itraconazole (400 mg/day)	Remission
2012, Songkhla[8]	46, male	Rubber planter	AIDS	Knee ulcer, anemia, thrombocytopenia, hepatosplenomegaly; few months	VL & CL, *L. siamensis*	No study	No study	ABd for 14 days, and itraconazole (400 mg/day)	Relapse 2 months after treatment
2012, Trang[8]	30, male	Pet store owner	AIDS	Skin papules and ulcers, hepatosplenomegaly, pancytopenia; 4 years	VL & CL, *L. siamensis*	No study	No study	ABd for 14 days, and itraconazole (400 mg/day)	Remission

NA: not applicable, ABd: amphotericin B deoxycholate, VL: visceral leishmaniasis, CL: cutaneous leishmaniasis, DAT: direct agglutination test for *Leishmania* antibody, HCV: hepatitis C virus

[59]

Table. Summary of 12 case reports of autochthonous leishmaniasis in Thailand (continued).

Year, province	Age (years), sex	Occupation	Underlying disease	Clinical features; duration	Form of leishmaniasis, species of *Leishmania*	Investigations		Treatment	Outcome
						Sandfly vectors	Animal reservoirs (DAT)		
2012, Trang[9]	32, female	NA	AIDS	Skin nodules, plaques, hepatomegaly, anemia; 1 month	VL & CL, *L. siamensis*	No study	No study	ABd for 14 days	Death 2 weeks after treatment
2012, Lop Buri[10]	3, female	No	No	Check ulcer, 1 month	CL, *L. siamensis*	No study	No study	Itraconazole (5 mg/kg/day) for 2 months	Remission
2013, Satun[11]	5, female	No	No	Hepatosplenomegaly, anemia, thrombocytopenia; 2 years	VL, *L. siamensis*	No study	No study	ABd for 2 times: 3 and 5 weeks, and ABd 5 day/mg/month for 6 months	Remission

NA: not applicable, ABd: amphotericin B deoxycholate, VL: visceral leishmaniasis, CL: cutaneous leishmaniasis, DAT: direct agglutination test for *Leishmania* antibody, HCV: hepatitis C virus

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APPENDIX

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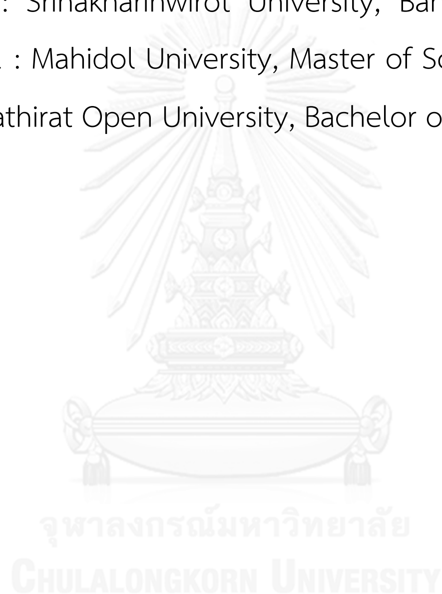
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