จำนวน การแพร่กระจายของเชื้อและพยาธิวิทยาด้านภูมิคุ้มกันของการติดเชื้อ *ลิชมาเนีย*
มาร์ตินิกเควนซิส ในหนู BALB/c ที่ถูกฉีดเชื้อเข้าเส้นทางและระยะเวลาที่ต่างกัน

นางสาวนวพรรษ สมบูรณ์พูลผล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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PARASITE BURDEN, DISTRIBUTION AND IMMUNOPATHOLOGY OF

LEISHMANIA MARTINIQUENSIS - INFECTED BALB/C MICE

IN DIFFERENT ROUTES AND TIME POINTS

Miss Nawaphat Somboonpoonpol

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	PARASITE BURDEN, DISTRIBUTION AND IMMUNOPATHOLOGY OF *LEISHMANIA MARTINIQUENSIS* - INFECTED BALB/C MICE IN DIFFERENT ROUTES AND TIME POINTS
By	Miss Nawaphat Somboonpoonpol
Field of Study	Veterinary Pathobiology
Thesis Advisor	Assistant Professor Woraporn Sukhumavasi, D.V.M., Ph.D.
Thesis Co-Advisor	Associate Professor Theerayuth Kaewamatawong, D.V.M., Ph.D., DTBVP

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D., DTBVP)

THESIS COMMITTEE

....Chairman

(Associate Professor Sonthaya Tiawsirisup, D.V.M., Ph.D., DTBVP)

....Thesis Advisor

(Assistant Professor Woraporn Sukhumavasi, D.V.M., Ph.D.)

....Thesis Co-Advisor

(Associate Professor Theerayuth Kaewamatawong, D.V.M., Ph.D., DTBVP)

....Examiner

(Associate Professor Padet Siriyasatien, B.Sc., M.D., Ph.D., DTMH)

....Examiner

(Associate Professor Nopadon Pirarat, D.V.M., Ph.D., DTBVP)

....External Examiner

(Professor Colonel Mathirut Mungthin, M.D., Ph.D.)

นวพรรษ สมบูรณ์พูลผล : จำนวน การแพร่กระจายของเชื้อและพยาธิวิทยาด้านภูมิคุ้มกันของการติดเชื้อ *ลิชมาเนีย มาร์ตินิกเควนซิส* ในหนู BALB/c ที่ถูกฉีดเชื้อเข้าเส้นทางและระยะเวลาที่ต่างกัน (PARASITE BURDEN, DISTRIBUTION AND IMMUNOPATHOLOGY OF *LEISHMANIA MARTINIQUENSIS -* INFECTED BALB/C MICE IN DIFFERENT ROUTES AND TIME POINTS) อ.ที่ปรึกษาวิทยานิพนธ์ หลัก: ผศ. สพ.ญ. ดร.วรพร สุขุมาวาสี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร.ธีระยุทธ แก้วอม ตวงศ์, 59 หน้า.

โรคลิชมาเนียเป็นโรคติดต่อจากสัตว์สู่คนที่อุบัติใหม่ในประเทศไทย และมีแมลงเป็นพาหะ การติดต่อโรค อาศัยการกัดของแมลงริ้นฝอยทรายเพศเมีย มีรายงานการติดเชื้อลิชมาเนีย *มาร์ตินิกเควนซิส* ในผู้ป่วยคนไทยที่ไม่ เคยเดินทางออกนอกประเทศอย่างต่อเนื่องตั้งแต่ พ.ศ. 2557 ซึ่งพบในผู้ป่วยที่มีภาวะภูมิคุ้มกันปกติและที่มีการติด เชื้อเอชไอวีร่วมด้วย รวมไปถึงผู้ป่วยที่มีหรือไม่มีโรคแทรกซ้อน และเด็ก วัตถุประสงค์ของการศึกษานี้เพื่อศึกษา จำนวน การแพร่กระจายและพยาธิวิทยาด้านภูมิคุ้มกันของการติดเชื้อลิชมาเนีย *มาร์ตินิกเควนซิส* ในหนูทดลองสาย พันธุ์ BALB/c โดยฉีดเชื้อระยะโปรแมสติโกทจำนวน 5x10⁶ ตัว เข้าทางหลอดเลือดดำ ช่องท้อง และใต้ผิวหนัง แก่ หนู BALB/c 16 ตัว ในแต่ละกลุ่ม ในวันที่ 7 14 28 และ 112 หลังการฉีดเชื้อ หนู 4 ตัว จากแต่ละกลุ่มจะถูกการุณย ฆาตเพื่อทำการเก็บตัวอย่าง เลือด ตับ ม้าม ไขกระดูก ต่อมน้ำลาย และไต เพื่อศึกษาการแพร่กระจายตัวของเชื้อใน แต่ละอวัยวะ ได้มีการใช้เทคนิคพีซีอาร์ที่จำเพาะกับยีน ITS1 ของเชื้อลิชมาเนีย พบดีเอ็นเอของเชื้อในตับของหนูทุก ตัวจากกลุ่มที่ฉีดเชื้อเข้าทางหลอดเลือดดำและในหนูเกือบทุกตัวในทุกช่วงเวลาที่ทำการทดลอง สำหรับม้าม พบสาร พันธุกรรมของเชื้อในหนูทุกตัวที่ได้รับเชื้อทางหลอดเลือดดำและช่องท้องในทุกช่วงเวลา ส่วนการนับจำนวนเชื้อใน ตับและม้ามจากการย้อม impression smear ด้วยสี Giemsa และคำนวณเป็น Leishman-Donovan unit (LDU) พบว่าการฉีดเชื้อเข้าทางหลอดเลือดดำพบจำนวนปรสิตสูงที่สุดจำนวน 101 LDU ในวันที่ 7 หลังฉีดเชื้อ ในขณะที่ใน ม้ามพบจำนวนเชื้อสูงสุด 3.1 LDU ในวันที่ 112 สำหรับการประเมินการตอบสนองทางภูมิคุ้มกันแบบพึ่งเซลล์ในตับ ได้ตรวจย้อมอิมมูโนฮิสโตเคมีโดยใช้ซีรั่มของผู้ป่วยที่ติดเชื้อลิชมาเนีย *มาร์ตินิกเควนซิส* จากการฉีดเชื้อทางหลอด เลือดดำ พบจำนวนแกรนูโลมาที่พัฒนาเต็มที่สูงกว่ากลุ่มที่ไม่ได้รับเชื้อและกลุ่มที่ได้รับเชื้อในเส้นทางอื่นในวันที่ 7 14 และ 28 หลังการฉีดเชื้อ (p< 0.05) การลดลงของแกรนูโลมาที่ยังไม่พัฒนาเต็มที่และชนิดที่พัฒนาเต็มที่แล้ว รวมทั้ง มีแกรนูโลมาแบบ involuting ในวันที่ 112 หลังการฉีดเชื้อในตับ ได้แสดงให้เห็นถึงความสามารถทางภูมิคุ้มกันแบบ พึ่งเซลล์ของหนู BALB/c ในการควบคุมการติดเชื้อลิชมาเนีย *มาร์ตินิกเควนซิส* ได้ โดยสรุป การศึกษานี้ได้แสดง หลักฐานครั้งแรกว่าเชื้อลิชมาเนีย *มาร์ตินิกเควนซิส* เป็นสาเหตุให้เกิดโรคลิชมาเนียแบบอวัยวะภายใน โดยการฉีด เชื้อในห้องทดลองเข้าทางหลอดเลือดดำและทางช่องท้องหนู BALB/c ซึ่งพบเชื้อระยะ amastigote และสาร พันธุกรรมของเชื้อในอวัยวะภายในเป้าหมายที่สำคัญ

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	....
ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม	....

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KEYWORDS: BALB/C MOUSE / LEISHMANIA MARTINIQUENSIS / PARASITE BURDEN / PARASITE DISTRIBUTION / STAGE OF HEPATIC GRANULOMA

NAWAPHAT SOMBOONPOONPOL: PARASITE BURDEN, DISTRIBUTION AND IMMUNOPATHOLOGY OF *LEISHMANIA MARTINIQUENSIS* – INFECTED BALB/C MICE IN DIFFERENT ROUTES AND TIME POINTS. ADVISOR: ASST. PROF. WORAPORN SUKHUMAVASI, D.V.M., Ph.D., CO-ADVISOR: ASSOC. PROF. THEERAYUTH KAEWAMATAWONG, D.V.M., Ph.D., DTBVP, 59 pp.

Leishmaniasis is an emerging zoonotic vector-borne disease in Thailand transmitted by the bite of a tiny female phlebotomine sandfly. Autochthonous *L. martiniquensis* infection in Thai patients has been continuously reported since 2014 in patients co-infected with HIV, immunocompetent patient with or without underlying diseases and children. The objective of this study was to investigate the parasite burden, distribution and immunopathology in *L. martiniquensis*-infected BALB/c mice. Sixteen mice were intraperitoneally, i.p., intravenously, i.v., and subcutaneously, s.c., infected with 5×10^6 promastigotes of *L. martiniquensis* each. On 7, 14, 28 and 112 days post infection, dpi, 4 inoculated mice were euthanized from each inoculation group. Blood, livers, spleens, bone marrows, salivary glands and kidneys were collected at each time points. To detect the distribution of parasite, PCR targeting *Leishmania*-specific ITS1 genes was performed. DNA amplicons were detected from all BALB/c livers inoculated via i.v. and almost all mice via i.p. in all time points. For spleen, *Leishmania* DNAs were present in all time points from both i.v. and i.p. routes. Giemsa-stained impression smear of liver and spleen was conducted to analyse parasite burden in Leishman-Donovan unit (LDU). Via i.v. route, the highest parasite burden in liver was found on 7 dpi, 101 LDU, whereas it was shown in the spleen on 112 dpi, 3.1 LDU. To evaluate cell-mediated immune responses in liver, indirect immunohistochemical staining using *L. martiniquensis*-infected human serum was performed. The number of mature granuloma on 7, 14 and 28 dpi from i.v. route was found significantly superior than the non-infected control and other routes ($p < 0.05$). Reduction of immature and mature granuloma with development of involuting granuloma on 112 dpi in livers revealed the ability of BALB/c cellular immunity to control *L. martiniquensis* infection. In conclusion, this study is the first to reveal that *L. martiniquensis* is a causative agent for visceral leishmaniasis in BALB/c mice by experimental inoculation via i.v. and i.p. routes based on the presence of amastigotes as well as genomic DNAs of *L. martiniquensis* in important target organs.

Department:	Veterinary Pathology	Student's Signature
Field of Study:	Veterinary Pathobiology	Advisor's Signature
Academic Year:	2016	Co-Advisor's Signature

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ANOVA	Analysis of Variance
bp	base pair
CL	cutaneous leishmaniasis
CR3	complement receptor 3
DAB	3,3'-Diaminobenzidine
DAT	direct agglutination test
DNA	deoxyribonucleic acid
dpi	day post infection
et al.	et alibi, and other
GP63	glycoprotein 63
H&E	hematoxylin and eosin
HIV	human immunodeficiency virus
IFN	interferon
IG	immature granuloma
IL	interleukin
i.p.	intraperitoneal route
ITS1	internal transcribed spacer 1
i.v.	intravenous route
IVG	involuting granuloma
LDU	Leishman-Donovan units

LPG	lipophosphoglycan
MAC	membrane attack complex
MCL	mucocutaneous leishmaniasis
MG	mature granuloma
MNC	mononuclear cell
NETosis	neutrophil extracellular trap
NG	no granuloma
NK	natural killer
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction technique
rpm	revolutions per minute
s.c.	subcutaneous route
SEM	standard error of the mean
SSU-rRNA	small subunit-ribosomal ribonucleic acid
TGF	transforming growth factor
Th	T helper cell
TNF	tumor necrosis factor
VL	visceral leishmaniasis

CHAPTER 1

INTRODUCTION

Leishmaniasis is a zoonotic vector-borne disease distributed globally in 4 continents and is endemic in 98 countries (Alvar et al., 2012). Female sand fly is the main biological vector for this protozoan (Dostalova and Volf, 2012). In human, at least 20 *Leishmania* species are responsible for different disease manifestation in which classified into 3 clinical forms including cutaneous, mucocutaneous, and visceral dependent upon *Leishmania* species and host immune responses (Banuls et al., 2007). According to the report from WHO Leishmaniasis Control Team in 2012, 1 million cases of cutaneous leishmaniasis were reported in the last 5 years whereas 300,000 estimated cases of visceral leishmaniasis were reported with over 20,000 deaths annually (Alvar et al., 2012).

In the past, Thailand was considered as non-endemic area for leishmaniasis. The majority of the reported cases were imported travelers or workers who relocated from endemic area, Middle East (Viriyavejakul et al., 1997; Wiwanitkit, 2011). In 1960, the first imported case in Thailand was a Pakistanian worker with a visceral form of leishmaniasis (Laohapaibul and Siampakdi, 1960) whereas, in 1981, the first imported case in Thai worker was succumbed to cutaneous leishmaniasis (Puavilai et al., 1981). However, the first autochthonous leishmaniasis in Thai 2 years old patient was reported in 1999 (Thisyakorn et al., 1999) hence making this disease no longer exotic. Since then, leishmaniasis cases have been rising periodically in human in Thailand predominantly in southern part of Thailand (Sukmee et al., 2011; Leelayoova et al., 2017). Consequently, leishmaniasis is considered to be an emerging infectious disease in Thailand due to 4 *Leishmania* species including *Leishmania donovani* (Kongkaew et al., 2007*), Leishmania infantum* (Maharom et al., 2008*), Leishmania siamensis* (Bualert et al., 2012; Supsrisunjai et al., 2017) and *Leishmania martiniquensis*. Autochthonous *L. martiniquensis* infection in Thai patients has been continuously reported since 2014 leading to visceral, disseminated cutaneous and combined clinical manifestation

(Pothirat et al., 2014; Chiewchanvit et al., 2015; Siriyasatien et al., 2016). Although the majority of leishmaniasis patients in Thailand were co-infected with HIV (Suankratay et al., 2010), immunocompetent patient with or without underlying diseases and children (Kattipathanapong et al., 2012) were reported to be infected with *Leishmania*. More importantly, potential vector and animal reservoir were identified in Thailand as DNAs of *Leishmania* were detected (Kanjanopas et al., 2013; Chusri et al., 2014).

Recurrent infection, side effect and resistance from treatment are challenging to control *Leishmania* infection (Roatt et al., 2014; Alemayehu et al., 2016; Singh et al., 2016). In order to develop effective anti-*Leishmania* drug and vaccine, understanding of disease mechanisms and immunopathology *in vivo* for particular *Leishmania* species are needed. So far, there is no murine experimental model specific for *L. martiniquensis* to answer aforementioned questions. Therefore, our aim of this study is to investigate the parasite burden, distribution and immunopathology in *Leishmania martiniquensis*-infected BALB/c mice, a susceptible mouse strain, via different routes and various time points.
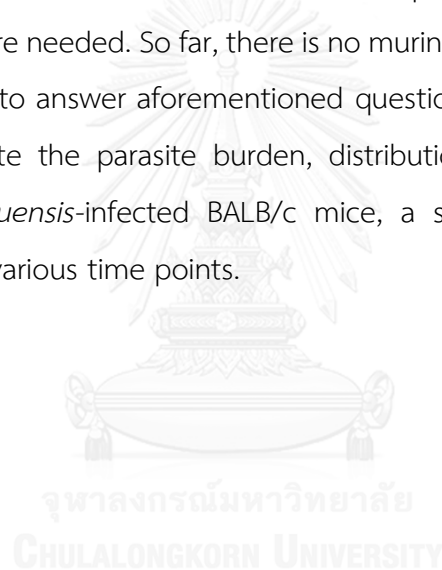
CHAPTER 2
OBJECTIVES

There were three objectives of this study

1. To detect the presence of *Leishmania martiniquensis* DNAs in different target organs of experimental BALB/c mice post-infection by various routes and time points

2. To compare *Leishmania martiniquensis* density in liver and spleen of experimental BALB/c mice post-infection by various routes and time points

3. To classify hepatic granuloma induced by *Leishmania martiniquensis* experimental infection in BALB/c mice by various routes and time points

CHAPTER 3

LITERATURE REVIEW

Leishmaniasis

Leishmaniasis is a vector-borne zoonotic disease caused by different species of protozoan parasite in genus *Leishmania*. The transmission occurs via biting of infected female phlebotomine sand fly belonging to the order Diptera, subfamily Phlebotominae (Dostalova and Volf, 2012). Although it is uncommon, venereal and vertical transmission were reported in both humans and animals (Naucke and Lorentz, 2012; Turchetti et al., 2014). In humans, there are 3 main clinical forms of leishmaniasis depending on the *Leishmania* species and host immune responses (World Health Organization, 2014). Cutaneous Leishmaniasis (CL), identified by nodules or ulcerative lesions of skin, is caused by *Leishmania major, L. tropica, L. aethiopica, L. mexicana, L. braziliensis, L. guyanensis, L. panamensis, L. peruviana, and L. amazonensis (Reithinger et al., 2007)*. Mucocutaneous Leishmaniasis (MCL) is caused by *L. braziliensis* and *L. panamensis* and infection leads to ulcer and necrosis on mucous membrane of mouth, nose and throat (Salam et al., 2014). Visceral Leishmaniasis (VL) is fatal if left untreated and characterized by hepatosplenomegaly, fever, muscle wasting, anemia, leukopenia and weight loss. Causative agents of VL include *L. donovani* and *L. infantum* (synonym *L. chagasi*). However, leishmaniasis can be asymptomatic in both humans and animal reservoirs making this disease neglected (Kaye and Scott, 2011; Esch and Petersen, 2013; Pace, 2014).

Biology of Leishmania

Leishmania has two morphological forms, amastigotes in vertebrate host and promastigotes in sand fly (Bates, 2007). The life cycle of *Leishmania* begins when female sand fly feeds on blood of an infected vertebrate host and *Leishmania* amastigotes are taken up. In sand fly mid gut, amastigote transforms to procyclic promastigotes and eventually become infective metacyclic promastigotes (Kamhawi,

2006). During the blood feeding, parasites are then move forward to the pharynx and regurgitated with saliva into the bite site entering dermis. Metacyclic promastigotes are phagocytosed by professional phagocytes where they transform into amastigotes (Handman and Bullen, 2002). When amastigotes replicate and occupy parasitophorous vacuole inside host cell's cytoplasm, infected cell is then ruptured. Amastigotes are subsequently released to infect neighboring phagocytes. The transmission cycle is complete when infected phagocytes or extracellular parasites are taken up by another sand fly (Kaye and Scott, 2011; Dostalova and Volf, 2012).

Epidemiology of leishmaniasis in Thailand

In Thailand, the number of leishmaniasis cases have been sporadically detected. In the past, Thailand was categorized as a non-endemic area as all cases occurred in returnee travelers from abroad (Viriyavejakul et al., 1997; Thisyakorn et al., 1999). Recently, the incidence of leishmaniasis in Thailand is mostly autochthonous cases caused by different species including *L. donovani*, *L. infantum*, *L. siamensis* and *L. martiniquensis* (Kongkaew et al., 2007; Maharom et al., 2008; Sukmee et al., 2008; Pothirat et al., 2014). Some of the previous reports of *L. siamensis*-infected cases were later confirmed and claimed to *L. martiniquensis* (Chiewchanvit et al., 2015). Many autochthonous cases were VL followed by combined VL and disseminated CL and CL (Leelayoova et al., 2017). Among these cases, majority of patients were immunocompromised by HIV co-infection followed by immunocompetent patients with underlying diseases and children. Interestingly, two of three cases in children were the first autochthonous case of VL (Thisyakorn et al., 1999) and CL (Kattipathanapong et al., 2012) in Thailand. For spatial distribution, a number of cases were mostly reported from southern followed by northern, central and eastern regions (Leelayoova et al., 2017).

Chronologically, the first autochthonous case of VL with an unidentified species was reported in Thailand in a 2-year-old girl with no history of travelling abroad (Thisyakorn et al., 1999). Later in 2007, another autochthonous VL was reported in a

37-year-old construction worker infected with *L. donovani*. In this report, a further investigation was also conducted by examining the environment. The *Sergentomyia* spp. sand flies was mostly found and Direct Agglutination Test (DAT) were positive in a cat and 3 cows around his habitat (Kongkaew et al., 2007).

In 2008, a suspected new species, *L. siamensis*, was reported to cause VL in a 55-year-old man who lived in Phangnga. In this study, DAT were also positive in 9 cats but negative by PCR (Sukmee et al., 2008). In the same year, VL caused by *L. infantum* was reported in a 66-year-old man who lived in Bangkok (Maharom et al., 2008). In 2010, the first autochthonous VL caused by *L. siamensis* in HIV-infected patient was reported in 37-year-old who lived in Chantaburi (Suankratay et al., 2010). Leishmaniasis caused by *L. siamensis* were reported again in 2012 in the southern part in three patients who lived in Songkla and Trang. Interestingly, all three cases were co-infected with HIV and presented in both CL and VL forms. (Bualert et al., 2012; Chusri et al., 2012). In the same year, a 3-year-old girl from Lopburi was also reported to have CL with unidentified *Leishmania* species (Kattipathanapong et al., 2012). Two years later, a 5-year-old girl from Satun presented with VL in which was initially diagnosed the causative agent as *L. siamensis*. She was also co-infected with HIV and had thalassemia (Osatakul et al., 2014).

In 2014, *L. martiniquensis* was firstly identified as a causative agent for autochthonous VL case in a 52-year-old man without underlying disease from Lampoon (Pothirat et al., 2014). Later in 2015 from the same province and Chiangmai, *L. martiniquensis* infection continued to emerge by causing 2 HIV-infected patients succumbed to disseminated CL and accompanying VL (Chiewchanvit et al., 2015). From a report of Siriyasatien et al (2016) using molecular detection of *Leishmania* spp., the prevalence of *Leishmania* infection in HIV-infected patients enrolling in the study at the Division of Infectious Diseases of Faculty of Medicine, Prince of Songkla University, located in the southern Thailand, was 0.95 % (3/316). All 3 cases were infected with *L. martiniquensis* detected from saliva and their clinical presentations were composed of disseminated CL, CL and aymptomatic (Siriyasatien et al., 2016).

Up to year 2017, the latest autochthonous leishmaniasis case in Thailand was reported in a 42-year-old woman co-infected with HIV. She had disseminated dermal leishmaniasis caused by *L. siamensis* (PCM2 Trang) (Supsrisunjai et al., 2017).

Leishmania martiniquensis

In 1995, *L. martiniquensis* was firstly isolated from HIV-infected patient, succumbing diffuse cutaneous nodular syndrome, from Martinique Island (French West Indies) (Dedet et al., 1995). Later in 2002, its taxonomical position was established (Noyes et al., 2002) and was named in 2014 (Desbois et al., 2014). *L. martiniquensis* is classfied in class Kinetoplastea, order Trypanosomatida and family Trypanosomatidae (Desbois et al., 2014). Apart from aforementioned cases of 4 *L. martiniquensis*-infected patients in Thailand between 2014 up to present, visceral form of Leishmaniasis was also reported in HIV-infected heterosexual Caribbean male patient (Liautaud et al., 2015).

Prior to characterization of *L. martiniquensis* in Thai patients, there were autochthonous cases reported from either unidentified species (Thisyakorn et al., 1999; Kattipathanapong et al., 2012) or suspected new species of *Leishmania* having identical sequences of ITS1 of rRNA using GenBank accession no. EF200011 (Sukmee et al., 2008), GQ226034 (Suankratay et al., 2010), JQ001751, JQ001752 (Chusri et al., 2012). The first evidence of using nomenclature *Leishmania* sp. *siamensis* appeared in a report in 9 horses showing *Leishmania*-induced cutaneous lesions in Germany and Switzerland due to the closed relationship of this *Leishmania*'s genotype (98% identity) (Muller et al., 2009) to the one in autochthonous human patient from Thailand (Sukmee et al., 2008). Later, Bualert et al., (2012) demonstrated that the novel species, the first two accession no., were *L. siamensis* by phylogenetic analysis. Also, the new case report from Trang province, Thailand, was declared as *L. siamensis* Trang strain. Since then, couple more articles were continuously published the findings of autochthonous infection both in Thailand (Phumee et al., 2013; Osatakul et al., 2014; Supsrisunjai et al., 2017) and in Myanmar (Phumee et al., 2013; Noppakun et al., 2014) by specifying the causative agent as *L. siamensis*.

Later in 2013, *L. siamensis* from previous 5 autochtonous VL patients in southern Thailand was further characterized to have 2 lineages including TR and PG (Leelayoova et al., 2013). For lineage PG, it was later claimed to be the same parasite from Martinique Island (French West Indies) by DNA sequence analysis (ribosomal RNA ITS-1 and large subunit of RNA polymerase II) (Pothirat et al., 2014). Thus the lineage PG was referred as the *Leishmania martiniquensis* while the lineage TR was identified as a new species, *Leishmania siamensis* (Leelayoova et al., 2017).

The true reservoir of *Leishmania martiniquensis* has not yet been identified (Desbois et al., 2014). The potential animal reservoir could be black rats (*Rattus rattus*), mongooses *(Herpestes auropunctatus)*, opossum (*Didelphis marsupialis)* and canids (Dedet et al., 1989; Zeledon, 1992; Chusri et al., 2014). Interestingly, the previous CL reports of 9 horses in Germany and Switzerland, a cow in Switzerland and a pregnant mare in United States (Muller et al., 2009; Lobsiger et al., 2010; Reuss et al., 2012) were later suggested by Leelayoova et al. (2017) to be *L. siamensis* PG lineage in which it eventually became *L. martiniquensis* due to the similarity between ITS1 of the SSU-rRNA gene of *Leishmania* DNA and the novel species of *Leishmania* reported in Thailand (Sukmee et al., 2008).

Potential vectors and host reservoirs for Leishmania spp. *in Thailand*

Potential vectors of leishmaniasis in Thailand were reported based on detection of *Leishmania* DNA amplicon in sand fly species namely *Sergentomyia (Neophlebotomus) gemmea,* and *Sergentomyia (Paratomyia) barraudi*. Also, black rat (*Rattus rattus*) was identified as potential animal reservoirs in southern region of Thailand (Kanjanopas et al., 2013; Chusri et al., 2014; Polseela et al., 2016). For seeking the possibility of pet animals to be potential reservoir host, Nimsuphan et al. (2014) found 0.84% (2/237) *Leishmania* seropositive cats in Surathani and Pangnga province in 2010 by DAT but negative for PCR. Also, seroprevalence of canine and feline leishmaniasis in major regions of Bangkok in 2014 was 8.4% (23/273) and 7.8% (21/269), respectively (Jitsamai and Sukhumavasi, unpublished data). Hence, true reservoir and true vector in Thailand have yet to be confirmed.

BALB/c mice

Inbred mouse strain BALB/c laboratory mice is one of the species commonly used in *Leishmania* infection experiment. This inbred strain could represent a model of *Leishmania* infection in both forms of visceral and cutaneous leishmaniasis. They are susceptible to visceralizing *Leishmania* spp., e.g., *L. donovani, L. infantum (L. chagasi)*, and cutaneous leishmaniasis-causing *L. major* (Loeuillet et al., 2016). The susceptibility is related to a predominant Th2 type immune response (Sacks and Noben-Trauth, 2002). Normally, early parasite growth in mouse tissue macrophages is determined by the phagosomal proton–cation antiporter encoded by the Slc11a1 gene. BALB/c mice have a nonfunctional gene, namely mutant Slc11a1 so it is subjected to uncontrollable parasite growth. (Wilson et al., 2005; Stanley and Engwerda, 2007; Loria-Cervera and Andrade-Narvaez, 2014)

Host-Leishmania interaction

While metacyclic promastigotes are being released into the dermis layer along with sand fly saliva, immunomodulatory and hemostatic properties of saliva allow the parasite to invade easily (Kamhawi, 2000). After sand fly bite, dermis structures and capillaries are mechanically disrupted and induced blood pool in dermis (de Almeida et al., 2003). During the first 5-10 minutes, neutrophils, the first line defense of immunity, are recruited to the site of infection. Phagocytosis and NETosis to trap up the pathogen are then triggered. Parasites can survive the killing effect of neutrophil by inhibiting the phagosome acidification. Later, professional phagocytes including macrophages and dendritic cells are recruited to take up the infected neutrophils by phagocytosis so called Trojan horse mechanism (Kaye and Scott, 2011). Free *Leishmania* promastigotes are also internalized by professional phagocytes. The surface lipophosphoglycan (LPG) and glycoprotein 63 (GP63 or major surface protease) of *Leishmania* have been identified to facilitate the phagocytosis and intracellular survival of parasite. LPG can inhibit the insertion of membrane attack complex (MAC) and cause a phagosomal hole that leads to failure of acidify and trigger microbicidal machinery. GP63 is able to degrade C3b to C3Bi (inactive form) which help the

internalization of parasite through complement receptor 3 (CR3). Moreover, GP63 can also degrade fibronectin and these fibronectin fragments can inhibit the reactive oxygen species formation from macrophage. Promastigotes can survive in the parasitophorous vacuole, transform to amastigote stage and replicate themselves without killing by macrophages. To avoid this mechanism, *Leishmania* triggers the alternative activation of macrophage by releasing Th2 cytokine, such as IL-4, that upregulates arginase activity, instead of the Th1 cytokine in which parasite will be killed by leishmanicidal activity through nitric oxide (NO) synthesis (Liu and Uzonna, 2012; Walker et al., 2014; de Menezes et al., 2016).

Organ-specific immunity

In the study of VL experiment in mice, the two major target tissues of infection are liver and spleen. The liver is a place of an acute resolving infection while spleen is a site of parasite persistence. After parasite inoculation, promastigotes invade the local macrophages, kupffer cells and dendritic cells where they transform and replicate into amastigote forms. At the beginning of infection, parasite growth is uncontrollable because inability of immune cell to produce IFN-γ and IL-12 and increased IL-10 and $TGF\beta$ level from spleen cell production leading to ineffective Th1 response. After the first 4 weeks, the performance of CD4$^+$ T, CD8$^+$ T cells and natural killer (NK) cells are recovered to produce IFN-γ, therefore the macrophage can kill the parasite through nitric oxide (NO) synthesis. Subsequently, infection of liver is controlled through granuloma formation characterized by parasitized Kupffer cells surrounded by a mantle lymphocyte. Finally, after 8 weeks, amastigotes in granulomas are absent and resolving the infection. In contrast, infection in the spleen and bone marrow are maintained through the whole course of infection. Stromal macrophages are the main target for the *L. donovani* infection. It can also induced the myelopoiesis in the bone marrow (de Freitas et al., 2016). TNF and TGF-β production are essential for control infection in liver but excessive production in splenic tissues can lead to immunopathology and immunological dysfunction. Under cytokine storm, splenic

architecture is damaged, making white pulp disorganized and decrease in size as well as red pulp hypertrophic. (Stanley and Engwerda, 2007; Loeuillet et al., 2016).

Diagnostic methods

Microscopy and culture *in vitro* are definitive methods to detect and confirm the *Leishmania* infection (Chargui et al., 2012; Stockdale and Newton, 2013). However, these methods need a specialist to perform and have poor sensitivity (Cuba Cuba et al., 1986; Myjak et al., 2009). Polymerase chain reaction (PCR), the method for DNA amplication, has proved highly sensitive and specific compared with standard methods (Chappuis et al., 2007; Chargui et al., 2012; Phumee et al., 2013). From the study of comparative of PCR methods for detection of *L. siamensis* infection (Hitakarun et al., 2014), the internal transcribed spacer 1 (ITS1) region of the small subunit ribosomal RNA (SSU-rRNA) gene is the common target gene for *L. siamensis* detection because of its great sensitivity and specificity. In a study reported from Italy, PCR was found to be more highly sensitive and specific than microscopic techniques for detection of *Leishmania* in peripheral blood and bone marrow samples from HIV-infected and uninfected patients (Antinori et al., 2007). There were many reports about the detection of *Leishmania* DNA in variety of samples by PCR such as bone marrow, blood, buffy coat, saliva, urine and tissues. Some papers also reported the positive results in asymptomatic patients (Phumee et al., 2013; Siriyasatien et al., 2016).

CHAPTER 4

MATERIALS AND METHODS

Animals

Parasite inoculation in mice was conducted by previous study so samples were obtained from storage in -20°C. Female specific-pathogen free BALB/c mice, 6-8 weeks old, were purchased from National Laboratory Animal Center, Mahidol University, acclimatized for 3 weeks and maintained in animal biosafety level 2 facility at National Institute of Animal Health, NIAH. Animal use protocol was approved by NIAH animal use committee (EA-001/57(R)).

Parasite preparation

L. martiniquensis (strain MHOM/TH/2011/PG) culture was obtained from Department of Parasitology, Phramongkutklao College of Medicine. Mid-log metacyclic promastigotes were washed twice with 1xPBS at 200xg for 5 min and then enumerated. Resuspension was performed in 200 μl 1xPBS.

Experimental infection and sample collection

Sixteen mice were intraperitoneally, i.p., intravenously, i.v., and subcutaneously, s.c., infected with $5x10^6$ promastigotes of *Leishmania martiniquensis* each. On 7, 14, 28 and 112 days post infection, dpi, 4 inoculated mice were euthanized from each inoculation group by ether jar. Control group were injected with 1xPBS via the same route and sacrificed on each time point. Mice were exanguineous from caudal vena cava and/or by cardiac puncture to collect blood. Heparinized blood was centrifuged at 3000 rpm for 10 min to isolate the buffy coat. Visceral organs including liver, spleen, kidney, salivary gland were harvested. Bone marrow was flushed with 1xPBS from femur then centrifuged to isolate pellet. All samples were stored at -20°C for further DNA extraction

DNA extraction and PCR

Genomic DNA of all tissue samples were extracted using DNA extraction kit (Genomic DNA Mini Kit (Tissue), Geneaid®, Taiwan). DNA from buffy coat samples were extracted using DNA extraction kit (DNeasy Blood and Tissue Kit, Qiagen®, Germany) according to the manufacturer's protocol. The concentration and purity of each DNA sample were evaluated by the ratio of absorbance at 260/280 nm using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific, Delaware, USA). The PCRs were performed on a GeneAmp® PCR system 2700thermal cycler (Applied Biosystems, Foster City, CA). The amplification was performed according to previously published article (Hitakarun et al., 2014). DNA was amplified from 500 nanogram sample DNA in a total reaction volume of 25 µL. Positive control *L. martiniquensis* DNA was obtained from promastigote culture. Distilled water was used as a template solution for negative control. The amplification product of the specific PCR assay is a 351 bp DNA fragment of a repetitive sequence of *L. martiniquensis* (Gene bank accession number: EF200012). PCR amplification products were analyzed by submerged gel electrophoresis with 100-bp DNA ladder (Vivantis, Malaysia) in 1.5% (w/v) agarose gel stained with SYBR®safe and were visualized under UV transilluminator.

Table 1. Primers for *Leishmania martiniquensis* detection by PCR

Target genes	Primer name	Oligonucleotide primer (5' →3')	Product sizes (bp)
ITS1	L5.8S	TGA TAC CAC TTA TCG CAC TT	~351
	LITSR	CTG GAT CAT TTT CCG ATG	

(Hitakarun et al., 2014)

Immunohistochemical Examination

Liver and spleen samples were fixed in 10% buffered formalin and then embedded in paraffin. Sections 4 µm were placed on positively-charged slides and processed to indirect immunohistochemical staining using *L. martiniquensis*-infected human serum (1:500 dilution) and Peroxidase-conjugated AffiniPure Goat Anti-human IgG heavy and light chain antibody (1:500 dilution; Jackson ImmunoResearch, USA). 3,3'-Diaminobenzidine (Dako REAL ™ DAB+chromogen, Denmark)-hydrogen peroxide was applied as the chromogen and Mayer's hematoxylin was used as the counterstain. Cell-mediated immune responses to parasitized Kupffer cells in the liver are categorized as following.

Table 2. Classification of hepatic immune responses

Grading	Description
No granuloma (NG)	No inflammation with no mononuclear cell (MNC) around the parasitized Kupffer cells
Immature granuloma (IG)	Less than 10 MNCs around the parasitized Kupffer cells
Mature granuloma (MG)	Epitheloid cells and more than 10 MNCs around the parasitized Kupffer cells
Involuting granuloma (IVG)	Devoid of amastigote and tissue inflammatory nearly resolved

The number of the each tissue response of no granuloma, immature granuloma, mature granuloma and involuting granuloma were counted for 25 consecutive microscopic fields per mouse liver at 400x magnification (Nieto et al., 2011; Tiwananthagorn et al., 2012).

Hepatic and splenic parasite burden

Impression smear of liver and spleen samples were previously stained with 10% Giemsa. Leishman-Donovan unit (LDU) index was used to evaluate the parasite burden. The LDU index was carried out under microscope to detect *Leishmania* amastigote form per 1000 nucleated cells multiplied by the organ weight. Counting was made to 100 organ cell nuclei per stamp (Stauber, 1956).

Statistical Analysis

Results were expressed in mean ± standard error of the mean (SEM). The statistical analysis was performed using the Two-way ANOVA and Kruskal-Wallis comparison test. Differences were considered significant when $p < 0.05$. The parasite distribution was using descriptive analysis.
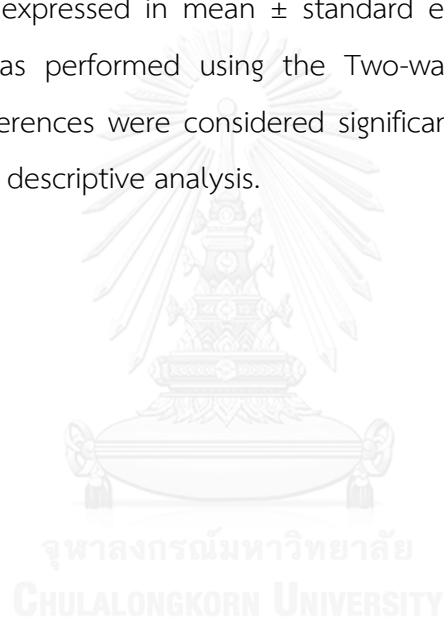
CHAPTER 5

RESULTS

Parasite detection by PCR

Molecular parasite identification and kinetics of distribution via each route of inoculation was conducted by conventional PCR technique. Various organs and tissues including liver, spleen, bone marrow, kidney, salivary gland and buffy coat, were collected.

DNA amplicons were detected in livers of all BALB/c mice (n=4/route/time point) experimentally inoculated with *L. martiniquensis* via i.v. and ip routes on 7, 14, and 28. On 112 dpi, this result was the same case for i.v. but DNAs were only detected from two mice (2/4) from i.p. group. For s.c., DNA amplicons were faintly detected on 14, 28, 112 dpi in some inoculated mice, 1/4, 2/4 and 1/4, respectively.

For spleen, DNAs of *L. martiniquensis* were present in all time points from every BALB/c mice inoculated by i.v. and i.p. route (n=4/route/time point). For s.c. route, the positive bands were found on 7, 28 and 112 dpi in certain mouse or mice (1/4, 1/4 and 2/4, respectively).

Regarding bone marrow, both i.v. and i.p. inoculation of *L. martiniquensis* in BALB/c mice on 7 dpi led to 50% of all mice to be infected (2/4). On 14 dpi, DNAs were present in all mice infected via both routes. On 28 dpi, all mice inoculated with parasite via i.v. and almost all mice with one mouse spared DNAs undetected had *L. martiniquensis* DNAs in bone marrow. For the last time point, DNAs of parasite were present in every inoculated mice for both i.v. and i.p. routes. Interestingly, none of *Leishmania* DNAs were amplified in all time course by s.c. inoculation.

Apart from target organ for leishmaniasis, DNAs of *L. martiniquensis* were found on 14 dpi via i.v. inoculation from buffy coat of one mouse (lane A) whereas DNAs

were detected on 28 and 112 dpi via the same routes from one mouse each (lane D and C, respectively). For kidney, 2 of 4 mice were positively found in i.v. & i.p.

Spleen samples were strongly found positive in all time points as liver samples were strongly positive on 7, 14, 28 dpi. Bone marrow samples were positively found since 14 dpi while Salivary gland samples were positively found 28 dpi onward. Moreover, buffy coat samples were found positive only on 14 dpi while kidney samples were found only on 28 dpi.
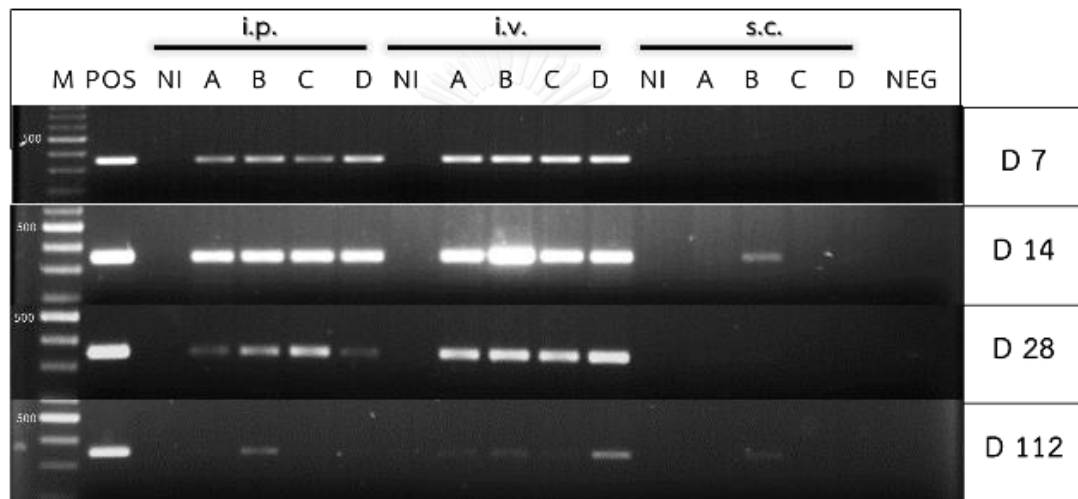
Figure 1. **PCR diagnosis of** *L. martiniquensis* **from** *L. martiniquensis***-infected BALB/c mouse liver using ITS1-specific primers on 7, 14, 28 and 112 dpi.**
Lane M: 100 bp DNA marker. Lane POS: Positive control. Lane NI: Non-infected control mouse. Lane A B C D: DNA from each inoculated mouse in each route. Lane NEG: Negative control

Table 3. Summary of PCR result from *L. martiniquensis*-infected BALB/c mouse liver using ITS1-specific primers on 7, 14, 28 and 112 dpi

Liver	i.p.				i.v.				s.c.			
	A	B	C	D	A	B	C	D	A	B	C	D
D 7	●	●	●	●	●	●	●	●				
D 14	●	●	●	●	●	●	●	●		●		
D 28	●	●	●	●	●	●	●	●	●	●		
D 112	●	●			●	●	●	●		●		

Figure 2. PCR diagnosis of *L. martiniquensis* from *L. martiniquensis*-infected BALB/c mouse spleen using ITS1-specific primers on 7, 14, 28 and 112 dpi.

Lane M: 100 bp DNA marker. Lane POS: Positive control. Lane NI: Non-infected control mouse. Lane A B C D: DNA from each inoculated mouse in each route. Lane NEG: Negative control

Table 4. Summary of PCR result from *L. martiniquensis*-infected BALB/c mouse spleen using ITS1-specific primers on 7, 14, 28 and 112 dpi

| Spleen	i.p.				i.v.				s.c.			
	A	B	C	D	A	B	C	D	A	B	C	D
D 7	●	●	●	●	●	●	●	●				●
D 14	●	●	●	●	●	●	●	●				
D 28	●	●	●	●	●	●	●	●				●
D 112	●	●	●	●	●	●	●	●		●		●

Figure 3. PCR diagnosis of *L. martiniquensis* from *L. martiniquensis*-infected BALB/c mouse bone marrow using ITS1-specific primers on 7, 14, 28 and 112 dpi. Lane M: 100 bp DNA marker. Lane POS: Positive control. Lane NI: Control mouse. Lane A B C D: DNA from each infected mouse in each route. Lane NEG: Negative control

Table 5. Summary of PCR result from *L. martiniquensis*-infected BALB/c mouse bone marrow using ITS1-specific primers on 7, 14, 28 and 112 dpi

Bone marrow	i.p.				i.v.				s.c.			
	A	B	C	D	A	B	C	D	A	B	C	D
D 7	●		●		●			●				
D 14	●	●	●	●	●	●	●	●				
D 28	●	●	●		●	●	●	●				
D 112	●	●	●	●	●	●	●	●				

Figure 4. PCR diagnosis of *L. martiniquensis* from *L. martiniquensis*-infected BALB/c mouse buffy coat on 14 dpi using ITS1-specific primers. Lane M: 100 bp DNA marker. Lane POS: Positive control. Lane NI: Non-infected control mouse. Lane A B C D: DNA from each infected mouse in each route. Lane NEG: Negative control

Table 6. Summary of PCR result from *L. martiniquensis*-infected BALB/c mouse buffy coat using ITS1-specific primers on 14 dpi

Buffy coat	i.p.				i.v.				s.c.			
	A	B	C	D	A	B	C	D	A	B	C	D
D 14					●							
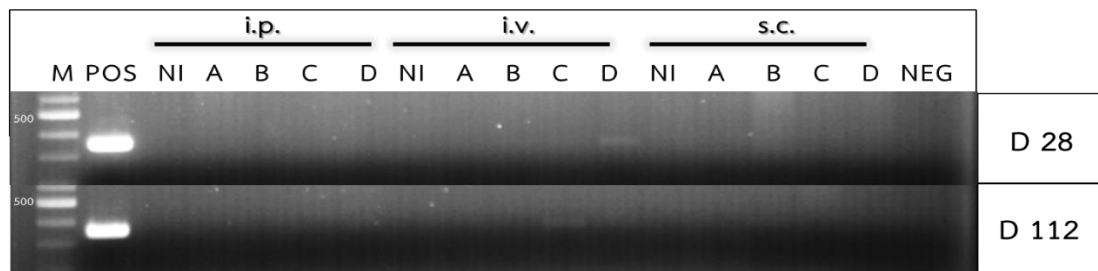
Figure 5. PCR diagnosis of *L. martiniquensis* from *L. martiniquensis*-infected BALB/c mouse salivary gland on 28 and 112 dpi using ITS1-specific primers. Lane M: 100 bp DNA marker. Lane POS: Positive control. Lane NI: Non-infected control mouse. Lane A B C D: DNA from each infected mouse in each route. Lane NEG: Negative control

Table 7. Summary of PCR result from *L. martiniquensis*-infected BALB/c mouse salivary gland using ITS1-specific primers on 14 dpi

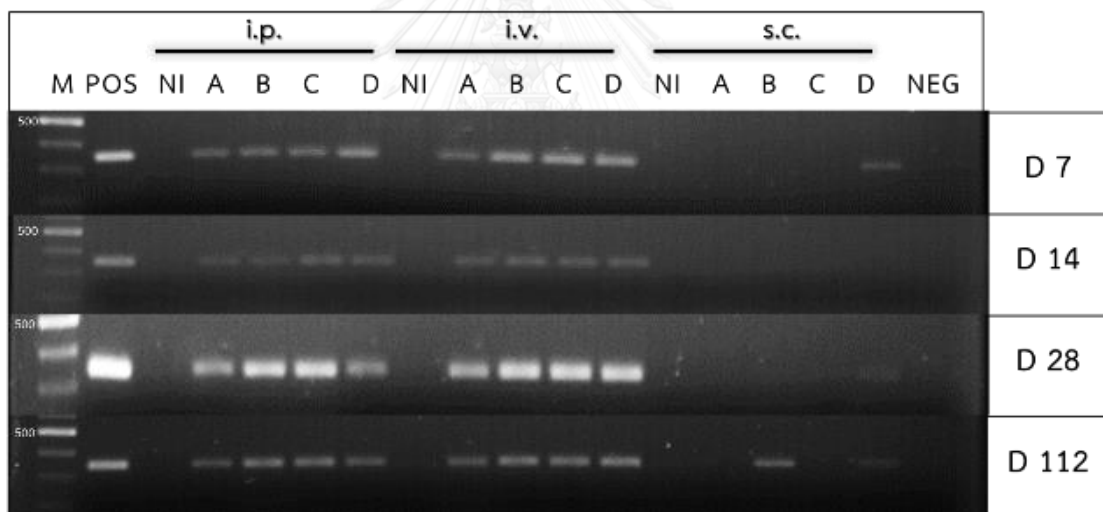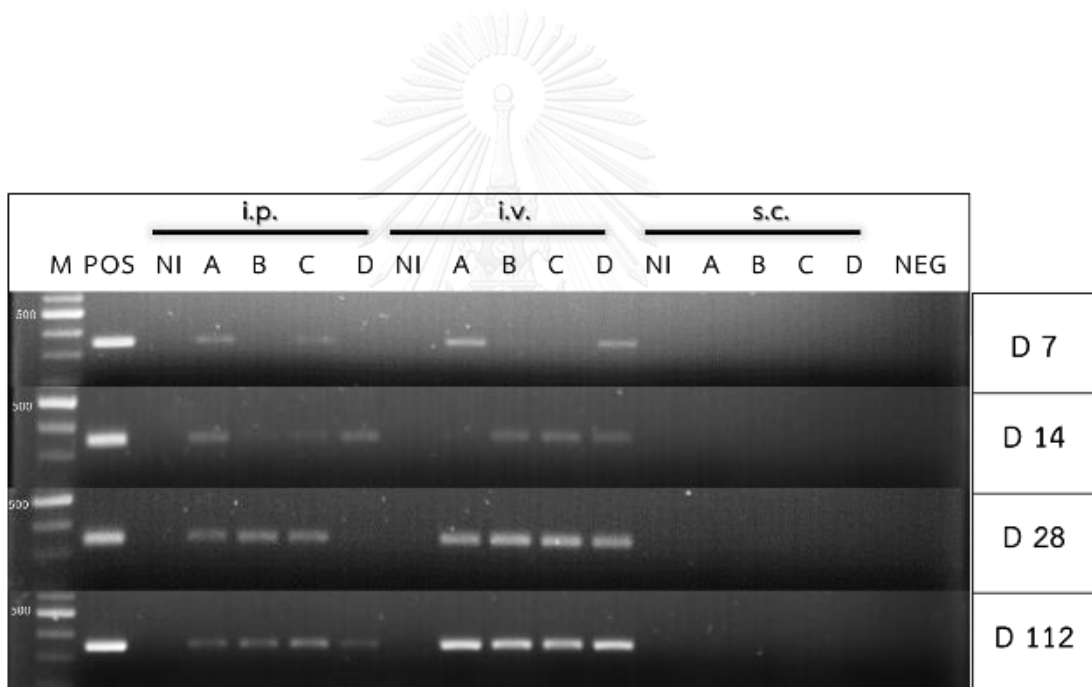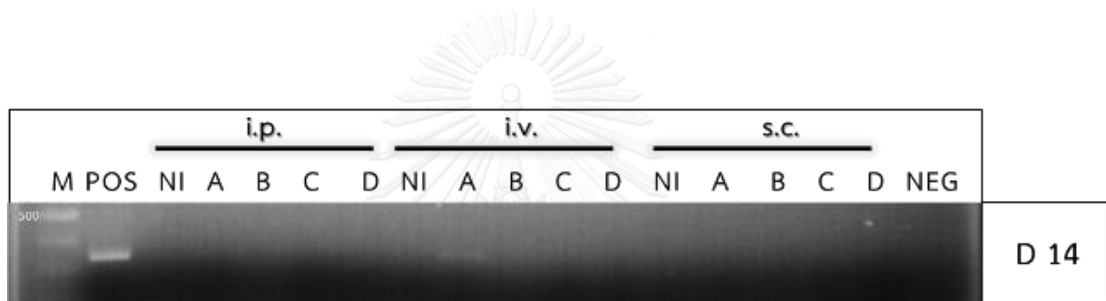| Salivary gland	i.p.				i.v.				s.c.			
	A	B	C	D	A	B	C	D	A	B	C	D
D 28								●				
D 112							●					

Figure 6. PCR diagnosis of *L. martiniquensis* from *L. martiniquensis*-infected BALB/c mouse kidney on 28 dpi using ITS1-specific primers. Lane M: 100 bp DNA marker. Lane POS: Positive control. Lane NI: Non-infected control mouse. Lane A B C D: DNA from each infected mouse in each route. Lane NEG: Negative control

Table 8. Summary of PCR result from *L. martiniquensis*-infected BALB/c mouse kidney using ITS1-specific primers on 28 dpi

Kidney	i.p.				i.v.				s.c.			
	A	B	C	D	A	B	C	D	A	B	C	D
D 28					●			●				

The parasite burden in liver and spleen

BALB/c mice were inoculated by *L. martiniquensis* promastigote via different routes including i.v., i.p. and s.c. then the parasite burden at 7, 14, 28, 112 dpi was observed. Number of amastigotes in liver and spleen impression smear was determined then followed by LDU analysis. Under the microscopic examination of Giemsa-stained liver impression smear, cell components included hepatocytes, mononuclear phagocytes, lymphocytes, neutrophils and erythrocytes. Majority of oval amastigotes without visible flagellum were found extracellular. Typical internal morphology was composed of basophilic round nucleus and rod-shaped kinetoplast oval (Fig. 7-8).
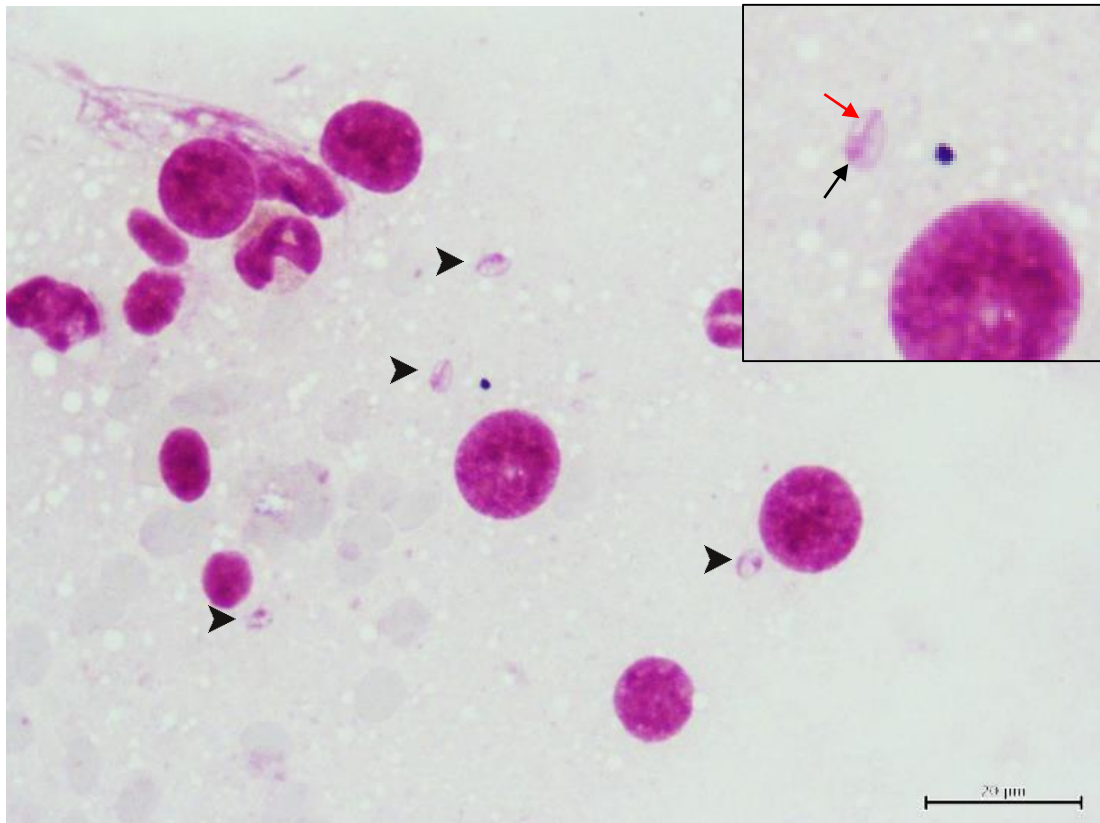
Figure 7. Giemsa-stained impression smear of *L. martiniquensis*-infected BALB/c mouse liver. Extracellular amastigotes were present (arrowheads) nearby hepatocytes (x 1,000 magnification). Inset showed typical internal structure of amastigote composed of round nucleus (black arrow) and rod-shaped kinetoplast (red arrow). Scale bar, 20μm.
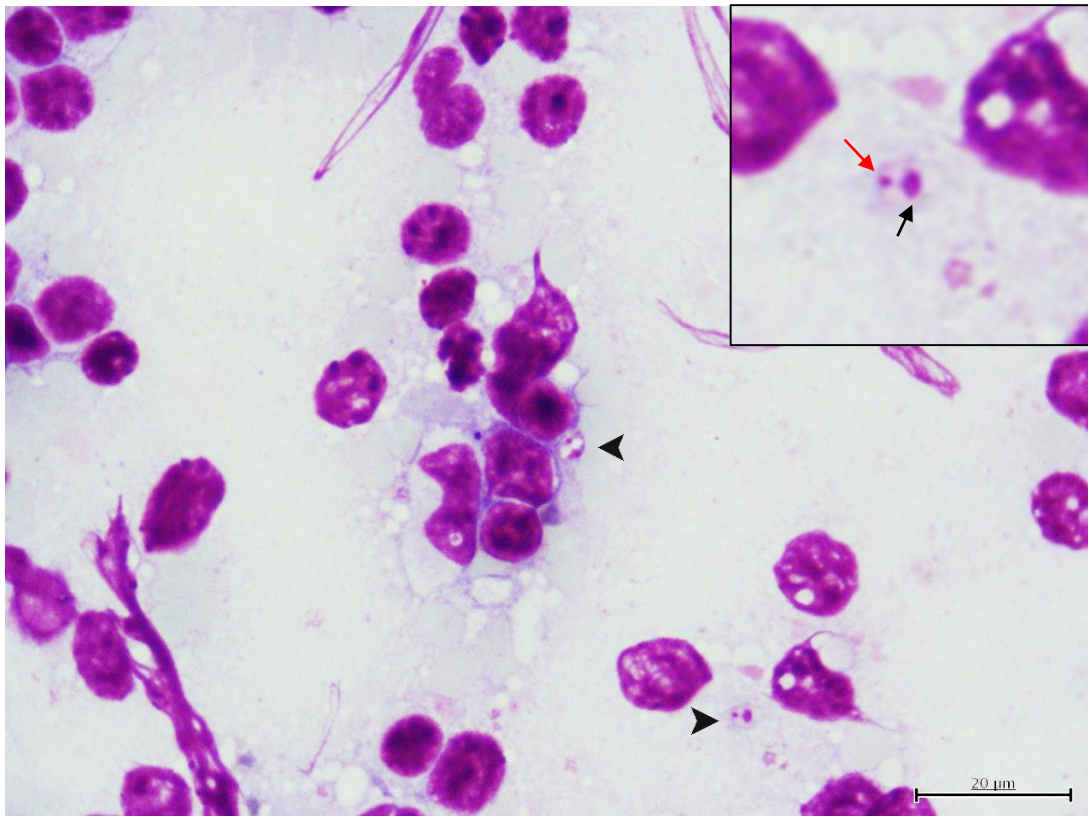
Figure 8. Giemsa-stained impression smear of *L. martiniquensis*-**infected BALB/c mouse spleen.** Extracellular amastigotes were present (arrowheads) next to leucocytes (x 1,000 magnification). Inset showed typical internal structure of amastigote composed of round nucleus (black arrow) and rod-shaped kinetoplast (red arrow). Scale bar, 20μm.

When the LDUs of infected BALB/c mouse liver were compared within the same time point, the LDU of i.v. route on 7, 14 and 28 dpi was significantly higher than non-infected control and other routes ($p < 0.05$) with the LDU value of 101, 71, 6, and 1 respectively. For 112 dpi, the number of LDU in liver of infected mice via all routes were negligible as low as 1 for i.v., 0.4 for i.p., and nondetectable for s.c. hence making insignificant difference between each group (Fig. 9).

Figure 9. The kinetics of parasite burdens were shown as LDU from the Giemsa-stained impression smear of liver of BALB/c mice during *L. martiniquensis* infection on 7, 14, 28 and 112 dpi by various routes of inoculation. Data represents mean of LDU ± SEM. Above the bars, different letters between each bar represented significant differences at p < 0.05 between different routes of parasite inoculation by comparing within the same time point.

The same data was plotted in different perspective in order to compare LDU throughout the time course but within the same route of inoculation. Interestingly, the only route that had significant reduction of parasite burden was infection via i.v. route. In details, LDU in liver of *L. martiniquensis*-infected mice on 7 dpi was 101 in which it was then significantly reduced on 14 dpi to the level of 71 ($p < 0.05$). On 28 dpi, the kinetic of LDU value was continuously decreased ($p < 0.05$) followed by steady decline from the value of 6 and 1 for 28 and 112 dpi, respectively, without significant difference. For i.p. route, parasite burdens on 7, 14, 28, and 112 dpi were 3, 3, 1, and 0.4, respectively, in which the changes between these values were not statistically significant. For s.c. route, amastigote was not detectable on 7, 28, 112 dpi whereas LDU was found 1.54 on 14 dpi (Fig. 10).

Parasite burden (LDU) - Liver

Figure 10. **The kinetics of parasite burdens were shown as LDU from the Giemsa-stained impression smear of BALB/c mouse liver during *L. martiniquensis* infection on 7, 14, 28 and 112 dpi by various routes of inoculation.** Data represents mean of LDU ± SEM. Different letters between each coordinate represented significant differences at p < 0.05 between different time points by comparing within the same route of inoculation.

As for the liver, the LDUs of infected BALB/c mouse spleen were compared within the same time point. For parasite burden in *L. martiniquensis*-infected BALB/c spleen, the LDU of iv route on 14, 28 and 112 dpi were significantly higher than non-infected control and also higher than other routes (*p* < 0.05) with the LDU value of 1.7, 2.5, 2.1 and 3.1 respectively, except for 112 dpi that no difference was detected between i.v. and i.p.. For i.p., LDU values were 0.1, 0.6, 0.7 and 2.4, respectively. For s.c., amastigote was not detected throughout the time point. (Fig. 11).
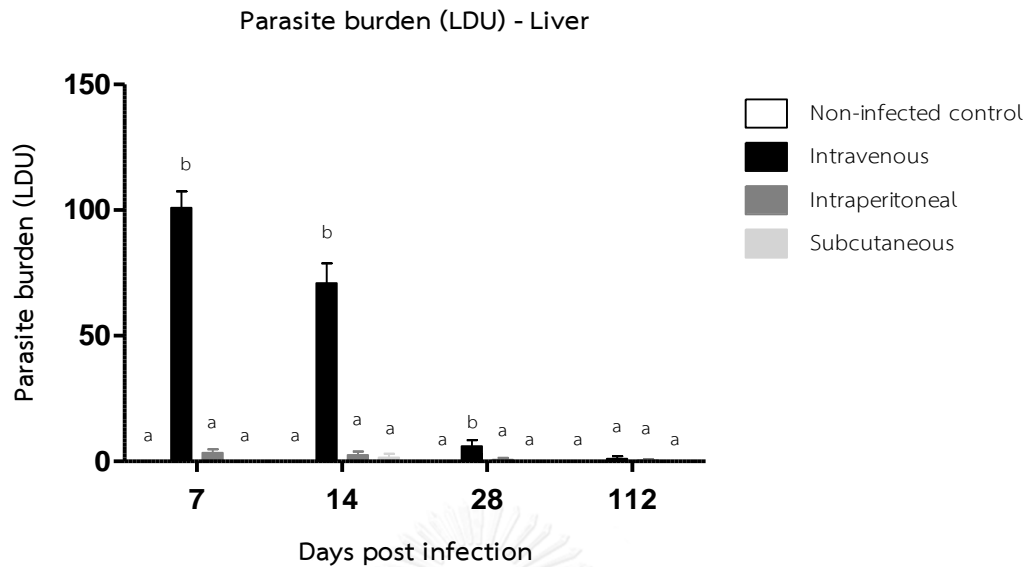
Figure 11. The kinetics of parasite burdens were shown as LDU from the Giemsa-stained impression smear of spleen of BALB/c mice during *L. martiniquensis* infection on 7, 14, 28 and 112 dpi by various routes of inoculation. Data represented mean of LDU ± SEM. Above the bars, different letters between each bar represented significant differences at $p < 0.05$ between different routes of parasite inoculation by comparing within the same time point.

Again, the same data was plotted in different chart in order to compare LDU throughout the time course but within the same route of inoculation. Although the trend of line chart for i.v. seemed to be in line with i.p., there was no significant difference between each time point. For i.p., the slight but insignificant increase of LDU for 7, 14 and 28 dpi was composed of 0.1, 0.6 and 0.7, respectively. Then the kinetic was sharply increased on 112 dpi at the level of 2.4. For s.c., the LDU was exactly overlapped with the non-infected control (Fig. 12).
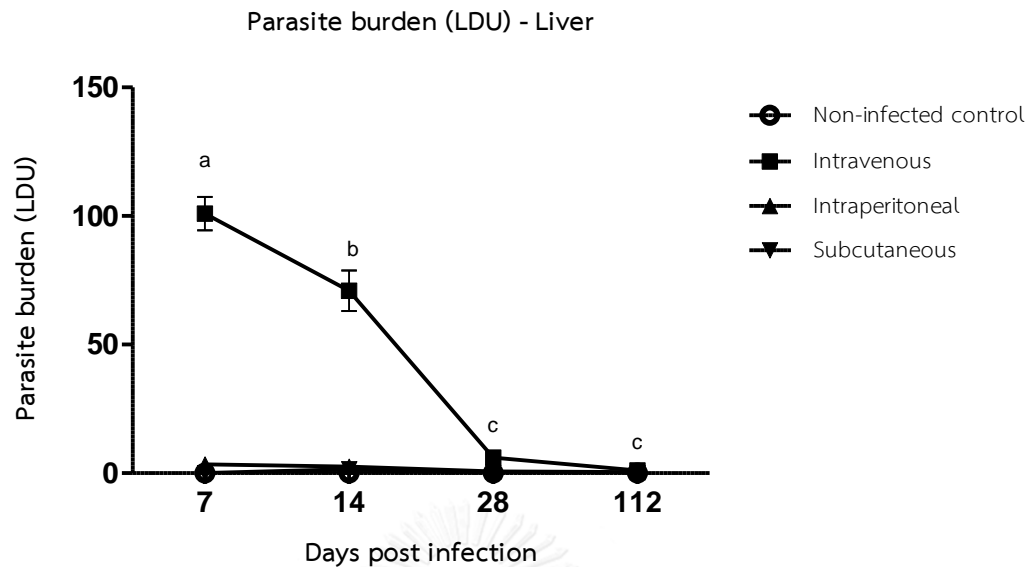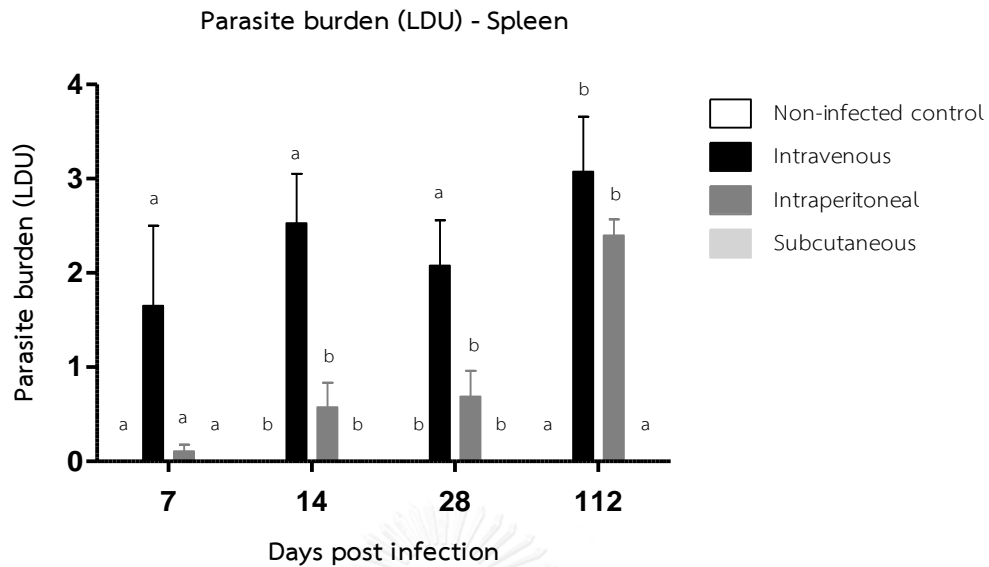
Parasite burden (LDU) - Spleen
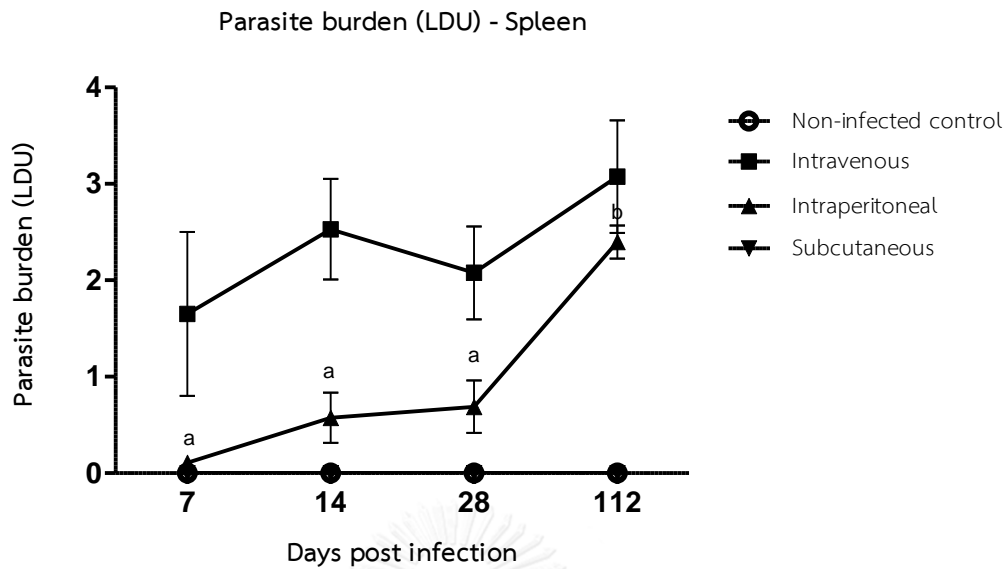
Figure 12. The kinetics of parasite burdens were shown as LDU from the Giemsa-stained impression smear of BALB/c mouse spleen during *L. martiniquensis* **infection on 7, 14, 28 and 112 dpi by various routes of inoculation.** Data represents mean of LDU ± SEM. Different letters between each coordinate represented significant differences at $p < 0.05$ between different time points by comparing within the same route of inoculation.

Hepatic immune responses

Before proceeding the immunohistochemistry technique, we need to ensure the absence of cross-reactivity of *L. martiniquensis*-infected human serum. Three sets of negative control were included in the experiment. Normal human serum was used as a primary antibody on intravenously *L. martiniquensis*-infected BALB/c mouse liver (Fig. 13A) and on non-infected control mouse liver (Fig. 13B). As a result, there was no brown staining in *Leishmania*-infected granuloma or any area of liver tissues, respectively. Another set of negative control, *L. martiniquensis*-infected human serum as a primary antibody was used on non-infected control mouse liver. Undoubtedly, brown staining was not also detected (Fig. 13C). To confirm the specificity of *L. martiniquensis*-infected human serum, the set of positive control *L. martiniquensis*-infected human serum was used as a primary antibody on intravenously *L.*

martiniquensis-infected BALB/c mouse liver conducted from irrelevant other experiment. Immunolabeled amastigotes were microscopically detected inside cytoplasm of macrophages as dark brown color liver tissue (Fig. 13D).
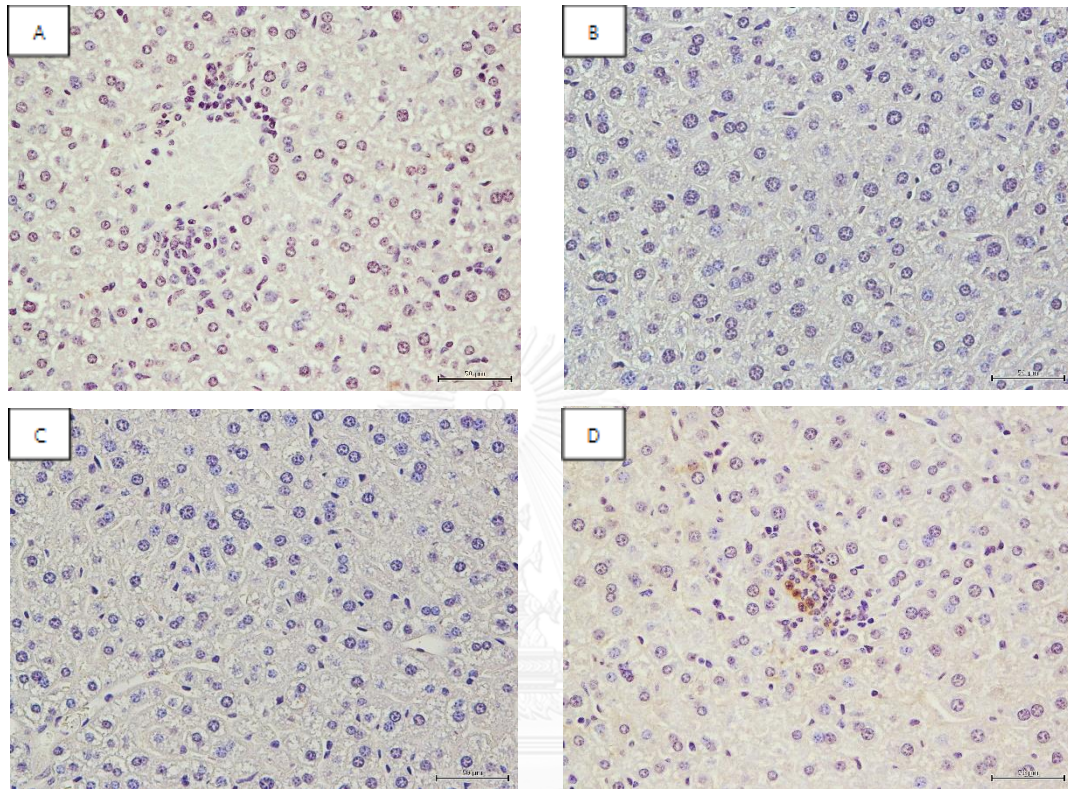
Figure 13. **The negative and positive control of indirect immunohistochemistry staining for *L. martiniquensis*-infected BALB/c mice experiment.** (A) The negative control of immunohistochemistry using normal human serum as a primary antibody on intravenously *L. martiniquensis*-infected BALB/c mouse liver on 14 dpi. (B) The negative control of immunohistochemistry using normal human serum as a primary antibody on non-infected control mouse liver. (C) The negative control of immunohistochemistry using *L. martiniquensis*-infected human serum as a primary antibody on non-infected control mouse liver. (D) The positive control of immunohistochemistry using *L. martiniquensis*-infected human serum as a primary antibody on intravenously *L. martiniquensis*-infected BALB/c mouse liver on 114 dpi conducted from irrelevant other experiment.

For the IHC experiment, the hepatic immune responses were graded into four types composed of no granuloma (NG), immature granuloma (IG), mature granuloma (MG), and involuting granuloma (IVG). NG was characterized by the presence of immunolabeled parasites in parasitized Kupffer cells without mononuclear cell (MNC) infiltrate (Fig. 14A). IG was graded by the presence of infected Kupffer cells, revealed in brown color of immunolabeled parasites, and enclosed by less than 10 MNCs with or without other inflammatory cell infiltrates. The internal structure of these granuloma were mostly arranged with poor-defined border (Fig. 14B). For MG, they were organized as round structure containing a central core of a large macrophage or epithelioid cell and surrounded by a couple of layers of greater than 10 MNCs associated with other inflammatory cell infiltrates (Fig. 14C). Lastly, IVG was revealed based on the absence of stained amastigote inside granuloma (Fig. 14D).
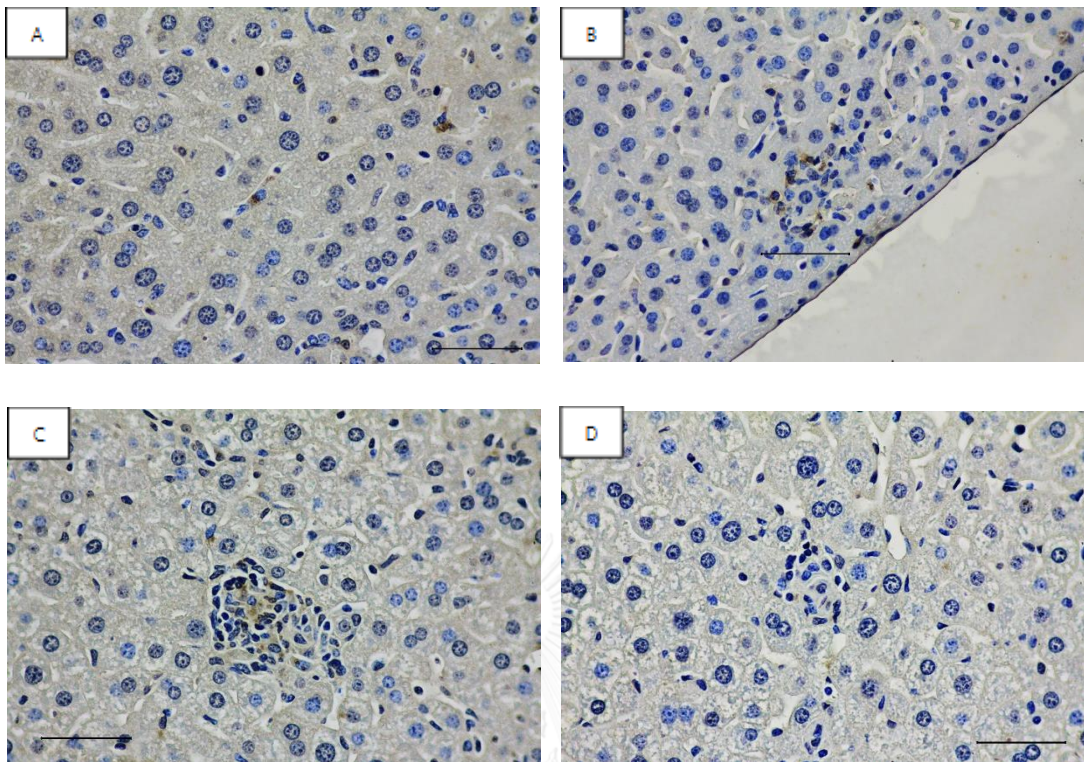
Figure 14. . **Four different stages of hepatic immune responses in the liver of** *L. martiniquensis*-**infected BALB/c mice.** (A) No granuloma. (B) Immature granuloma. (C) Mature granuloma. (D) Involuting granuloma. Dark brown color represents intracellular *L. martiniquensis* amastigotes by indirect immunohistochemical staining using *L. martiniquensis*-infected human serum. Scale bar, 50 μm.

Within the same time point, the number of NG on 7 dpi via iv route was found significantly higher than the non-infected control and other routes ($p < 0.05$) whereas the number of NG within the same time point on 14, 28 and 112 dpi was not statistically significant (Fig. 15A). The number of IG from both i.v. and i.p. routes were significantly greater than non-infected control and s.c. route ($p < 0.05$). However, infection via i.v. route seemed to induce higher number of IG than did i.p. route but the difference were not statistically significant. For 14 dpi, the number of IG in i.v. route was significantly higher than the non-infected control and other routes ($p < 0.05$) but i.p. and s.c. routes were not statistically significant from non-infected control. For 28 and 112 dpi, the number of IG were not statistically significant compared to non-infected control mice (Fig. 15B). Considering the MG, the number of granuloma on 7, 14 and 28 dpi from i.v. route was found significantly superior than the non-infected control and other routes ($p < 0.05$) (Fig. 15C). For IVG, the number of granuloma induced by i.v. inoculation on 14, 28 and 112 dpi were found significantly higher than the non-infected control and other routes ($p < 0.05$). Also, the number of IVG by i.p. inoculation was significantly higher than non-infected control and s.c. group (Fig. 15D).

Figure 15. **The kinetics of hepatic immune responses in BALB/c mice during** *L. martiniquensis* **infection on 7, 14, 28 and 112 dpi by various routes of inoculation.** Data represents mean ± SEM. The letters "a", "b" and "c" represents significant differences at $p < 0.05$ between different routes of parasite inoculation by comparing within the same time point.

Compared within the same route, the number of NG via i.v. route on 7 dpi was 42.5 per 25 fields at 400x magnification. Then it was precipitously decreased on 14 dpi ($p < 0.05$) in which the number was plateau for the rest of the observation period. For i.p. route, pattern of the curve was similar to the i.v. but the number of NG was found 21.2. However, the difference between 7 and 14 dpi was not statistically significant whereas the difference between 7 or 14 dpi and 28 or 112 dpi was in contrast ($p < 0.05$) (Fig. 16A). The number of IG via i.v. route at 7 and 14 dpi were 31.5 and 46, respectively. However, this increase was not statistically different then the number was suddenly dropped to 4 on 28 dpi ($p < 0.05$) and sustained to the level of 3 on 112 dpi (Fig. 16B). For the infection via i.p. route, the kinetic of IG number was not statistically different throughout the time course. The number of MG via i.v. route on 7, 14 and 28 dpi were 43, 57 and 59.3, respectively, in which the difference of increment was not statistically significant. Then the value was abruptly decreased on 112 dpi. For i.p. route, the number of MG on 7, 14, 28 and 112 dpi were 9.2, 6.2, 3.8 and 2.4, respectively, in which there was no statistically different for this declined value. For the liver of *L. martiniquensis*-infected BALB/c mice via s.c. route, no MG was detected. The number of IVG via i.v. route on 7, 14, 28 and 112 dpi were 0, 14.5, 43 and 24.5, respectively, in which the number was significantly peaked at 28 dpi ($p < 0.05$). For i.p. routes, the number of IVG on 7, 14 and 28 dpi were 0.2, 0.2, and 0, respectively. However, these stable value during these periods were not statistically different. On 112 dpi, the number of IVG were significantly increased to 9.4 and 3.2 in infected livers via i.p. and s.c. routes, respectively ($p < 0.05$). (Fig. 16D). Microscopic changes were not observed in non-infected control BALB/c mice.
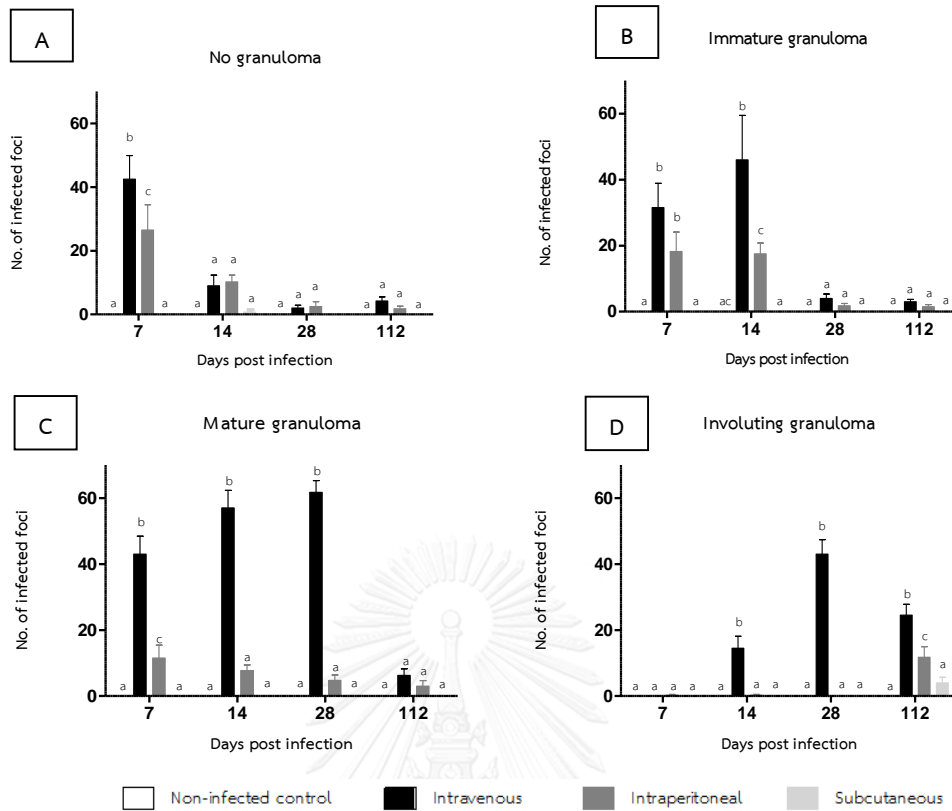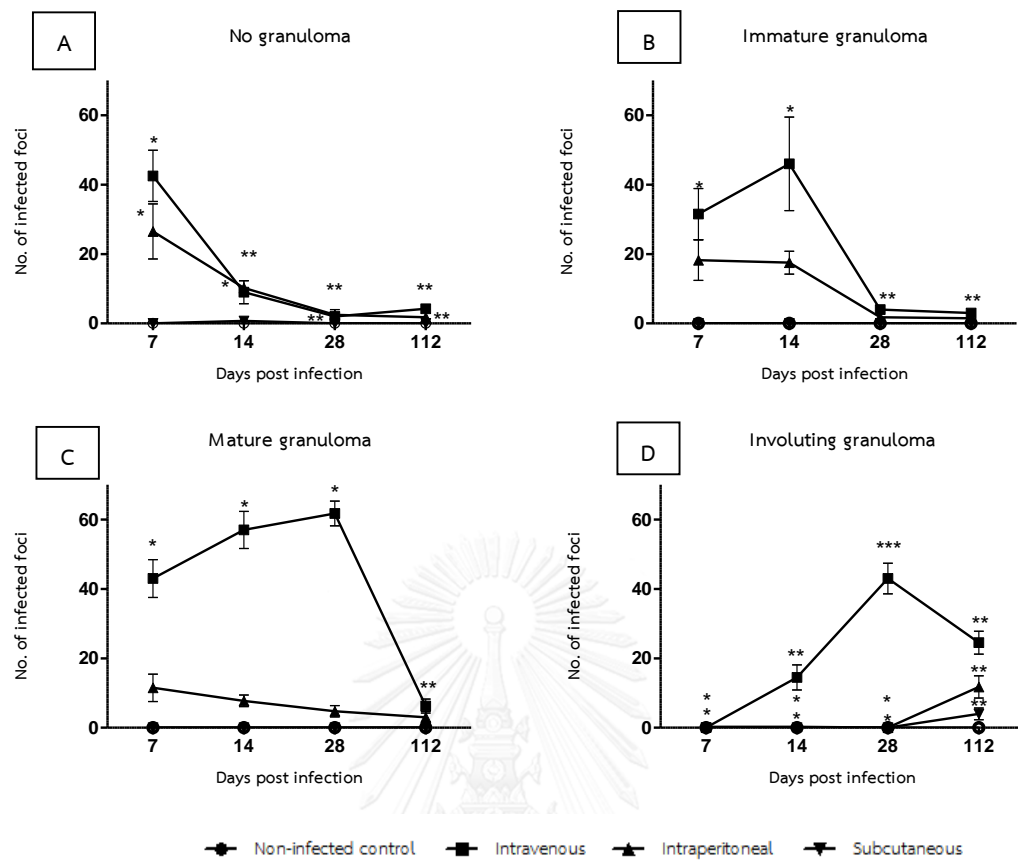
Figure 16. The kinetics of hepatic immune responses in BALB/c mice during *L. martiniquensis* infection on 7, 14, 28 and 112 dpi by various routes of inoculation. Data represents mean ± SEM. The asterisk (*, **, ***) represents significant differences at $p < 0.05$ between different time points by comparing within the same route of parasite inoculation.

CHAPTER 6

DISCUSSION

L. martiniquensis induces emerging visceral and cutaneous leishmaniasis in human both in foreign countries (Dedet et al., 1995; Desbois et al., 2014; Liautaud et al., 2015) and autochthonous cases in Thailand in the past 10 years (Pothirat et al., 2014; Chiewchanvit et al., 2015; Leelayoova et al., 2017). Although, so far, the evidence of potential animal reservoir for this disease in Thailand only demonstrated black rat (*Rattus rattus*) for *Leishmania siamensis* (Chusri et al., 2014), experimental study using murine to elucidate as a host for *L. martiniquensis* has not yet been conducted. Based on our data, this study is the first to conclude that *L. martiniquensis* infection caused visceral leishmaniasis in BALB/c mice by experimental inoculation via intravenous and intraperitoneal routes. The evidences were composed of the presence of amastigotes in hepatic and splenic impression smears as well as genomic DNAs of *L. martiniquensis* in both organs.

From previous studies, BALB/c mice commonly used as an animal model to study pathogenesis and immune responses of leishmaniasis caused by *L. infantum* (synonym as *L. chagasi*) (Prianti et al., 2007; Malafaia et al., 2011; Oliveira et al., 2012), *L. panamensis* (Rojas et al., 1993), *L. Mexicana* (Guevara-Mendoza et al., 1997), *L. major* (Ghaffarifar et al., 2015; Sassi et al., 2015). *L. infantum* is a causative agent for visceral leishmaniasis in human commonly affecting children and dogs in which also serve as a reservoir host (Gramiccia and Gradoni, 2005; Chappuis et al., 2007). Based on higher splenic parasite burden in BALB/c mice infected with *L. martiniquensis* via i.v. route compared to i.p. route from our study, this results were correlated with a study using the same mouse strain but different *Leishmania* species, *L. infantum*. They also inoculated mice with almost equivalent number of parasites, 10^7 promastigotes. However, they suggested i.p. route over i.v. route as a murine model for leishmaniasis due to higher homogeneity of infection for cellular immune study (Rolao et al., 2004). For higher liver parasite burden in i.v. route compared to other routes, our results were correlated with the study using *L. donovani* but they used intracardiac instead of

intravenous route (Kaur et al., 2008). Experimental infection of *L. martiniquensis* via i.v. route should be suitable model for evaluation of therapeutic efficacy or vaccine studies as this route caused evident pathology with high parasite burden (Ahmed et al., 2003; Rosypal et al., 2005; Carrion et al., 2006; Peine et al., 2014).

By subcutaneous route of *L. martiniquensis* inoculation, we rarely found parasite burden in both liver and spleen as well as negligible hepatic granuloma development. This data were in line with previous studies using *L. donovani* experimental infection in BALB/c mice. They compared parasite load in liver using various routes of inoculation composed of intracardiac, intraperitoneal, intradermal and subcutaneous. Subcutaneous infection regardless of inoculum dose yielded the lowest parasite load compared to other routes on 15, 30, 45 dpi. Also, Th1 cytokines, IgG2a in serum and IFN-g from splenic culture, were detected at the highest level on 30 dpi (Kaur et al., 2008). So, with both species of *Leishmania*, this route of inoculation was most likely to be able to induce strong Th1 immune responses in BALB/c mice. In order to address if this route of inoculation could lead to immune-protection for this mouse strain, challenging these mice with the correspondent species of *Leishmania* would be able to test this hypothesis. Further experiment from our samples would be beneficial to determine the type of immune responses and the level of cytokines from both target organ and systemic circulation.

To detect parasite distribution, we performed conventional method by microscopic examination of amastigotes from Giemsa-stained impression smear of liver and spleen. For molecular detection, we intended to load equivalent amount of 500 ng genomic DNAs in each lane. So the intensity of amplicons was most likely to be interpreted semi-quantitatively. Surprisingly, by i.v. inoculation, microscopic method was able to demonstrate the presence of parasite in liver and spleen with identical frequency compared to PCR on 7, 14, 28 dpi and 14, 28, 112 dpi, respectively. The discrepant results only occurred on 112 dpi in liver. Amastigotes of *L. martiniquensis* was only detected in 1 out of 4 mice whereas PCR could detect DNAs of parasite in all mice (4/4). Interestingly, the intensity of all amplicons derived from all infected-mice' liver on 112 dpi was very faint associated with undetectable amastigote from microscopic method (3/4) thus demonstrating higher sensitivity of PCR technique in

case of low parasite burden. The advantage of using PCR is able to overcome limitation of microscopic examination due to negative detection if parasite number is below the detection limit. Further study using real time PCR should provide kinetics of parasite burden in which we have a collaborative project in process.

For qualitative evaluation of liver and spleen via i.p. inoculation, PCR was able to detect DNAs of parasite more uniformly in all mice on 7, 14, 28 dpi whereas the results from impression smear were slightly discrepant. On 112 dpi in liver, amastigote was not found in all mice by imprint smear whereas 2 out of 4 mice had parasite DNAs. In contrast, microscopic and molecular detection results from spleen on 112 dpi were exactly in line. These results revealed the advantage of both conventional and advanced approach in the aspect of high specificity and high sensitivity, respectively, to support each other. It seemed that our criterion to observe the parasite burden, by examining 100 nucleated cells/stamp for 10 stamps using oil immersion at 1000x magnification, yielded slightly less sensitive result compared to PCR. From previous published works, comparative study between PCR and microscopic examination also correlated with our results in regards of higher sensitivity of PCR (Boelaert et al.; Ozerdem et al., 2009; Abd El-Salam et al., 2014).

In terms of kinetics of parasite load derived from LDU quantitation throughout our observation period, the highest parasite burden in liver of *L. martiniquensis*-infected BALB/c via i.v. was found on 7 dpi then gradually decreased to negligible level at the end of experiment. This may be due to Kupffer cells have reduced innate ability to contribute to leishmanicidal activity hence leading to increased parasite burden during the first week as seen in *L. infantum* (Leclercq et al., 1996). In contrast, the study of experimentally *L. donovani*-infected BALB/c mice found that parasite rapidly increased during the first two to four weeks of infection instead (Murray et al., 1987). After 3 - 4 weeks post-infection, acquired resistance were developed so the liver parasite burden were decreased (Engwerda et al., 1998; Murray, 2001).

In contrast to the liver, splenic *L. martiniquensis* burden on 7 dpi were found lesser than did the liver up to 50 times. The low parasite burden in spleen during acute phase (< 4 weeks) of *Leishmania* infection may be similar to the fact that marginal zone macrophages were phagocytosed more than 95 percent of *L. infantum* in BALB/c

infected by i.v. route. In addition, more than 50 percent of the parasite number of inoculation were killed within 24 hours (Engwerda and Kaye, 2000). From our results, the kinetic of parasite burden in spleen infected via i.p. route were sharply increased after 28 dpi. In experimental and clinical leishmaniasis, the immune system in spleen was unsuccessful to clear parasite so the lifelong chronic infection usually persisted in the body (Kaye et al., 2004). If we happen to extend the observation period, we might be able to determine how long parasite can really persist in spleen of BALB/c mice despite the presence of acquired immunity.

As a result of host-parasite interaction, liver plays role to control and limit *Leishmania* parasite replication by formation of granulomas (Engwerda et al., 2004). Our evidence also supported this findings since the development of mature granuloma was peaked on 28 dpi in the fashion of reverse relationship with kinetics of parasite burden. Chronic phase of infection was found to begin by 28 dpi for *L. infantum* in mice (Nieto et al., 2011). Since the formation of a granuloma is not always associated with parasite control and effectiveness of granulomas to kill *Leishmania* depends on their type of granuloma maturation (Murray, 2001), mature granuloma on 28 dpi of BALB/c mice may be able to enhance parasite killing and reached effective antileishmanial activity (reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs)) and control infection upon final development of involuting granuloma on 112 dpi (Murray, 2001; Stanley and Engwerda, 2007; Nieto et al., 2011). The development of hepatic granuloma is the strategic process to control the *L. martiniquensis* infection (Murray, 2001; Stanley and Engwerda, 2007) which is the main histopathology changed in host whom infected with all species of *Leishmania* (Gutierrez et al., 1984; Kaye and Beattie, 2016).

Regarding the presence of parasite by PCR detection, our study was designed the first collection time point on 7 dpi in which parasites were firstly detected in liver and spleen via i.v. and i.p. infection. DNAs were still present throughout the observation period up to 112 dpi except for the absence of amplicons in half of the 112 dpi group (2/4 mice). However, from previous study that used 10^7 promastigotes *L. infantum*-infected BALB/c mice by i.p. route, *Leishmania* DNAs were found from liver and spleen as early as 1 dpi by real-time PCR. This parasitism were continuously detected

throughout the observation period as well, 1, 3, 7, 14, and every 2 weeks until 84 dpi (Rolao et al., 2007) in which this was in line with our study. Further study should be performed by designing the collection period within the first week post-infection in order to see how early *L. martiniquensis* amastigotes would be recruited to the target organs and to better elucidate the kinetics of parasite burden including development of hepatic granuloma during acute phase of infection.

In recent years, the application of saliva specimen is increasingly popular and widely used as a tool for diagnosis of infectious diseases. The saliva can be used as a substitute for blood or urine for detection of antigen, antibody, and nucleic acid (Lima et al., 2010). For instance, infectious systemic diseases including rabies, human immunodefiency virus (HIV), hepatitis B virus and human papilloma virus (Xavier Santos et al., 2015; Corstjens et al., 2016). Besides these diseases, the saliva also used for diagnosis of bacterial infections such as *Helicobactor pylori* (Jankowski et al., 2016; Medhat et al., 2016; Young and Luo, 2016) and tuberculosis (Jacobs et al., 2016).

For parasitic infections, apart from *Plasmodium falciparum* (Nwakanma et al., 2009) and *Entamoeba histolytica* (Khairnar and Parija, 2008; Haque et al., 2010), *Leishmania* DNAs were also detected from saliva of subclinical and clinical leishmaniasis patients (Galai et al., 2011; Phumee et al., 2013; Siriyasatien et al., 2016). However, unlike convenient collection of saliva from pigs by letting them chew a bunch of rope, this method has limitation to use with rodents due to gnawing behavior. Therefore, instead of collecting secreted saliva, we directly dissected salivary glands to address if saliva gland plays role in parasite harboring. Surprisingly, only a quarter of BALB/c mice (1/4) from 28 dpi and 112 dpi via i.v. route were found positive for *L. martiniquensis* DNAs with weak amplicon intensity hence this data is the first evidence showing the presence of *L. martiniquensis* DNAs from salivary glands of infected BALB/c mice. Related to the previous study in gold hamster, they found amastigotes in salivary gland tissues infected with *L. infantum* by H&E staining histopathology (Martin-Martin et al., 2015). Since amastigote mostly invades mononuclear cells, it may be possible to speculate that we happened to collect intracellular parasites from associated lymph nodes of these 2 mice despite strong attempt to dissect them carefully. In contrast, the negative results from the majority of infected mice might be due to low amount

of salivary gland DNA yields in which this also occurred with non-infected salivary glands. In order to clarify this point, further study is needed by collection of additional major salivary glands composed of parotid and sublingual glands apart from submandibular glands to increase DNA yield.

Alternatively, the induction of salivary secretion could be enhanced by injection of secretory stimulant (pilocarpine) to collect the oral cavity fluid (Blanchard et al., 2015) since it has the possibility to contain leukocytes due to bleeding from oronasal tract (Kaufman and Lamster, 2002). Indeed, the components of oral cavity fluid are saliva secreted from major and minor salivary gland including fluid from other secretory tissues such as labial gland and crevicular fluid. The leukocytes were found predominantly from gingival crevicular fluid (Golub and Kleinberg, 1976; Lamster and Ahlo, 2007) in which this may be source of pathogen's DNAs contributing to non-invasive diagnosis technique.

Indeed, mice have major and minor salivary glands comprising of parotid gland, sublingual gland and submandibular gland. For minor gland, mice have anterior buccal gland, anterior lingual gland and palatine gland (Amano et al., 2012). For our experiment, we were collected the submandibular salivary gland instead of the whole saliva. Among these major salivary glands, the saliva most coming from the submandibular glands (de Almeida Pdel et al., 2008). In mice, the submandibular and sublingual glands are along together within the same encapsulate and covered up by fat tissues and submandibular lymph nodes (Amano et al., 2012).

Apart from the target organs, kidney is another organ that can be involved by VL or CL. Abnormality signs of kidney from leishmaniasis patients can found proteinuria, hematuria, abnormalities in urinary concentration and acidification and acute and chronic renal insufficiency. Immune complex deposition has been found to cause a glomerulonephritis in VL. Patients can be an immunocompetent or immunocompromised person such as HIV infection or organ transplant (Clementi et al., 2011; Vassallo et al., 2014).

The positive band from kidney sample on 28 dpi can be assumed that *L. martiniquensis* can impair the kidney function. Pathogen can reach to glomerular of kidney at that time and were caught by the antibody. Then, a deposition of immune

complexes are formed and inducing the glomerulonephritis (Silva Junior et al., 2014). Related to the previous study, they were found glomerulonephritis with IgG deposit in *L. chagasi*-infected mice (Prianti et al., 2007). Renal PCR positively detected DNAs in both of symptomatic and asymptomatic seropositive in *Leishmania*-infected dogs (Soares et al., 2005). Also, future study should be performed in urine specimen. Many previous study reported the successful of detection DNA of *Leishmania* and antigen in patient urine even though the sensitivity of urine PCR was poorer than the other methods (Veland et al., 2011; Phumee et al., 2013; Ben-Abid et al., 2016).

CHAPTER 7
CONCLUSION

L. martiniquensis can cause the pathogenicity in the liver and spleen of infected BALB/c mice. Experimental infection of *L. martiniquensis* via intravenous route should be suitable model for evaluation of therapeutic efficacy or vaccination study as this route cause evident pathology with high parasite burden. In contrast to i.v. route, parasite burden of *L. martiniquensis* mouse inoculation via subcutaneous route hardly detected in both liver and spleen. Also, negligible hepatic granuloma was developed via s.c. inoculation. The distribution of *L. martiniquensis* DNAs can be found in many organs including liver, spleen, bone marrow, buffy coat, salivary gland and kidney.

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REFERENCES

APPENDIX

VITA

Miss Nawaphat Somboonpoonpol was born in Bangkok, Thailand. She has been graduated with Bachelor degree of Veterinary science with Second Class Honors in academic year 2013.   For Master of Science Program in Veterinary Pathobiology, Faculty of Veterinary Science, Chulalongkorn University, she received Chulalongkorn University Graduate Scholarship to commemorate the 72 nd anniversary of his Majesty King Bhumibol Adulyadej. She, also, received The 90 th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund) for her M.Sc. Her field of interest is Leishmania, laboratory animal and animal experimentation.