การเหนี่ยวนำให้เกิดออโต้ฟาจีโดยเลปโตสไปราสายพันธุ์ก่อโรคในแมคโครฟาจของมนุษย์และหนูไมซ์

นางสาวฉัตรทิพย์ ศรีปทุมทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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AUTOPHAGY INDUCTION BY PATHOGENIC *LEPTOSPIRA* IN HUMAN AND MURINE MACROPHAGES

Miss Chattip Sripatumtong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

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Field of Study	Medical Microbiology
Thesis Advisor	Assistant Professor Kanitha Patarakul, M.D., Ph.D.
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นางสาว ฉัตรทิพย์ ศรีปทุมทอง : การเหนี่ยวนำให้เกิดออโต้ฟาจีโดยเลปโตสไปราสายพันธุ์ก่อโรคในแมคโครฟาจของมนุษย์และหนูไมซ์. (AUTOPHAGY INDUCTION BY PATHOGENIC *LEPTOSPIRA* IN HUMAN AND MURINE MACROPHAGES) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.พญ. กนิษฐา ภัทรกุล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.ธนาภัทร ปาลกะ, 79 หน้า.

โรคเลปโตสไปโรซิสเป็นโรคที่ระบาดจากสัตว์สู่คนสามารถพบได้ทั่วโลก สาเหตุเกิดจากการติดเชื้อเลปโตสไปราสายพันธุ์ก่อโรค ในมนุษย์เชื้อสามารถทำให้เกิดพยาธิสภาพในหลายระบบและอาจเกิดอาการที่รุนแรงจนทำให้เสียชีวิตได้ แต่ในสัตว์รังโรคมักไม่ทำให้เกิดพยาธิสภาพและไม่แสดงอาการ ปัจจุบันยังไม่ทราบกลไกของความแตกต่างนี้ การศึกษานี้มีจุดมุ่งหมายเพื่อศึกษาและเปรียบเทียบการเกิดออโต้ฟาจีโดยเชื้อเลปโตสไปราในแมคโครฟาจของหนูไมซ์และของมนุษย์ โดยใช้เชื้อเลปโตสไปราซีโรวาร์ Pomona และเซลล์ไลน์แมคโครฟาจ RAW264.7 ของหนูไมซ์และเซลล์ไลน์โมโนไซต์ THP-1 ของมนุษย์ ผลการตรวจหาการเกิดออโต้ฟาจีโดยดูการเปลี่ยนแปลงของ LC-3II ซึ่งใช้เป็นเครื่องหมายติดตามการเกิดออโต้ฟาจีโดยวิธี Western blot พบว่าทั้งแมคโครฟาจของมนุษย์และหนูไมซ์ที่ติดเชื้อเลปโตสไปราสามารถกระตุ้นให้เกิดออโต้ฟาจีได้ แต่รูปแบบของ LC3-II นั้นมีความแตกต่างกันขึ้นกับปริมาณและระยะเวลาในการติดเชื้อ ในการศึกษาโดยใช้กล้องจุลทรรศน์อิเลกตรอนพบว่าในแมคโครฟาจของหนูไมซ์เชื้อเลปโตสไปราอยู่ภายในถุงหุ้มสองชั้นซึ่งเป็นลักษณะจำเพาะของออโต้ฟาโกโซม ส่วนในแมคโครฟาจของมนุษย์พบเชื้อเลปโตสไปราในถุงหุ้มชั้นเดียวและอยู่ในไซโตพลาสซึม เมื่อศึกษาด้วยกล้องคอนโฟคอลในชั่วโมงที่ 8 ภายหลังการติดเชื้อพบว่าเชื้อเลปโตสไปราไม่อยู่ในไลโซโซมของแมคโครฟาจของมนุษย์ซึ่งแตกต่างจากของหนูไมซ์ การใช้สาร 3-methyladenine เพื่อยับยั้งกระบวนเกิดออโต้ฟาจีแสดงให้เห็นว่ากระบวนเกิดออโต้ฟาจีอาจเกี่ยวข้องกับการกำจัดเชื้อเลปโตสไปราในแมคโครฟาจของหนูไมซ์ แต่ไม่ได้เป็นกลไกหลักในการกำจัดเชื้อในแมคโครฟาจของมนุษย์ นอกจากนี้ยังพบว่าการเกิดออโต้ฟาจีในแมคโครฟาจของหนูไมซ์หลังติดเชื้อเลปโตสไปราอาจผ่านทาง TLR4 ดังนั้น กระบวนการเกิดออโต้ฟาจีที่แตกต่างกันในแมคโครฟาจของหนูไมซ์และของมนุษย์อาจเป็นกลไกหนึ่งที่ทำให้เชื้อเลปโตสไปราก่อโรคได้แตกต่างกันในสัตว์รังโรคและมนุษย์

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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CHATTIP SRIPATUMTONG : AUTOPHAGY INDUCTION BY PATHOGENIC *LEPTOSPIRA* IN HUMAN AND MURINE MACROPHAGES.

ADVISOR : ASST. PROF. KANITHA PATARAKUL, M.D., Ph.D.,

CO-ADVISOR : ASSOC. PROF. TANAPAT PALAGA, Ph.D., 79 pp.

Leptospirosis is a zoonotic disease with a worldwide distribution, caused by pathogenic *Leptospira* species. Pathogenic leptospires may evoke severe systemic infection in humans but only cause mild chronic disease or asymptomatic carrier state in several animals. The reasons for this difference in infection and related natural defense mechanisms remain unclear. In this study, we aimed to study autophagy induction by pathogenic *Leptospira* in murine and human macrophages. *L. interrogans* serovar Pomona was used to infect RAW264.7 murine macrophage cell line and THP-1 human monocytic cell line. Autophagy formation in murine and human macrophages was detected as the appearance of autophagic markers, LC3-II, in Western blot. Furthermore, the leptospires in the murine macrophages were observed under transmission electron microscopy to be located within double membrane vesicles, which is the characteristic of autophagosomes. Leptospires in human macrophages, however, were found in the single membrane compartment and/or in the cytosol. In addition, in contrast to murine macrophages, co-localization of leptospires with lysosome was not observed by confocal microscopy in human macrophages at 8 hour post infection. Treatment with an autophagy inhibitor, 3-methyladenine, the autophagy process seemed to participate in the killing of pathogenic *Leptospira* in murine macrophages but was not the major mechanism in the clearance of pathogenic *Leptospira* in human macrophages. Furthermore, TLR4 may contribute to autophagy after pathogenic *Leptospira* infection in murine macrophages. Therefore, the distinct autophagic process between human and murine cells may explain the nature of infection in different hosts of *Leptospira*.

Field of Study : Medical Microbiology Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

APS	Ammonium peroxodisulfate
Bp	Base pair
BSA	Bovine serum albumin
ºC	Degree Celsius
$C_3H_3NaO_3$	Sodium pyruvate
$C_8H_7N_3O_2$	3-Aminophthalhydrazide, 5-amino-2,3-dihydro-1,4-phthalazinedione
$C_{24}H_{39}O_4Na$	Sodium deoxycholate
$CaCl_2$	Calcium chloride
CH_3COONa	Sodium acetate
$CuSO_4$	Copper sulfate
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EMJH	Johnson and Harris modification of the Ellinghausen and McCullough medium
et al.	et alii
FBS	Fetal bovine serum
FeSO4	Ferrous sulfate
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid

HCl	Hydrochloric acid
$C_{18}H_{26}ClN_3O^{\cdot}H_2SO_4$	Hydroxychloroquine sulfate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$HOC_6H_4CH=CHCO_2H$	*trans*-4-Hydroxycinnamic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IFA	Immunofluorecent assay
IPTG	Isopropyl-D-thiogalactopyranoside
KCl	Potassium chloride
kDa	Kilodalton
$KH_2\ PO_4$	Potassium dihydrogen phosphate
LB	Luria-Bertani
LC-3	Microtubule-associated protein light chain 3
LipL32	*Leptospira* lipoprotein 32 kDa
LPS	Lipopolysaccharide
MAT	Microscopic agglutination test
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Milimolar
MOI	Multiplicity of infection
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
Na_2CO_3	Disodium carbonate
$NaHCO_3$	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulfate

ng	Nanogram
NH_2CH_2COOH	Glycine
NP-40	Tergitol-type NP-40
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p.i.	post infection
PMA	phorbol-12-myristate-13-acetate
Raw264.7	Murine macrophage-like cell line
RPMI	RPMI1640 medium
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
TAE	Tris-acetate buffer
TBS	Triethanolamine-buffered-saline
TEM	Transmission electron microscopy
TEMED	N,N,N,N,-Tetramethylethylenediamine
THP-1	Human monocytic cell line
Tween 20	Polysorbate 20
Tween 80	Polysorbate 80
µg	Microgram
µl	Microliter
$ZnSO_4$	Zinc Sulfate

CHAPTER I
INTRODUCTION

Leptospirosis is the most widespread zoonosis in the world [1]. Leptospires are spirochete bacteria, including pathogenic and saprophytic species comprising the genus *Leptospira*, which belongs to the family Leptospiraceae [2, 3]. Leptospires are helically coiled spirochetes that are about 0.1 μm in diameter and 6-20 μm in length, and the wavelength is approximately 0.5 μm [2, 4]. Leptospires are thin, highly motile, obligately aerobic spirochetes that share features of both Gram-positive and Gram-negative bacteria [3, 5, 6]. Leptospiral lipopolysaccharide (LPS) is similar to LPS from Gram-negative bacteria but with less endotoxic activity [7]. Leptospires have distinctive hooked ends. Two periplasmic flagella with polar insertions are located in the periplasmic space and responsible for motility [8]. Since these bacteria are poorly Gram stained, the leptospires are best visualized by dark-field or phase-contrast microscope. They grow in simple media enriched with vitamins B1 and B12, long chain fatty acid, and ammonium salt [9]. Leptospires can be stained by carbon fuchsin counterstain [4, 10]. They produce both catalase and oxidase. The optimal growth condition of leptospires is at pH 6.8-7.4 and at temperature between 28°C-30°C [11-14]. They are initially separated into two groups; *Leptospira interrogans* sensu lato (pathogenic species) and *L. biflexa* sensu lato (saprophytic species) [4, 15, 16]. *L. interrogans* have been classified into more than 260 serovars, whereas over 60 serovars of *L. biflexa* have been reported [4, 17]. Conventional serological typing based on immunodominant surface-exposed epitopes of the LPS and more recent genotyping based on DNA relatedness demonstrated by genomic DNA-DNA hybridization are used to categorized *Leptospira* spp. into serovars and genospecies, respectively [7].

Leptospirosis is believed to be the most widespread zoonosis in the world. It can be transmitted, in humans, by direct or indirect contact with urine of an infected animal. In most tropical areas, leptospirosis is seasonal with peak incidence occurring in summer (February to March) and during rainy seasons (August to September) [4, 8, 18].

In tropical countries, farming activities, contact with animals (rats, other rodents, and livestock), poor waste disposal, rainfall, and floods are mostly related to infection [19-21]. The sources of infection are soil or water contaminated with infected urine of maintenance hosts or accidental (incidental) hosts. The disease is maintained in nature by asymptomatic reservoir hosts [8]. Reservoir hosts can be domestic and wild animals such as rat, rodents, pigs, cattle and dogs [4, 22]. Rat and other rodents are the most important sources for human infection. Leptospires enter the body through mucous membrane and skin cuts or abrasions by contact with urine of infected animals [4, 23]. *Leptospira* can spread into the bloodstream and target organs such as kidneys, liver, heart, causing inflammation of various organs. The disease can cause non-specific symptoms such as fever, chills, headache, severe myalgia, conjunctival suffusion, anorexia, nausea, vomiting and prostration [4, 24]. Severe leptospirosis may manifest as jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis, or meningitis with mortality rate up to 50% [4, 25, 26]. The mechanisms by which leptospires cause disease are not well understood [4]. Leptospires and their lipoprotein extracts stimulated monocytes leading to intracellular signaling such as p38 phosphorylation, NF-kB activation, and release of IFN-γ and nitric oxide [27, 28]. LPS of leptospires stimulated mouse macrophages by Toll-like receptor 2 (TLR2) and TLR4 [29] but in human macrophages, LPS of leptospires stimulated by TLR2 instead of TLR4 [30]. However, the humoral immunity is the primary protective immune response against *Leptospira* [31]. LPS extract lacking endotoxic activity have been shown to stimulate innate immunity, induce a serovar-specific antibody, and induce protective immunity in rabbits, guinea pigs, mice, hamsters and dogs [32-36]. Many bacteria have evolved mechanisms to invade eukaryotic cells and survive intracellularly [37, 38]. After cell invasion, bacteria use a variety of mechanisms to evade degradation. For example, *Listeria monocytogenes*, *Rickettsia*, and *Shigella*, were demonstrated to be able to lyse the vacuole and enter the cytoplasm [39, 40]. However, some intracellular pathogens reside within vacuoles that mature through the endocytic pathway and are modified to

prevent lysosomal fusion and acquisition of hydrolytic enzymes [38], but some bacteria can be destroyed by a process called autophagy.

Autophagy is the process of physiologically and immunologically controlled intracellular pathway that sequesters and degrades cytoplasmic targets including macromolecular aggregates, lipids and nucleotides from damaged proteins, cellular organelles, and intracellular pathogens such as viruses, bacteria, and protozoans [41-43]. There are three types of autophagy that occur in a cell, macroautophagy, microautophagy, and chaperone-mediated autophagy [41]. Macroautophagy, more commonly known as autophagy, is important for degrading protein aggregates [41], and clearing intracellular pathogens [44]. Autophagy can also be activated by other physiological stress stimuli (e.g., energy depletion, endoplasmic reticulum stress, high temperature, and high cell-density conditions), hormonal stimulation, pharmacological agents (e.g., rapamycin and other compounds discussed below), innate immune signals, and in diseases such as bacterial, viral, and parasitic infections [45-47].

Macroautophagy is the conserved pathway from yeast to mammals, and is mediated by a special organelle termed the autophagosome. Upon induction, a small vesicular sac called the isolation membrane and subsequently encloses a portion of cytoplasm, which results in the formation of a double-membrane structure, the autophagosome. Then, the outer membrane of the autophagosome fuses with a lysosome (autolysosome), leading to the degradation of the enclosed materials together with the inner autophagosomal membrane. The endosome can also fuse with the autophagosome before fusion with the lysosome. Amino acids and other small molecules that are generated by autophagic degradation are delivered back to the cytoplasm for recycling or energy production [42, 45, 48-50].

The process of a double-membrane structure or the autophagosome formation involves two major steps: initiation step or nucleation step, and elongation step of the isolation membrane [45]. Initiation step or nucleation step, called the phagophore (isolation membrane), which expands through the assembly of the multimer complex of Atg lipid (Atg8-phosphatidylethanolamine); known as LC3-II) [51], and protein-protein

conjugates (Atg12-Atg5, complexed with Atg16) [52]. The Atg12-Atg5/Atg16 and Atg8 (LC3)-PE conjugation systems are important for the elongation step [52]. Elongation step (growth of isolation membrane and its closure to generate a double membrane autophagosome). Maturation step of the double-membrane autophagosome into autophagosome-lysosomal fusion (called the autolysosome), lysosomal digestion, and lysosomal acidification. The regulatory signals that integrate environmental cues with the autophagic machinery are involved in autophagy [45]. The activated form of LC3, LC3-II, binds to isolation membranes (phagophores) and autophagosomes, and can be used as a specific marker for autophagy [45, 53, 54].

Previous study reported distinct fates of pathogenic *Leptospira* within macrophages of human or mouse origin [55]. It was demonstrated that pathogenic *Leptospira* had similar ability to adhere to and enter primary and immortal macrophages from human and mouse, THP-1 and J774A.1, respectively. However, their intracellular fate in human macrophages differed markedly from that in mouse macrophages. The leptospires resided within membrane-bound vacuoles in the murine macrophages, whereas they lived freely in the cytosol of human macrophages, with no surrounding vesicular membrane (Figure 1).

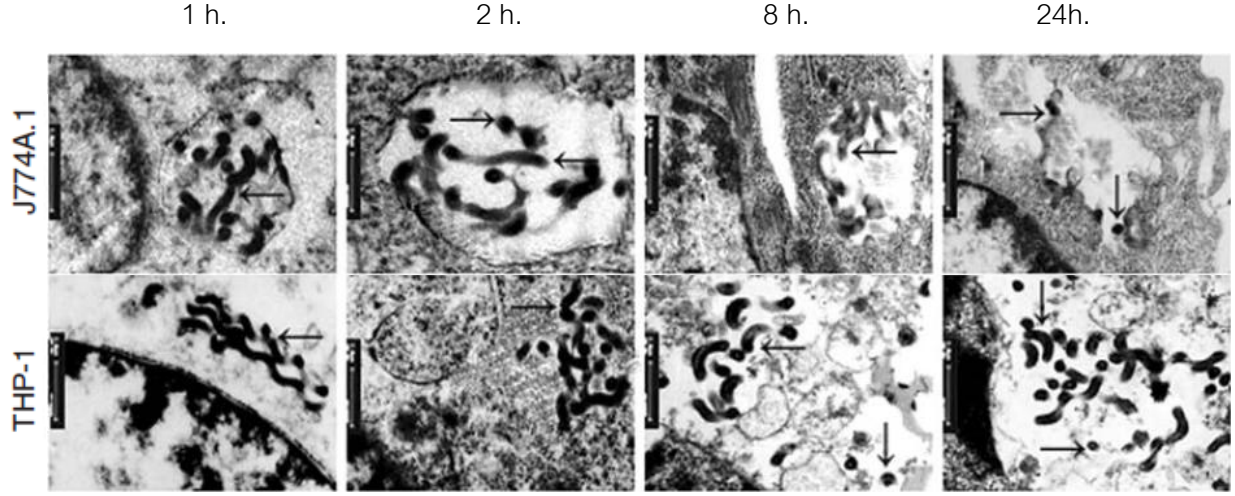


Figure 1. Intracellular localization of pathogenic *Leptospira* in human and murine macrophages [55].

In contrast to human macrophages (THP-1), murine macrophages (J774A.1) contained pathogenic *Leptospira* in a structure similar to autophagosome as observed by transmission electron microscopy. However, autophagy induction in macrophages by *Leptospira* has never been studied. Therefore, this study aimed to determine whether infection with pathogenic *Leptospira* can differently induce autophagy in human and murine macrophages.

CHAPTER II
OBJECTIVES

Hypothesis

Pathogenic *Leptospira* induces autophagy differently in infected human and murine macrophages

Objectives

1. To study autophagy induction by pathogenic *Leptospira* in infected human and murine macrophages
2. To study the role of TLR4 in autophagy induction in pathogenic *Leptospira*-infected murine macrophages

CHAPTER III
REVIEW OF RELATED LITERATURES

Leptospira and Leptospirosis

Characteristic of *Leptospira*

Leptospira are spirochete bacteria, dividing into pathogenic species (*L. interrogans*), which can cause disease in humans and some animals, and saprophytic (*L. biflexa*) species, which are free-living organisms found in water and soil [4, 15, 16]. Genus *Leptospira* belongs to the family Leptospiraceae [2, 3]. Leptospires are helically coiled spirochetes that are about 0.1 μm in diameter and 6-20 μm in length, and the wavelength is approximately 0.5 μm (Figure 2) [2, 4]. Leptospires are thin, highly motile, aerobic spirochete whose membrane shares features of Gram-positive and Gram-negative bacteria [3, 5, 6]. The spirochetes have pointed end of which one or both ends are usually hook or question mark-shaped ends [3, 4]. Since these bacteria are poorly Gram stained, the leptospires are best visualized under dark-field or phase-contrast microscope. Leptospires can be stained by carbon fuchsin counterstain [4, 10]. They produce both catalase and oxidase.

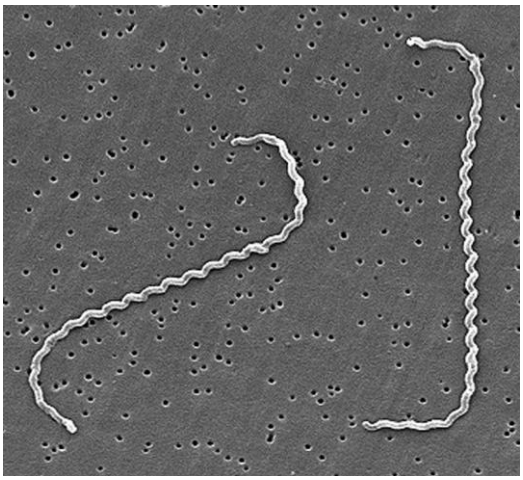


Figure 2. Scanning electron micrograph of *L. interrogans* bound to a 0.2μm membrane filter [4].

Leptospires have a typical double-membrane structure containing the outer membrane (OM) and inner membrane (IM) [6, 56]. The inner membrane (IM) is closely associated with the peptidoglycan (PG) cell wall, which is overlaid by the outer membrane (OM) [56]. The outer membrane is composed of phospholipids, outer membrane proteins (OMPs) and lipopolysaccharide (LPS), which is the major antigen of *Leptospira* (Figure 3). LPS of leptospires has a lower endotoxic potential compared with that of other Gram-negative bacteria [57-59]. The periplasmic space contains two axial filaments or periplasmic flagella with polar insertion, which are responsible for the shape and motility of leptospires (Figure 4) [60, 61]. Leptospires show two forms of movement, translational and non-translational movement [4].

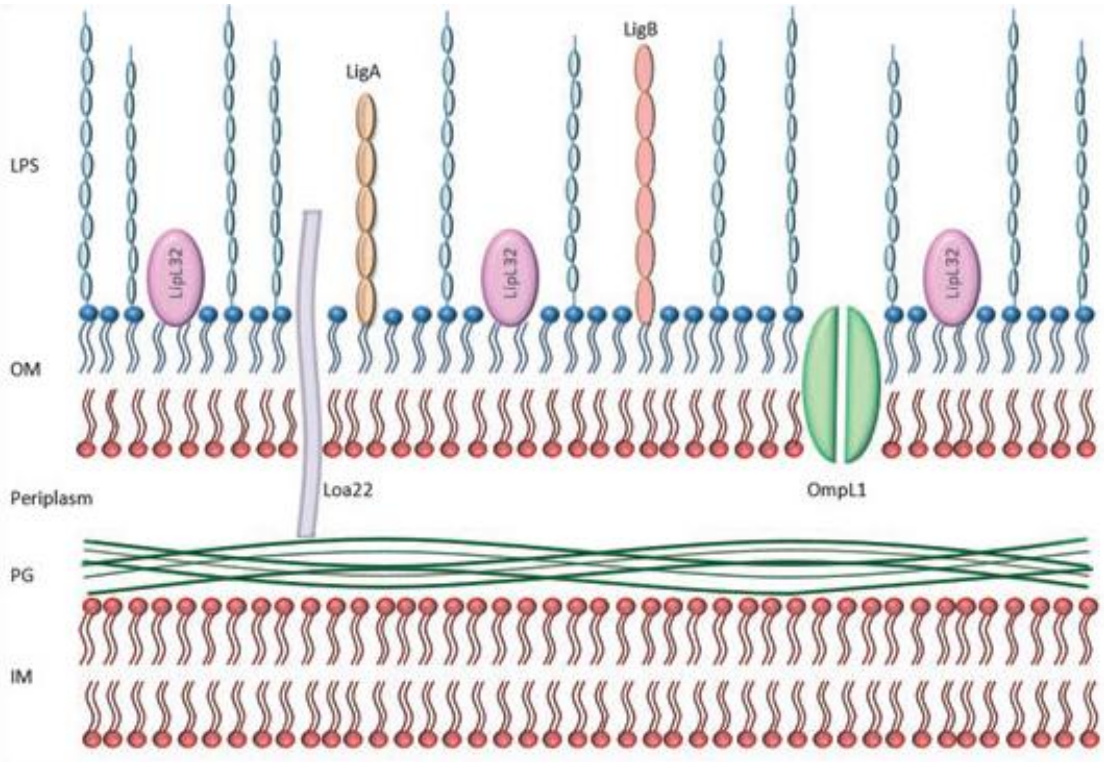


Figure 3. Structure of leptospiral cell wall containing the outer membrane (OM), periplasm, peptidoglycan (PG), and inner membrane (IM) [62].

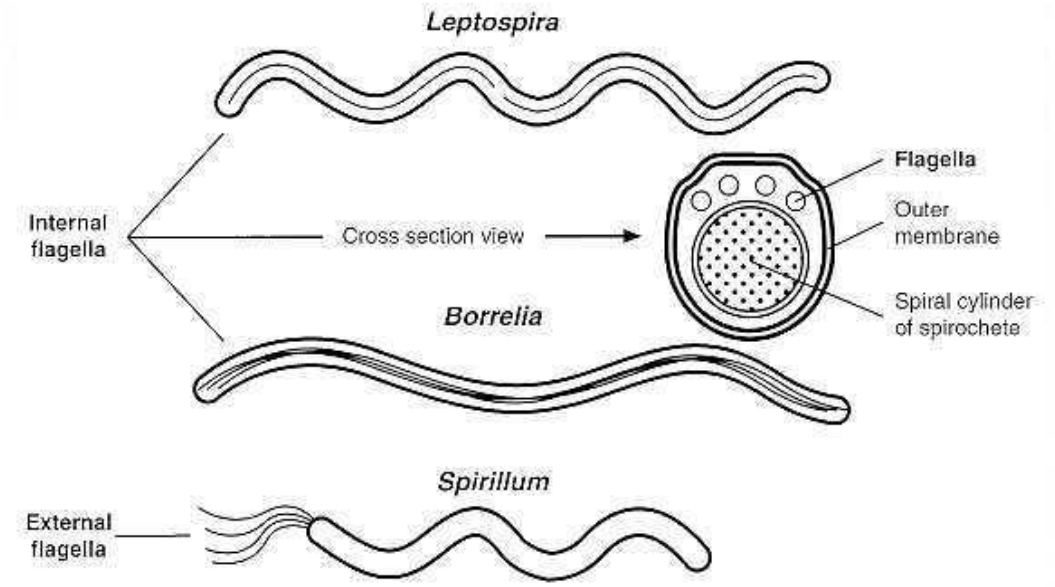


Figure 4. The schematic shows *Leptospires* and spirochetes structure. Cross section view indicates position of flagella, outer membrane and spiral cylinder of spirochete [63].

Cultivation Method

Leptospira is an obligately aerobic bacterium that requires carbon and energy during *in vitro* growth. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium is commonly used medium for leptospires. The culture medium is supplied with 10% rabbit serum or 0.2-1% bovine serum, vitamin B12, long chain fatty acids, ammonium salt, and sodium pyruvate [4, 8, 64]. Long-chain fatty acids obtained from polysorbate (Tween 80) are carbon and energy sources which are metabolized by the beta-oxidation pathway [65, 66]. Growth factors of leptospires are vitamin B1 and B12 [65, 67] .

The optimal growth condition of leptospires is at pH 6.8-7.4 and at temperature between 28°C-30°C [11-14]. The minimal growth temperature of pathogenic and saprophytic leptospires is at 13-15°C and 5-10 °C, respectively. The ability of pathogenic leptospires to grow at 13°C can be used to differentiate pathogenic from

saprophytic leptospires [68, 69]. Growth of leptospires is generally slow on primary isolation, and cultures are retained for up to 13 weeks before being discarded whereas pure subculture in liquid media usually grows within 10 to 14 days to reach log phase. Leptospires are maintained in semisolid media, containing 0.1-0.2 % agar. Its growth reaches a maximum density in a discrete zone beneath the surface of the medium, which is known as a Dinger's ring or disk [4]. The maintenance of stock cultures can be achieved by repeated subculture or storage in liquid nitrogen for long-term preservation to maintain its virulence and give good yields [4, 70]. Colony morphology of leptospires on solid agar is dependent on agar concentration and serovars [71, 72]. The solid media have been used for isolation of leptospires from mixed cultures, on which hemolysin production can be detected [4, 10].

Molecular biology

The leptospiral genome is approximately 5,000 kb [73, 74]. The genome is comprised of two circular chromosomes, a 4,400-kb and a smaller 350-kb chromosome [74, 75]. The chromosome of *Leptospira* is characterized by a G+C content of 35–41 mol% [2]. Leptospiral genome contains two sets of 16S rRNA and 23S rRNA genes and one set of 5S rRNA gene [76, 77]. Previous studies comparing whole genome sequences of 2 pathogenic and one saprophytic species has identified 2,052 genes (61% of whole genome) common to all (Figure 5) [2, 78]. Comparative genomics of *L. interrogens* and *L. biflexa* showed that 627 genes are unique to *L. interrogens* and are absent in *L. biflexa* and over 500 of these genes (80%) encode hypothetical proteins [2]. These findings support the idea of a common origin for leptospiral pathogenic strains and saprophytic strain [78].

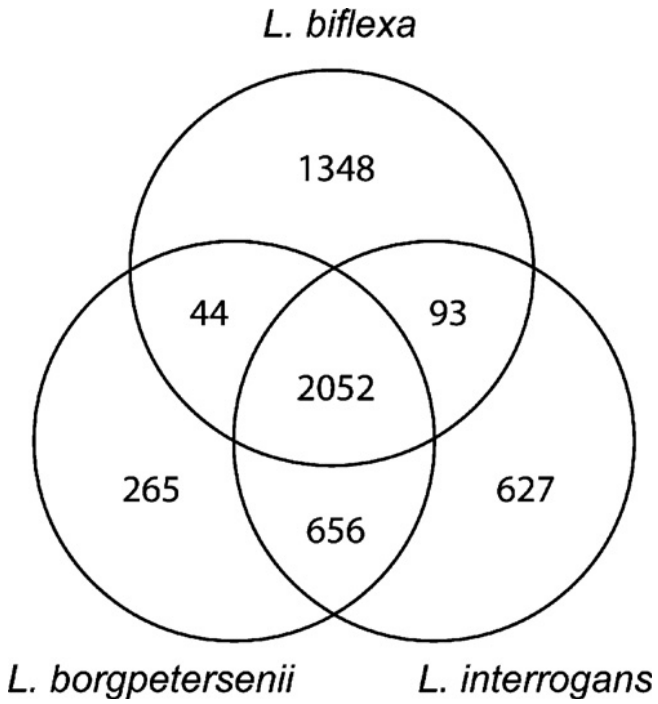


Figure 5. Comparative genomics of *Leptospira* spp. [2]

Taxonomy and classification

The genus *Leptospira* was divided into two major species, *L. interrogans* and *L. biflexa*. *L. interrogans* are pathogenic strains, whereas *L. biflexa* are saprophytic strains isolated from the environment [2, 4]. The genus *Leptospira* belongs to the kingdom Monera, phylum Spirochetes, class Spirochetes, order Spirochetales, family Leptospiraceae [4, 79].

Serological classification

The pathogenic leptospires (*L. interrogans*) and non-pathogenic, free-living organisms found in water and soil (*L. biflexa*) are divided into various serovars based on cross-agglutinin adsorption test (CAAT) with homologous, serovar-specific antibodies [4, 80, 81]. Leptospiral strains are commonly referred to serovar. *L. interrogans* have been currently classified into more than 250 serovars, whereas there are over 60

serovars of *L. biflexa* (Figure 6) [4, 17]. The examples of serogroups and serovars of *L. interrogans* are shown in table 1

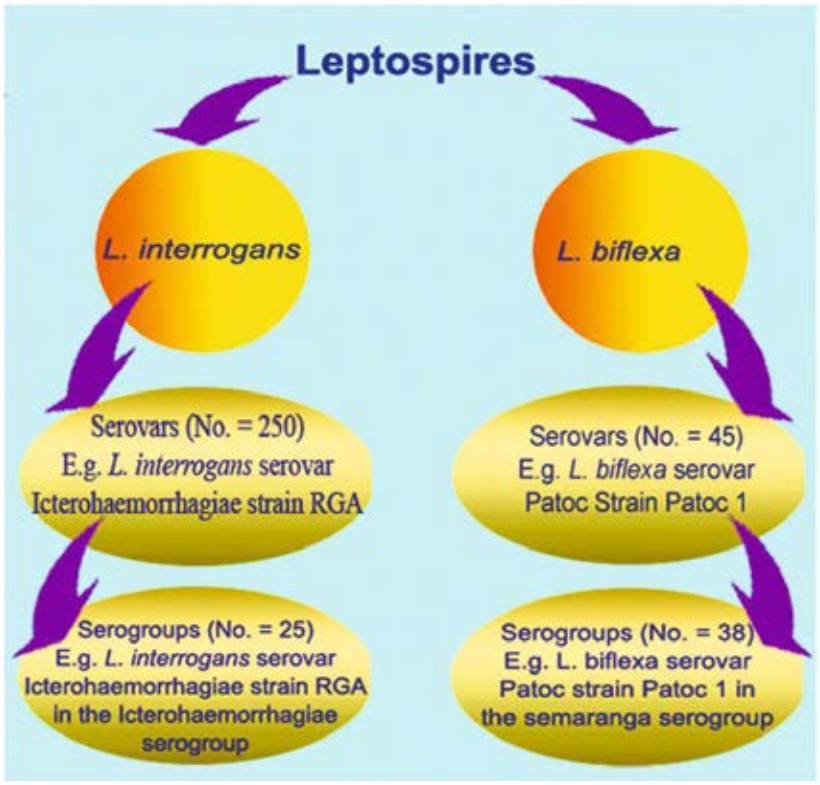


Figure 6. Serological classification of *Leptospira* spp. [17]

Genotypic classification

The phenotypic classification has currently been replaced by genotypic classification [82, 83]. The genotypic classification of the *Leptospira* is based on at least 70% DNA-relatedness whose related DNA sequences contain no more than 5% unpaired bases [7, 83, 84]. However, genotypic classification is not corresponding to serological classification since some serovars are found in more than one species of *Leptospira* and vice versa [4].

Table 1. Serogroups and serovars of *L. interrogans* sensu lato.

Serogroup	Serovar(s)
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai, Zimbabwe
Hebdomadis	Hebdomadis, Jules, Kremastos
Autumnalis	Autumnalis, Fortbragg, Bim, Weerasinghe
Pyrogenes	Pyrogenes
Bataviae	Bataviae
Grippotyphosa	Grippotyphosa, Canalzonae, Ratnapura
Canicola	Canicola
Australis	Australis, Bratislava, Lora
Pomona	Pomona
Javanica	Javanica
Sejroe	Sejroe, Saxkoebing, Hardjo
Panama	Panama, Mangus
Cynopteri	Cynopteri
Djasiman	Djasiman
Sarmin	Sarmin
Mini	Mini, Georgia
Tarassovi	Tarassovi
Ballum	Ballum, Aroborea
Celledoni	Celledoni
Louisiana	Louisiana, Lanka
Ranarum	Ranarum
Manhao	Manhao
Shermani	Shermani
Hurstbridge	Hurstbridge

Modified from [4]

Epidemiology

Leptospirosis is the most widespread zoonosis in the world [1]. It can be transmitted to humans by direct or indirect contact with urine of infected animals. The incidence of leptospirosis in tropical or subtropical areas is significantly higher than that of temperate regions [4] due mainly to the ability of leptospires to live in the warm environment longer than in the temperate environment. However, in most tropical areas leptospirosis is seasonal, with peak incidence occurring in summer and during rainy seasons occurring during August to September and February to March [4, 8, 18].

The sources of infection are soil or water contaminated with infected urine of maintenance hosts or accidental (incidental) hosts. The disease is maintained in nature by asymptomatic reservoir hosts [8]. Reservoir hosts can be domestic or wild animals such as rodents, small marsupials, pigs, cattle and dogs [4, 22]. Rat and other rodents are the most important sources for human infection. Leptospires enter the body through mucous membrane and skin cuts or abrasions by contact with urine of infected animals (Figure 7) [4, 23].

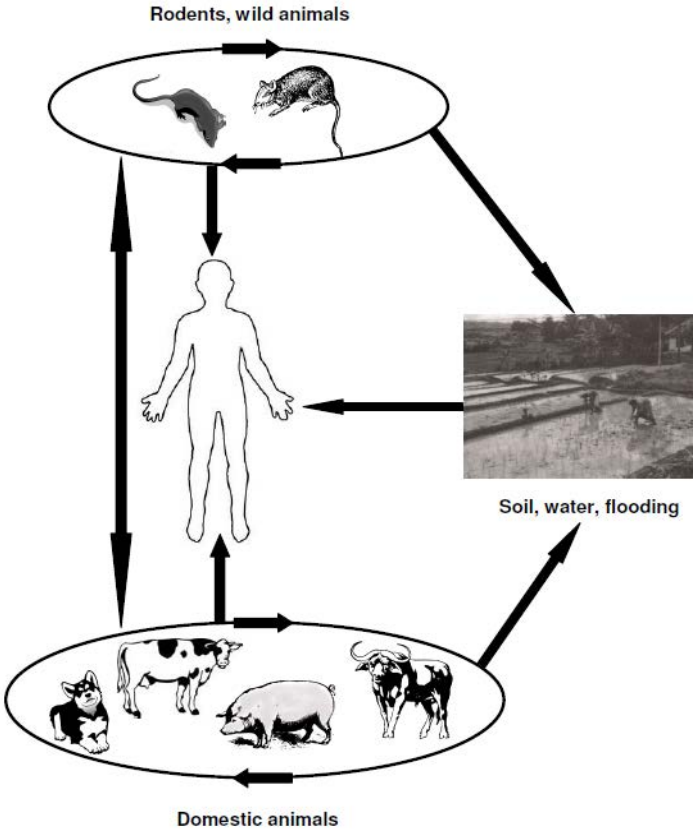


Figure 7. Epidemiology of leptospirosis. Pathogenic *Leptospira* are shedded into urine of reservoir hosts. Infection in human most commonly occurs from direct contact with infected urine or indirect contact with contaminated soil or water [85].

Leptospirosis is an increasingly recognized cause of unspecified febrile illnesses in Southeast Asia including Indonesia, Lao and Vietnam [86]. In Thailand, leptospirosis is an emerging infectious disease that was first reported in 1942 (8, 7 (M)). The number of cases was 859 per 5 years between 1990 and 1995, Data from the disease notification reports indicates a drastically increased from 385 cases in 1996 to 2,334 cases in 1997, and a report of outbreak of 14,285 cases in 2000. Since then there are reports of cases Thailand as shown in Figure 8 and Thailand especially in the Northeastern part is considered to be an endemic area of leptospirosis [87] .

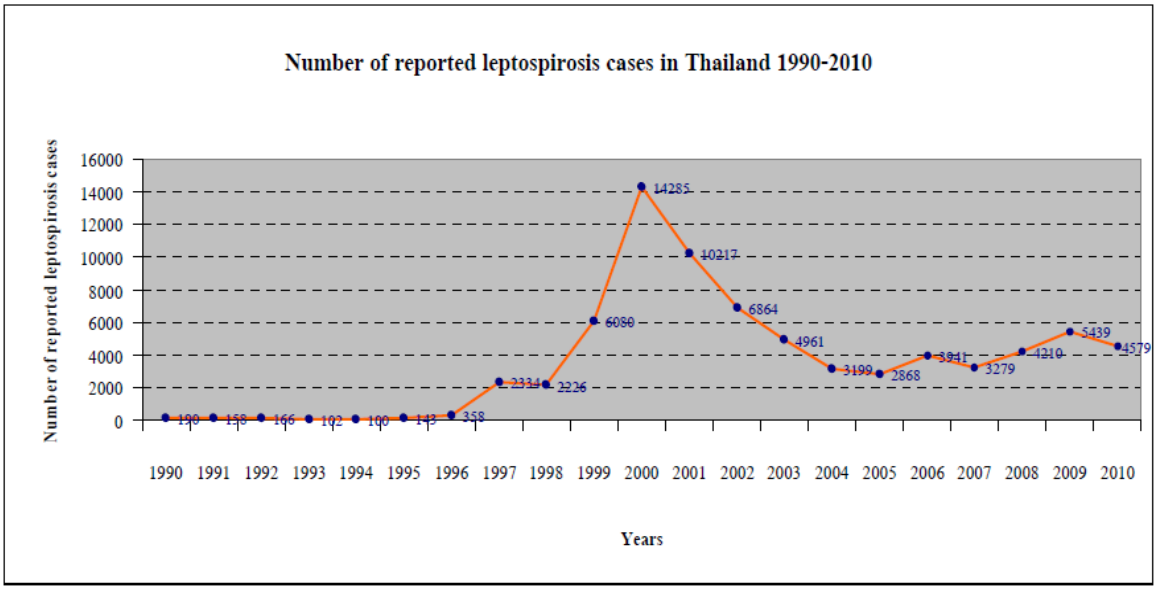


Figure 8. Reported cases of leptospirosis in Thailand from 1990 to 2010 [87].

Clinical features of leptospirosis

Clinical features of leptospirosis are diverse ranging from subclinical infection to fever, chills, headache, severe syndrome of multiorgan failure with high mortality rate [4, 8, 18, 24]. Two forms of leptospirosis have been described, which are anicteric leptospirosis and icterohaemorrhagic leptospirosis or Weil's disease [15].

Anicteric form

The great majority of leptospirosis is anicteric leptospirosis. Symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion, and less often a skin rash [88]. If present, the rash is typically transient or present temporary for less than 24 hours. This anicteric syndrome usually lasts about a week, and the resolution corresponds to the development of antibodies. The differential diagnosis must include influenza [89], HIV seroconversion [90] and, in the tropics, dengue fever [91-93].

Icterohaemorrhagic form or Weil's disease

Icterohaemorrhagic leptospirosis or Weil's disease is a much more severe form of the illness and often rapidly progresses. It is described as jaundice, renal failure, and haemorrhage of target organs. The icterohaemorrhagic form contributes to the high mortality rate, which ranges between 5 and 15% [4, 8, 94]. Weil's disease represents between 5 and 10% of all patients with leptospirosis [94]. It is a common cause of the acute renal failure (ARF), which occurs in 16-40 % of cases [95-98]. The occurrence of pulmonary symptoms is one of major causes of death in leptospirosis [26, 99-101]. It is presented with a ranging of symptoms, such as cough, dyspnea, hemoptysis to respiratory distress syndrome in adults [102-110]. Cardiac involvement is common but may be underestimated. A mortality rate of 54% was reported in severe leptospirosis cases with myocarditis [111, 112]. Ocular involvements of severe leptospirosis were noted in early reports [4]. Conjunctival suffusion is seen in the major complaints of patients with leptospirosis [113]. Uveitis may present weeks, months, or occasionally years after the acute stage of infection (Figure 9) [114, 115].

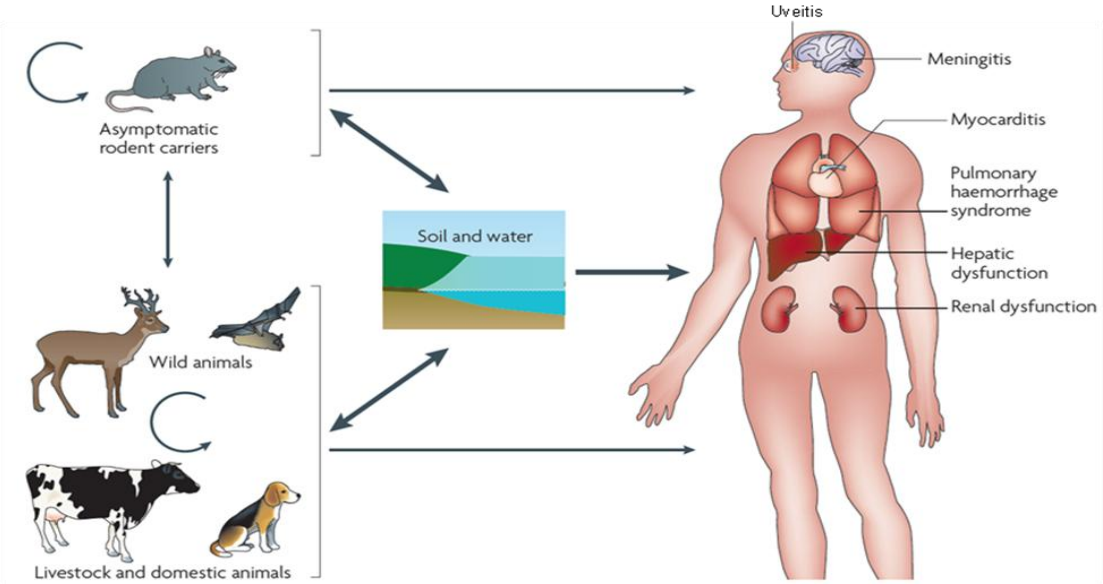


Figure 9. The symptoms of leptospirosis are multisystemic involvement such as uveitis, meningitis, myocarditis, pulmonary haemorrhage syndrome, hepatic dysfunction, and renal dysfunction [3].

Laboratory diagnosis

The laboratory diagnosis is very important, because the clinical symptoms are not specific in both humans and animals.

Microscopic demonstration

Leptospires can be seen under dark-field microscopy. The samples such as, blood, urine, cerebrospinal fluid, or peritoneal dialysis fluid was used. Minimal number of leptospires that is necessary for observation by dark-field microscope is about 10^4 cells/ml. Immunofluorescence staining and immunoperoxidase staining has been applied to increase sensitivity of direct microscopic examination. The leptospires can be detected in histophatological tissue stained with silver staining or immunohistochemical staining [116, 117].

Cultivation

Cultures isolated from clinical specimens, such as serum, blood, and, cerebrospinal fluid (CSF) in 7-10 days of infection. After the first week until about 30 days of the disease, leptospires may be isolated from urine, fluid in the eyes, and kidneys. The sample culture was considered as the gold standard for diagnosis. However, culture is not used as a routine test for diagnosis, since the growth rate of leptospires is slow [4, 118, 119].

Molecular diagnosis

DNA of leptospires can be detected in blood, cerebrospinal fluid (CSF), urine, and aqueous humor by polymerase chain reaction (PCR). The PCR is a method with high sensitivity and has been reported to detect at its earliest stages. Various primer pairs for detection by PCR of leptospires have been designed to target at the 16S rRNA or 23S rRNA genes, pathogenic *Leptospira*-specific genes such as *lipL32*, and the repetitive sequences [76, 120]. Recently, a real-time PCR is demonstrated as the rapid detection method of pathogenic leptospires [121]. The limitation of PCR is an expensive and a requirement of high skill [122-132].

Serological diagnosis

Serological methods aim to determine the level of antibody in serum typically about 5-7 days after the patient begins to show symptoms. The microscopic agglutination test (MAT) is currently the standard method for diagnosis of leptospirosis. It uses live leptospires as antigens to react with antibodies in serum. The standard criteria for positive MAT are four-fold increase of antibody titers of paired sera or a conversion from seronegativity to a titer of 100 or more. The MAT is read under dark-field microscope and result interpretation is complicated [4, 8, 133-137]. In addition, detection of antibodies against leptospires can be done in several ways, such as ELISA [138-143] , complement fixation test [133] , macroscopic slide agglutination [144-146], latex agglutination [147-150], dipstick ELISA [151-153] and immunofluorescence [154, 155].

Pathogenesis

The primary step of infection is bacterial adherence to host cells and then replication and dissemination to target organs. Moreover, the interactions trigger host innate and adaptive immunity. Therefore, host-pathogen interactions may be crucial for pathogenesis and outcome of infection [156]. In leptospirosis, after pathogenic leptospires enter a host, they spread via bloodstream. Although, their reproductive rate is slightly slower than some other pathogens, leptospires may be able to produce a larger number within a few days. Leptospires are non-pyrogenic bacteria, since their presence does not cause strong inflammation. They do not localize at the point of infection, so entry site does not become inflamed [4, 8]. However, understanding of mechanisms of leptospirosis is limited. Several putative virulence factors have been described, but most of their functions remain unknown.

It is generally accepted that the initial interaction between microbe and host cell is an important step for many infectious disease [157]. Comparison study of colonization of fibroblasts and macrophages by high and low-virulent *Leptospira* strains found that both strains adhere to fibroblasts and macrophages using one or both ends of spirochete, followed by phagocytosis of both strains. Both strains adhered to macrophages more strongly than fibroblasts. The more virulent strain could invade host cell nucleus, while the lower virulent strain remained in phagosome [158]. Genomic sequencing revealed that *L. interrogans* contains several genes that are related to attachment to and invasion of eukaryotic cells [159]. The function of these leptospiral proteins required elucidation. An understanding of mechanism by which *L. interrogans* attaches and invades mammalian cell would be a great step forward in knowledge of pathogenesis of leptospirosis [159, 160].

Autophagy

Introduction

Autophagy is the process of physiologically and immunologically controlled intracellular pathway that sequesters and degrades cytoplasmic targets including macromolecular aggregates, lipids, and nucleotides from damaged proteins, cellular organelles, and intracellular pathogens such as viruses , bacteria, and protozoans [41-43]. There are three types of autophagy that occur in a cell, macroautophagy, microautophagy, and chaperone-mediated autophagy [41]. Macroautophagy, more commonly known as autophagy, is important for degrading protein aggregates [41], and clearing intracellular pathogens [44]. Macroautophagy is conserved in mammals, and is mediated by a special organelle termed the autophagosome [45].

Autophagic Pathway

Autophagy is a general process by which cytoplasmic materials including organelles and lysosomes used for degradation [45, 161]. The process of autophagy involves a set of evolutionarily conserved gene products, known as Atg proteins including Atg 5, 7, 10, and 12, which are required for the formation of isolation membrane and autophagosome [45, 51]. The process of a double-membrane structure or the autophagosome formation composes of two major steps: initiation step or nucleation step, and elongation step of the isolation membrane [45]. Initiation step or nucleation step, is the formation of the phagophore or isolation membrane, which expands through the assembly of the multimer complex of Atg lipid [51] (Atg8-PE; known as LC3-II), and protein-protein conjugates (Atg12-Atg5 in complex with Atg16) (Figure 10) [52]. The Atg12-Atg5/Atg16 and Atg8 (LC3)-PE conjugation systems are important for the elongation step [52]. Elongation step is the growth of isolation membrane and its closure to generate a double membrane structure called autophagosome. Maturation step is the transition of the double-membrane

autophagosome into autophagosome-lysosomal fusion (called the autolysosome), followed by lysosomal digestion, and lysosomal acidification [45]. The contents of the autolysosome are released into the cytoplasm of the cell, where they are reused in many cellular processes.

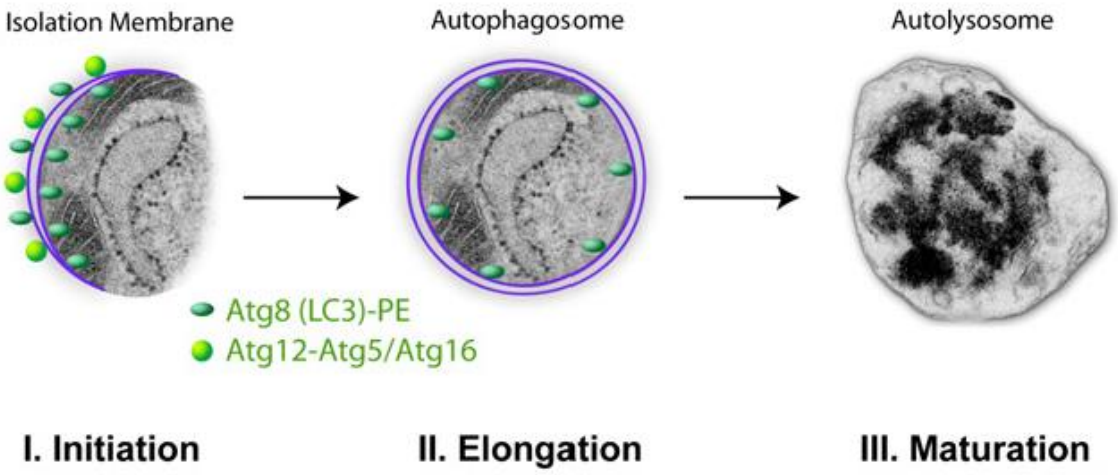


Figure 10. The molecular machinery of macroautophagy [52].

Autophagy activation

Autophagy is induced in mammalian cells by stress, cell damage, and amino acid starvation [41, 162]. The mammalian cells-target of rapamycin protein (mTOR), is a phosphotidylinositol-3' kinases (PI3K)-related kinase that signals cell health and promotes cell division [163]. Inhibition of mTOR by rapamycin simulates amino acid starvation under nutrient depleted conditions there by inducing autophagy [164]. Assembly of the autolysosome is disrupted by the PI3K inhibitors, wortmannin [165], and 3-methyladenine (3MA) [166]. Both wortmannin and 3MA act by inhibiting type III PI3K, cytosolic enzymes required for the early stages of autophagosome development (Figure 11) [167]. Loss of the proton gradient mutes the degradative activity of the autolysosome and prevents additional lysosomes from fusing with the organelle [168].

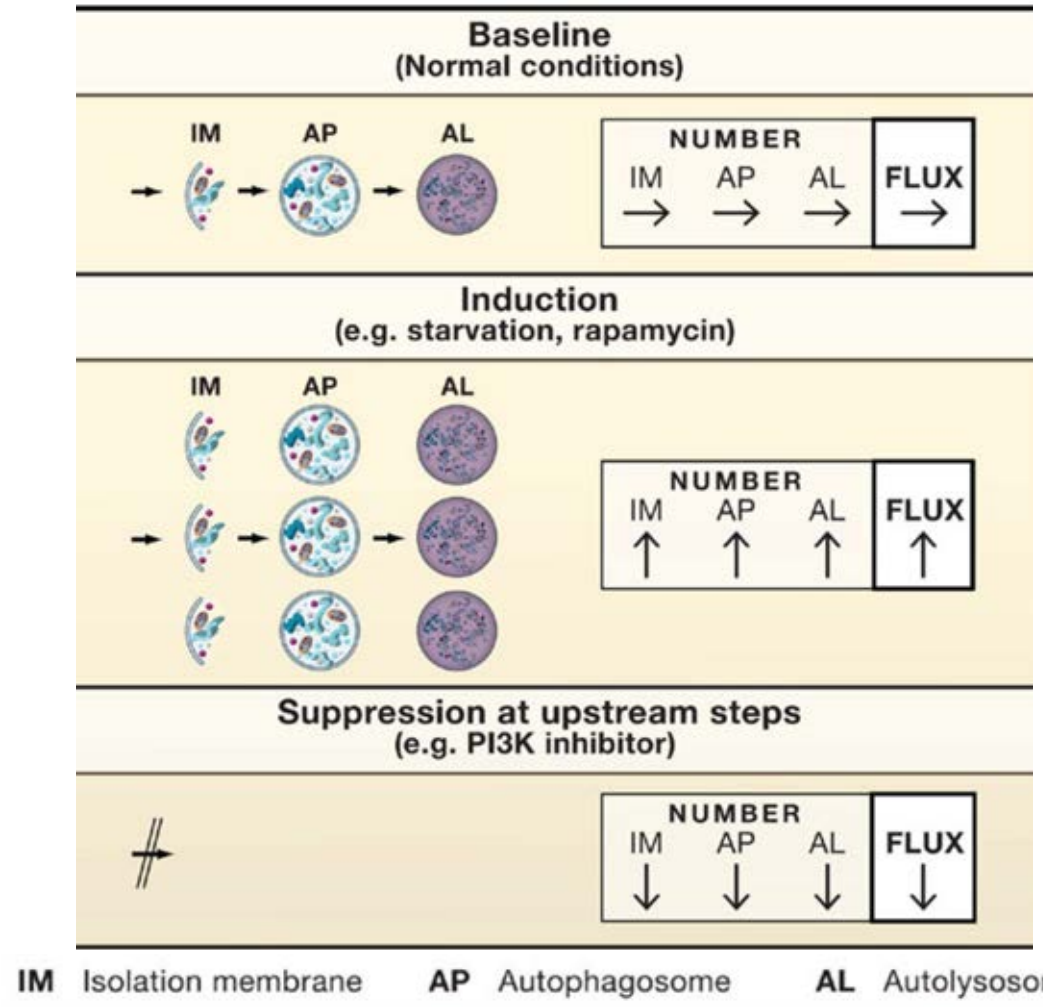


Figure 11. The regulators of autophagy and cellular accumulation of different autophagic structures [45].

Methods for detection of autophagy

Three principal methods are presently used to monitor of the autophagosomes, including electron microscopy, light microscopy detection of the subcellular localization of LC3, and biochemical detection of the membrane-associated form of LC3.

Electron Microscopy

The conventional method is to observe a double-membrane structure defined as an autophagosome at the ultrastructural level by electron microscopy (Figure 12) [45]. In the past, electron microscopy has been used extensively to quantify the number of autophagosomes in a variety of cells and tissues. Basically, the number or area within autophagosomes is quantified from an unbiased sampling of grids and images. The disadvantage of electron microscopy is its long procedure and requirement of experience. However, the advantage is that autophagosomes can be unequivocally identified based on their morphology as well as by the presence of specific markers in the membrane [45, 52].

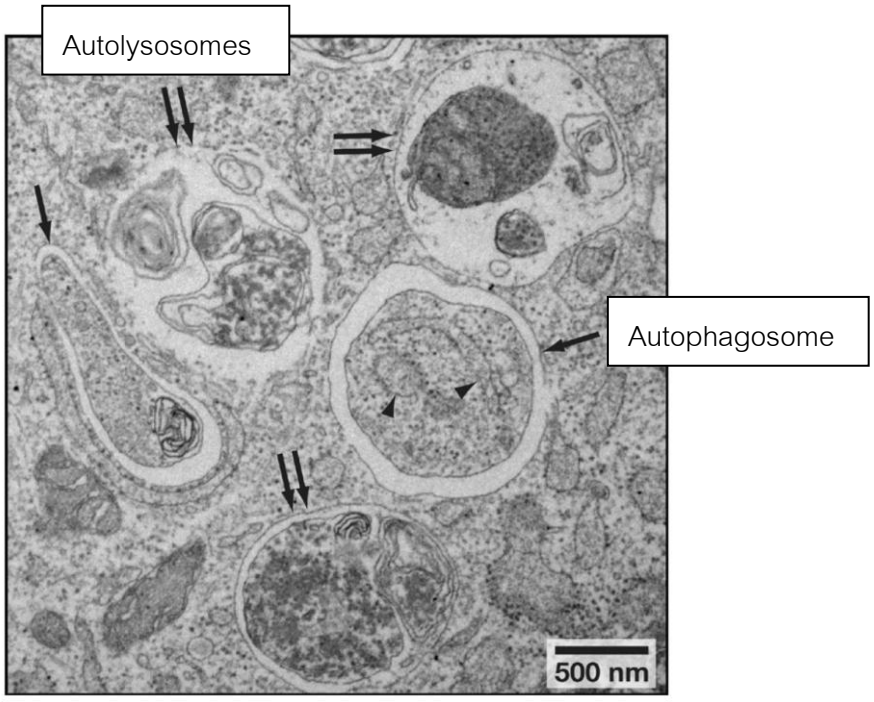


Figure 12. Morphology of Autophagosome (double-membrane structure), and Autolysosome [45].

Fluorescence Microscopy

The assessment of autophagosome number by electron microscopy requires considerably specialized expertise, and is becoming increasingly replaced by light microscopy and biochemical methods that are more widely accessible to researchers in different fields. As noted above, the mammalian autophagy protein, LC3, is a marker of autophagosomes [45, 48]. Atg8 is the only specific marker for autophagosomes found so far which remains bound after the closure of the sequestering isolation membrane. Mammalian cells contain several homologs, LC3 [169], GABARAP and GATE16 [170], all of which have been shown to bind to the membrane of autophagosomes. Of these three proteins, LC3 [171], has been investigated the most. Pro-LC3 gets cleaved at the position after a highly conserved glycine residue in the C-terminus by the protease Atg4 [172] to give rise to the cytosolic form LC3-I. Upon induction of autophagy LC3-I is modified in a ubiquitin-like process, which involves Atg7 and Atg3 and the attachment of a lipid residue, presumably phosphatidylethanolamine (PE) to the C-terminal glycine residue [173, 174], in an Atg5–Atg12-dependent manner [175]. The resulting form, LC3-II, binds to isolation membranes (phagophores) and autophagosomes, and can be used as a specific marker for autophagy.

Biochemical Assays

In addition to its utility in fluorescence microscopy assays, LC3 is also useful in biochemical assays to assess autophagosome numbers. The conversion from endogenous LC3-I to LC3-II can be detected by immunoblotting with antibodies against LC3 in Western blots. Although the actual molecular weight (MW) of LC3-II (a PE-conjugated form) is larger than that of LC3-I, LC3-II (apparent MW is 14 kD) migrates faster than LC3-I (apparent MW is 16 kD) in sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) because of extreme hydrophobicity of LC3-II (Figure 13) [45, 176]. The amount of LC3-II usually correlates well with the number of autophagosomes.

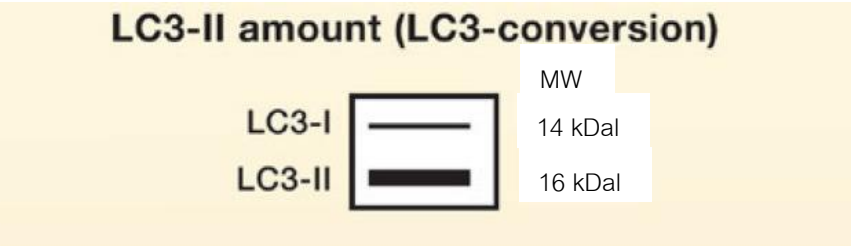


Figure 13. The conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound lipidated form) by immunoblotting [45, 174].

Autophagy and Immunity

One of the most important functions of autophagy appears to be its role in the host defense against cellular pathogens [177]. In general, bacterial pathogens enter the cell via an endocytosis pathway, enclosed within a vesicle called a phagosome that ultimately fuses with and is degraded by the lysosome [177]. In contrast, it has recently been shown that certain bacteria undermine the autophagic machinery to promote their replication and survival [178, 179]. This evasion is accomplished in different ways by different bacteria. In the case of *Listeria monocytogenes*, *Shigella* and certain other bacteria, the microbes induces lysis of the phagosome, causing their release into the cytoplasm and enabling them to replicate in that environment [180, 181]. Other invasive bacteria including *Mycobacterium tuberculosis* modify the phagosome in which they are contained, to prevent fusion with the lysosome [182]. Still other pathogens such as *Legionella pneumophila* induce the autophagic pathway and replicates within autophagosome [183]. In organisms such as *L. pneumophila*, induction of autophagy enhances the replication and survival of the invading bacteria [184].

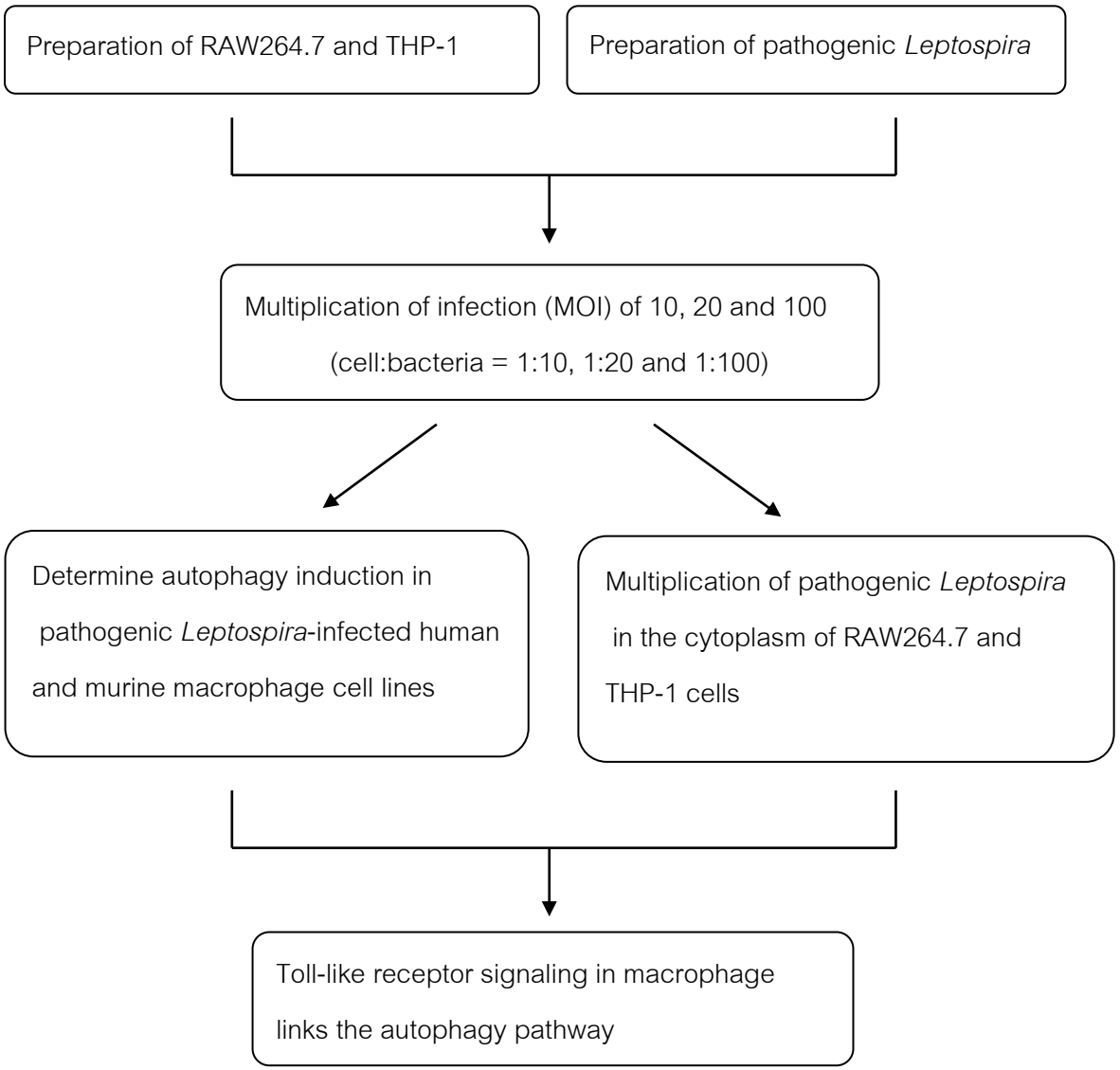
From these recent discoveries, it is now clear that autophagy can aid in defense against pathogens, but microbes can also employ this pathway to promote their viability. This is similar to the role of autophagy in cancer and other diseases – depending on the

progression of the disease, autophagy may be a protective mechanism, eliminating damaged organelles or even damaged cells, or it may have harmful effects by causing cell death or in the case of cancer by promoting the survival of tumor cells under limiting nutrient conditions [185, 186]. These varying effects of autophagy only serve to emphasize the importance of being able to control the activity of this degradative pathway, if it is ever to be used therapeutically.

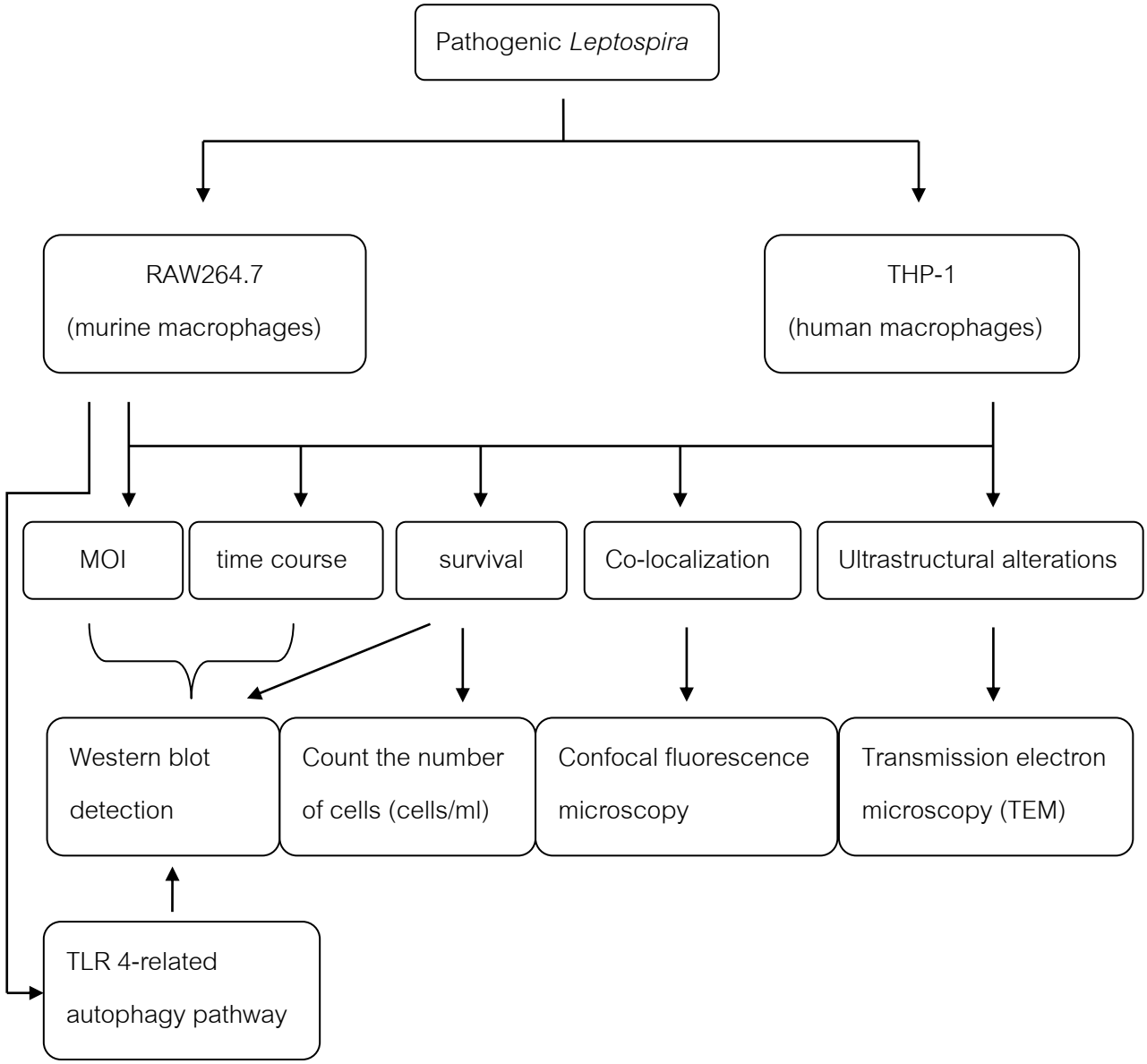
Investigations of autophagy will aid in our understanding of this important process, and hopefully lead to treatments for the human conditions and diseases in which autophagy is implicated.

CHAPTER IV

MATERIALS AND METHODS



Experimental Flow Chart



Bacterial cultivation

Leptospira

Leptospira interrogans serovas Pomona was obtained from Khon Kaen University. *Leptospira* was cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) media at 29ºC for approximately 7 days until cell density reached approximately $1x10^8$cells/ml which was counted with a Petroff-Hausser counting chamber under dark-field microscopy. Low-passaged *L. interrogans* serovas Pomona, which was subcultured not more than five passages *in vitro*, was used in the study to prevent the loss of virulence of leptospires.

Cell lines and media

The murine macrophage-like cell line (RAW264.7: ATCC No. TIB-71) and the human monocytic cell line (THP-1: ATCC No. TIB-202) were cultured in DMEM (Invitrogen, USA) and RPMI1640 medium (Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen, USA), 100 U/ml penicillin (Invitrogen, USA), 100 µg/ml streptomycin (Invitrogen, USA), 1% sodium pyruvate (Invitrogen, USA) and 1% HEPES (Invitrogen, USA), respectively at 37 ºC in humidified 5% CO_2 incubator (Sanyo, Japan). To differentiate cells into macrophages, THP-1 cells were treated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) for 24 hours before each experiment.

Cell preservation and storage

Freezing media were prepared by adding 10% DMSO (Calbiochem, Germany) to DMEM or RPMI-1640 complete media. After collecting cells by centrifugation (1,500 rpm for 5 minutes), $5x10^6$cells were resuspended in 1 ml of cold freezing media and stored in cryogenic vial (Corning, Mexico). The cells were immediately stored at -80 ºC overnight before long term storage in liquid nitrogen.

Before use frozen cells in cryogenic vials obtained from liquid nitrogen were thawed in 37 ºC water bath (Memmert, Germany). Cell suspensions were added to 9 ml serum-free media and centrifuged at 1,500 rpm for 5 minutes. Freezing media was removed followed by adding DMEM or RPMI-1640 complete media.

Western blot

Protein extraction

Cells infected with *L. interrogans* serovar Pomona were washed twice in PBS and then lysed in RIPA buffer [50mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0% NP-40, 0.5% $C_{24}H_{39}O_4Na$, 0.1% SDS, and protease inhibitor was added (Roche Diagnostics, Sweden)]. Cells were incubated on ice for 5 minutes, transferred to tube on ice and centrifuged at 8,000xg for 10 minutes at 4ºC. The supernatant containing soluble proteins was carefully transferred to a tube on ice and stored at -80ºC for future analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were heated at 95ºC for 10 minutes. Heated samples and prestained molecular weight markers (Fermentas, Canada) were loaded to the gels. The samples were separated at 100 volt for at least 90 minutes in Western blot running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS) using Mini-PROTEAN® system (BioRad, USA).

Protein transfer

After separation process, the gels were equilibrated in transfer buffer (48 mM Tris pH 9.2, 39 mM glycine, 1.3 mM SDS, 20% methanol) for 5 minutes. Polyvinylidene fluoride membranes (PVDF; Millipore, Germany) were soaked in absolute methanol

(Burdick & Jackson, Korea) and rinsed with deionized water twice and immersed in transfer buffer. Proteins on the gel were transferred to PVDF membranes using a semi-dry transfer Trans-Blot® SD (BioRad, USA) apparatus at 150 mA for 90 minutes.

Antibody probing

After protein transfer, the PVDF membranes were blocked in blocking solution (1X Tris buffered saline (TBS) + 3 % skim milk) at room temperature for 1 hour followed by washing 3 times, 10 minutes each with washing buffer (1X TBS + 0.1% Tween 20). After blocking, the PVDF membranes were incubated with 1:5,000 dilution of rabbit anti-LC3-II (Sigma aldrich, USA) and 1:5,000 dilution of moues anti ß-actin (Merck, Germany) at 4ºC overnight. The probed membranes were further incubated on a rocker for 1 hour at room temperature. The primary antibody solution was discarded, and the membrane was washed 3 times with 1X TBS + 0.1% Tween 20 for 10 minutes each. After washing buffer was discarded, 1:5,000 dilution of donkey anti rabbit IgG (GE) and 1:5,000 dilution of goat anti-mouse IgG (KPL, USA) conjugated with horseradish peroxidase (HRP) were added. The PVDF membranes were incubated for 1 hour with rocking before washing 3 times with 1X TBS + 0.1% Tween 20 for 10 minutes each.

Signal detection by chemiluminescence and autoradiography

The signal was detected by ECL detection system (company) according to manufacturer's instructions. The mixture of solution A and B was poured directly onto the membranes and incubated for 1 minute. The membranes were wrapped with the plastic wrap and placed in a film cassette (Life Technologies™, USA) to expose to High Performance Chemiluminescence Film: Amersham Hyperfilm™ ECL (Amersham Biosciences, England) in the dark. Exposure time for LC3II and ß-actin was 15-30 minutes and 5-10 seconds, respectively. The film was developed for 5-10 seconds in x-ray film developer, washed with tap water, fixed for 3-5 minutes in the fixer and finally washed with tap water before air dry.

Infection of macrophages with *Leptospira*

Freshly cultured *L. interrogans* serovar Pomona in EMJH liquid medium were counted by dark-field microscopy with a Petroff-Hausser counting chamber. The RAW264.7 cells and the THP-1 were cultured in DMEM and RPMI1640 medium, respectively. After pre-incubation, the cells were washed 3 times with Phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM $Na_2HPO_4 \cdot 2\ H_2O$ and 2mM KH_2PO_4). The leptospires were incubated with RAW264.7 and THP-1 at a multiplicity of infection (MOI) of 10, 20, and 100 (10, 20, and 100 leptospires per host cell) for 1, 2, 4, 8 and 24 hours. Then, cells were washed twice in PBS and lysed in RIPA buffer. After centrifugation at 8,000xg for 10 minutes at 4ºC, proteins of infected cells were analyzed using 12% SDS-PAGE and Western blot analyses as described above.

Determination of *Leptospira* survival

Enumeration of leptospires in macrophages

The tested RAW264.7 and THP-1 cells ($3x10^5$) were infected with *L. interrogans* serovar Pomona at MOI of 1:100 at 37ºC for 1 hour and treated with gentamicin at a final concentration of 50 μg/ml to kill the remaining extracellular leptospires. Then, the cell samples were treated with 200nM of rapamycin or 5mM of 3-methyladenine (3-MA), which is a stimulator and inhibitor of autophagy, respectively. After incubation for 1 or 8 hours, the cells were lysed with ice-cold PBS containing 0.05% sodium deoxycholate (Sigma, USA). The lysates were centrifuged at 1,500xg for 2 minutes to remove large cell debris. The supernatant was centrifuged at 15,000xg at 4ºC for 10 minutes to precipitate the leptospires. The leptospiral pellets were inoculated in EMJH media and then incubated at 29 ºC for 4 weeks. Viable leptospires were counted under dark-field microscopy with a Petroff-Hausser counting chamber.

Determination of co-localization of *Leptospira* and lysosome by confocal microscopy

RAW264.7 and THP-1 cells ($3x10^5$) were seeded on a glass coverslip each well in 24-well plates and then infected with *L. interrogans* at MOI of 1:100 at 37ºC for 1 hour, then the cells were incubated for 1 hour or 8 hours, and fixed with acetone at 30 ºC for 5 minutes before washing three times in 2xPBS, and then permeabilized with cold methanol at -20ºC for 10 minutes before washing three times in 2xPBS. The cell samples were primarily labeled with 1:500 diluted mouse antiserum against *L. interrogans* serovar Pomona, 1:200 rabbit LAMP-2a (Abcam, Englang) at 30 ºC for 1 hour before washing three times in 2xPBS, the samples were secondarily labeled with 1:60 fluorescein isothiocynate (FITC)-conjugated goat anti-mouse IgG (Sigma, USA), 1:2000 diluted Alexa Fluor® 555-conjugated goat anti-rabbit IgG (H+L), $F(ab')_2$ (Cell Signaling Technology, USA) at 30ºC for 1 hour before washing three times in 2xPBS. Finally, the samples were stained with 1:1,000 dilution of 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma aldrich, USA) at 30ºC for 5 minutes. The prepared cells were mounted with mounting medium, and then examined under a confocal microscope (Olympus).

Determination of ultrastructural alterations with transmission electron microscopy

After the RAW264.7 and THP-1 cells were infected with *L. interrogans* at 37ºC for 1 hour, the cultures were washed 3 times with PBS and then 50 µg/ml of gentamicin. Then, the cells collected at 4 hour only were fixed with 3% glutaradehyde in 0.1M phosphate buffer, pH 7.3, at 4ºC for 2 hours, then washed 3 times for 10 minutes each with 0.1M phosphate buffer before postfixing in 1% osmium tetroxide 0.1M phosphate buffer, pH 7.3, at room temperature for 1 hour. Next, the cells were washed twice for 5 minutes each with distilled water and dehydrated sequentially with 50% alcohol, 70% alcohol, 85% alcohol, 95% alcohol and absolute alcohol. Cells were embedded in Epon, cut with a diamond knife, and collected on 100 mesh nickel grids. The cell sections were

observed under transmission electron microscopy (TEM) to visualize the leptospires and autophagosomes in macrophages.

Toll-like receptor signaling in macrophage links the autophagy pathway

RAW264.7 and THP-1 cells ($3x10^5$) were seeded on glass coverslips each well in 24-well plates and treated with 5 μg/ml of rabbit anti-TLR 4, or 5 μg/ml of rabbit IgG (control) at 37ºC for 1 hour, then infected with *L. interrogans* at MOI of 1:100 at 37ºC for 1 hour. After 1 hour or 8 hours incubation, the cultures were washed twice with PBS and centrifuged at 1,500xg for 5 minutes at 4ºC and then analyzed using 12% SDS-PAGE and Western blot as described earlier.

Statistical analysis

Results are expressed as means ± SD. The one-way ANOVA analysis of variance with Tukey–Kramer's post-hoc test was used to determine the statistical significance for all pairwise multiple-comparison procedures. A P-value of $p<0.05$ was considered as statistical significance. Data were analyzed and plotted as graphs using GraphPad Prism software.

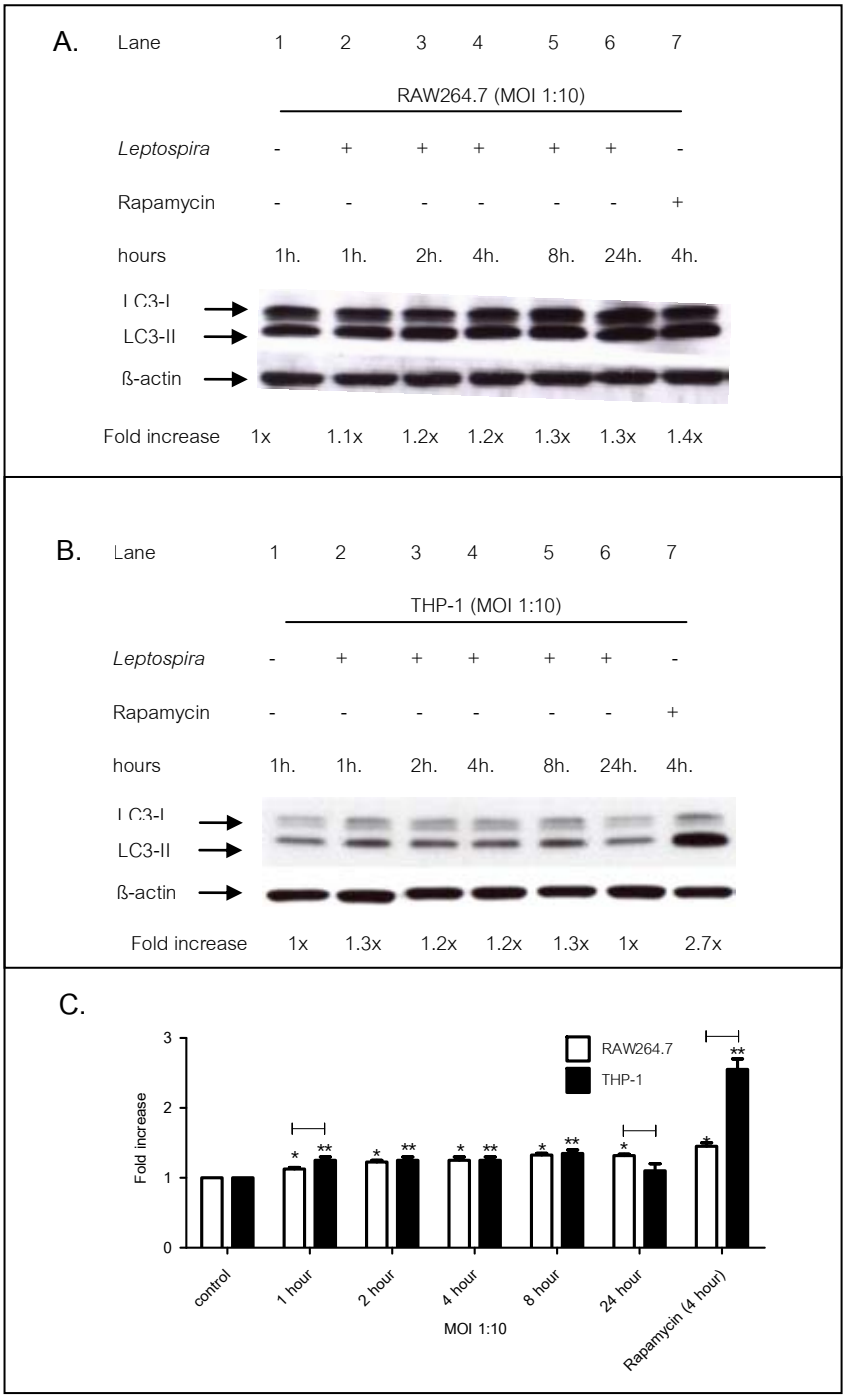
CHAPTER V
RESULTS

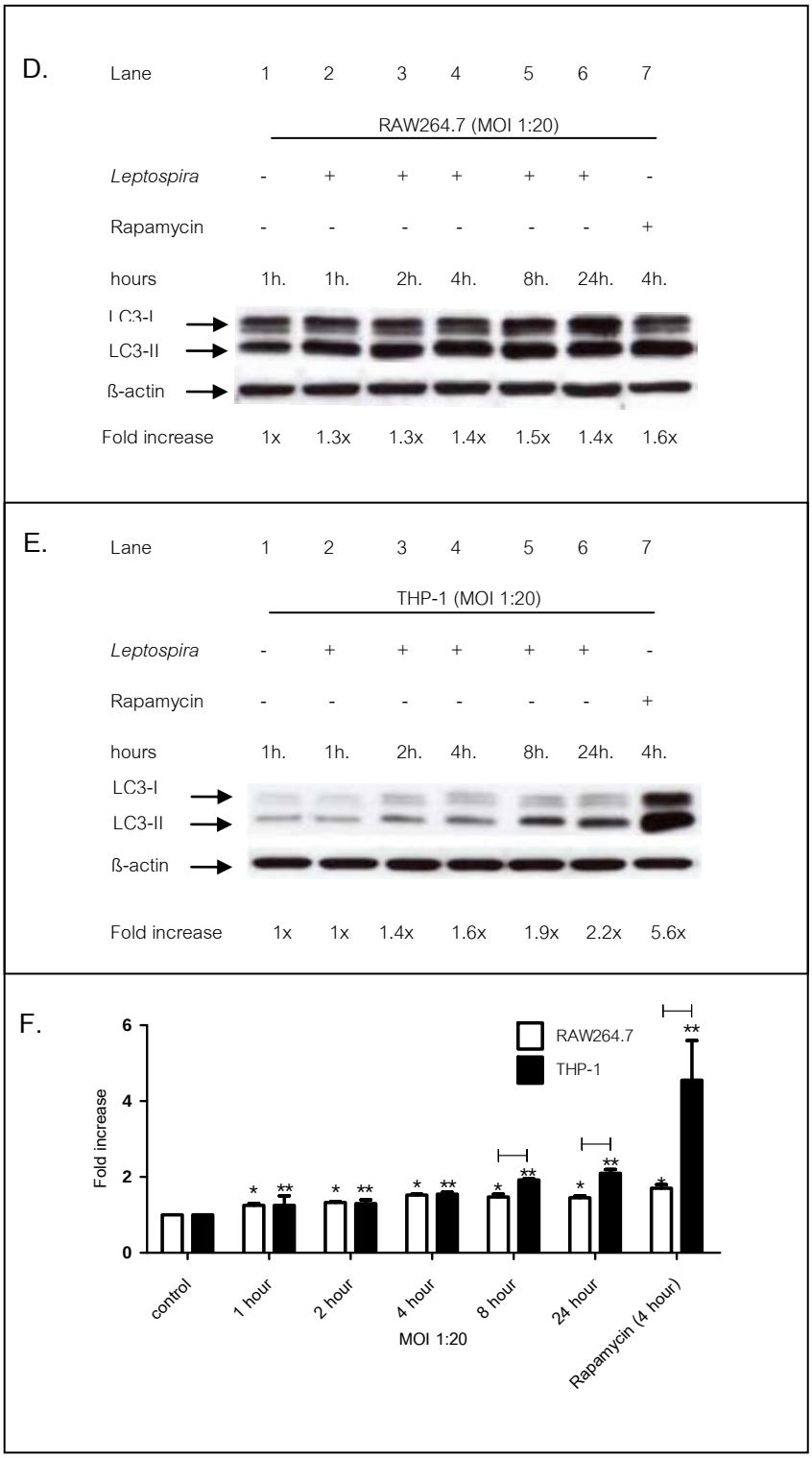
Autophagy in human and murine macrophages induced by pathogenic *Leptospira* was investigated and compared in this study

Detection of LC3II in murine and human macrophages infected with pathogenic *Leptospira*

To test if pathogenic *Leptospira* infection induced autophagy in murine macrophages (RAW264.7) and human macrophages (THP-1), we examined microtubule-associated protein light-chain 3 (LC3), an essential component and a well characterized marker of the autophagy system. There are two forms of LC3, which can be differentiated by their mobility on SDS-PAGE. LC3-II is directly conjugated to phosphatidylethanolamine on the membrane of forming autophagosomes and has a faster electrophoretic mobility than that of unconjugated LC3-I, which is localized in the cytosol. It has been shown that LC3-II formation is corresponding to an increase in autophagy [1]. Following infection of RAW264.7 and THP-1 cells with pathogenic *Leptospira* at different multiplicity of infection (MOI), the conjugated LC3-II form was significantly higher than that of uninfected cell control at 1, 2, 4, 8, and 24 hours post infection (p.i.) (Figure 14A, 14B, 14D, 14E, 14G, 14H; lane 2-6). At MOI of 1:10 , the fold increase of LC3-II in THP-1 cells was significantly higher than that of RAW264.7 cells at 1 hour p.i. but was less than that of RAW264.7 cells at 24 hours p.i. (Figure 14A, 14B, and, 14C). At MOI 1:20, the increment of LC3-II in THP-1 cells was significantly higher than that of RAW264.7 cells at 8 and 24 hours p.i. (Figure 14D, 14E, and, 14F). However, after 1, 4, and 8 hours of leptospiral infection at MOI 1:100, LC3-II in RAW264.7 cells was increased at a significantly higher level than that in THP-1 cells (Figure 14G, 14H, and, 14I). These findings suggest that pathogenic *Leptospira* can induce autophagy in both murine and human macrophages with different kinetic patterns. In response to rapamycin treatment, an autophagy inducer, a strong signal of LC3-II was observed

indicating autophagosome formation (lane 7 of Figure 14A, 14B, 14D, 14E, 14G, 14H) and therefore was served as a positive control. Notably, rapamycin was able to induce LC3-II in THP-1 cells better than that in RAW264.7 cells.




D.
Lane 1 2 3 4 5 6 7
RAW264.7 (MOI 1:20)
Leptospira - + + + + + -
Rapamycin - - - - - - +
hours 1h. 1h. 2h. 4h. 8h. 24h. 4h.
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.3x 1.3x 1.4x 1.5x 1.4x 1.6x
E.
Lane 1 2 3 4 5 6 7
THP-1 (MOI 1:20)
Leptospira - + + + + + -
Rapamycin - - - - - - +
hours 1h. 1h. 2h. 4h. 8h. 24h. 4h.
LC3-I
LC3-II
ß-actin
Fold increase 1x 1x 1.4x 1.6x 1.9x 2.2x 5.6x
F.
Fold increase
RAW264.7
THP-1
control
1 hour
2 hour
4 hour
8 hour
24 hour
Rapamycin (4 hour)
MOI 1:20

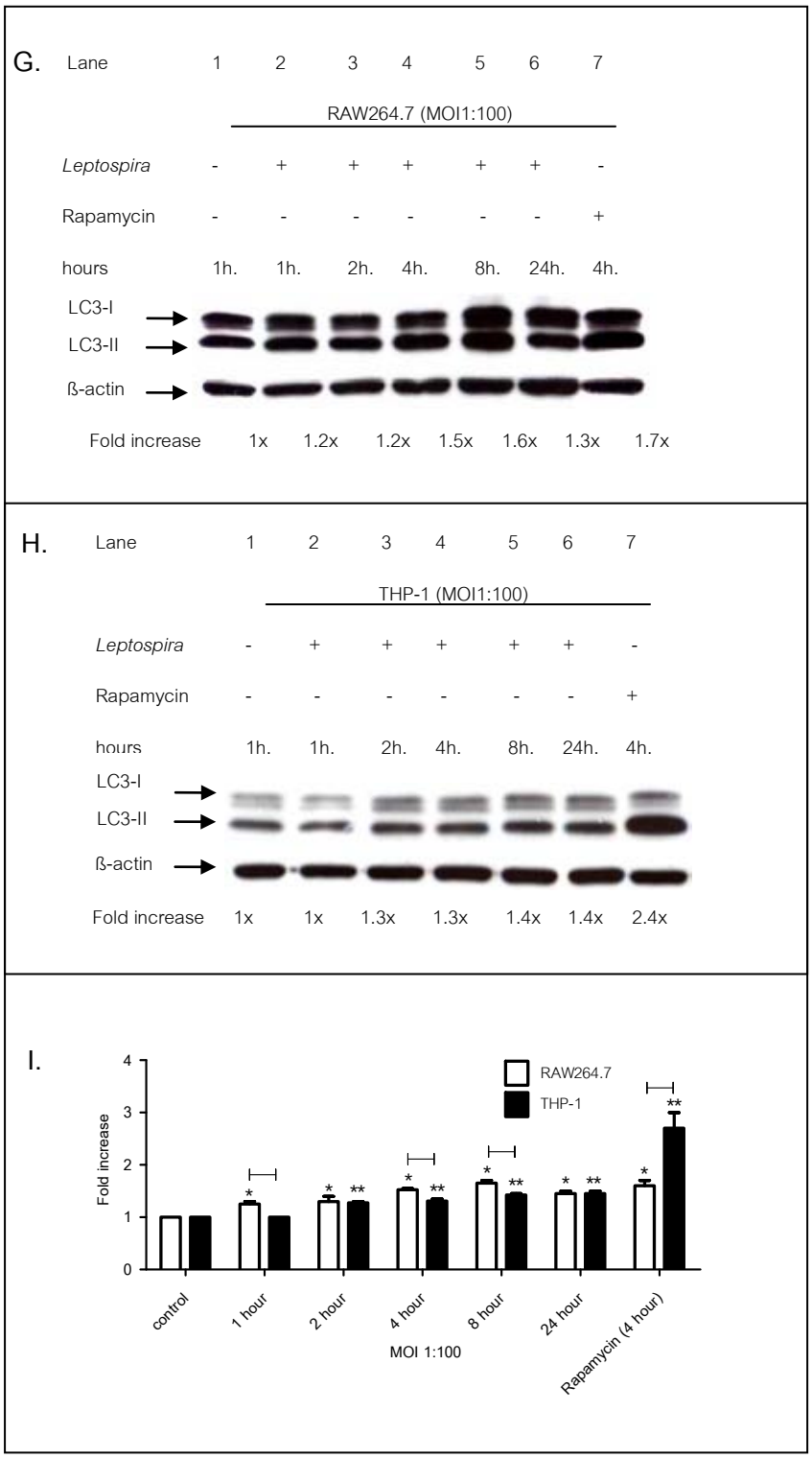

G.
Lane 1 2 3 4 5 6 7
RAW264.7 (MOI1:100)
Leptospira - + + + + + -
Rapamycin - - - - - - +
hours 1h. 1h. 2h. 4h. 8h. 24h. 4h.
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.2x 1.2x 1.5x 1.6x 1.3x 1.7x
H.
Lane 1 2 3 4 5 6 7
THP-1 (MOI1:100)
Leptospira - + + + + + -
Rapamycin - - - - - - +
hours 1h. 1h. 2h. 4h. 8h. 24h. 4h.
LC3-I
LC3-II
ß-actin
Fold increase 1x 1x 1.3x 1.3x 1.4x 1.4x 2.4x
I.
RAW264.7
THP-1
Fold increase
0
1
2
3
4
control
1 hour
2 hour
4 hour
8 hour
24 hour
Rapamycin (4 hour)
MOI 1:100

Figure 14. Detection of LC3-II conversion in pathogenic *Leptospira*-infected RAW264.7 cells (A, D, H) and THP-1 cells (B, E, H). RAW264.7 and THP-1 cells were infected with pathogenic *Leptospira* at MOI of 1:10 (A-C), 1:20 (D-F), and 1:100 (G-I), or treated with 200 nM rapamycin (control) for 4 hour. The extracellular bacteria were killed with gentamycin. At 1, 2, 4, 8 and 24 hour post infection, infected cells were collected for analyses by Western blot to detect autophagic markers. The result was reported as fold increase of LC3-II intensity of each sample (lane 2-6) in comparison with LC3-II in uninfected cell control (lane 1) as shown in the bottom line (Figure 14A, 14B, 14D, 14E, 14G, 14H) and bar graphs (Figure 14C, 14F, 14I); open bars represent fold increase in RAW264.7 cells, solid bars represent fold increase in THP-1 cells, $*P$ value < 0.05, pathogenic *Leptospira*-infected RAW264.7 cells in comparison with uninfected cell control ; $**P$ value < 0.05, pathogenic *Leptospira*-infected THP-1 cells in comparison with uninfected cell control; and $\vdash P$ value < 0.05, pathogenic *Leptospira*-infected RAW264.7 cells in comparison with infected THP-1 cells.

Visualization of autophagosome in pathogenic *Leptospira*-infected murine and human macrophages by transmission electron microscopy (TEM)

The formation of pathogenic *Leptospira*-associated autophagosome, whose characteristics is a double-membrane vesicle, in RAW264.7 and THP-1 cells were observed under TEM. The double-membrane compartment containing pathogenic *Leptospira* was found at MOI of 100 for 4 hours p.i. in RAW264.7 cells (Figure 15A). However, the leptospires resided within a single-membrane vacuole (Figure 15B) or lived freely and multiplied in the cytosol, with no surrounding vesicular membrane (Figure 15C) in THP-1 cells at 4 hours p.i. This finding suggests that double-membrane autophagosomes are formed after pathogenic *Leptospira* infection in RAW264.7 cells. However, leptospires were associated with single-membrane compartment or evade out the vesicle into the cytosol in THP-1 cells.

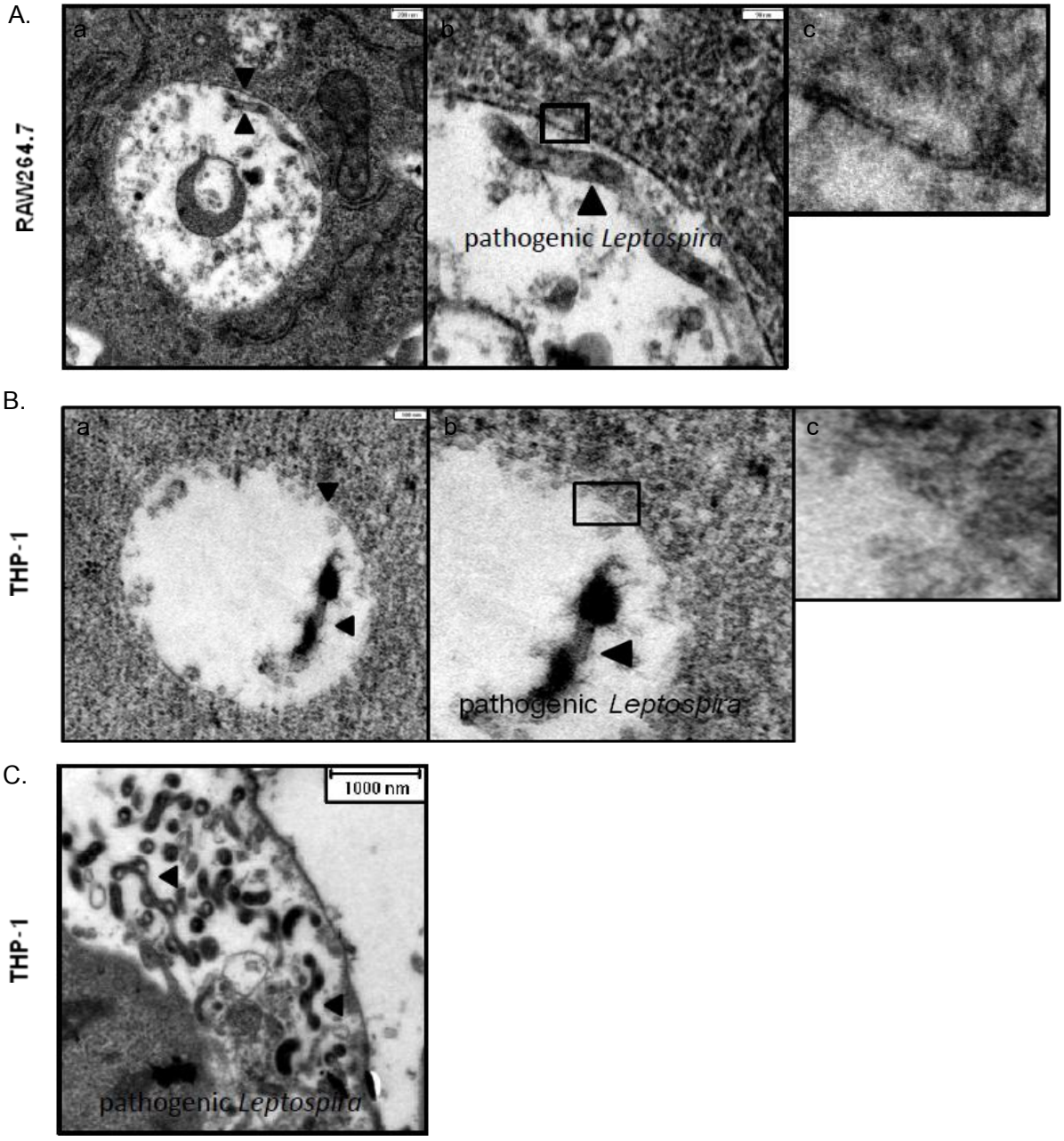

A.
RAW264.7
a
b
c
pathogenic Leptospira
B.
THP-1
a
b
c
pathogenic Leptospira
C.
THP-1
1000 nm
pathogenic Leptospira

Figure 15. Ultrastructural alterations of pathogenic *Leptospira*-infected RAW264.7 (A) and THP-1 cells (B and C). RAW264.7 and THP-1 cells were infected with pathogenic *Leptospira* at MOI of 100 for 1 hour. The extracellular bacteria were killed with gentamycin. At 4 hour post infection, infected cells were collected for TEM examination. (A) RAW264.7 cell contained pathogenic *Leptospira* within a double-membrane vesicle at 4 hours p.i. The enlarged pictures in 'b' and 'c' were derived from the square of 'a'. The arrow indicates spiral-shaped pathogenic *Leptospira* while the triangle indicates double-membrane formation. (B and C) shows the location of pathogenic *Leptospira* in THP-1 cells at 4 hours. The enlarged pictures in 'b' and 'c' were derived from the square of 'a'. (B) shows leptospires resided within a single-membrane vacuole. (C) shows dividing leptospires lived freely in the cytosol.

Determination of location of pathogenic *Leptospira* in murine and human macrophages by confocal microscopy

RAW264.7 and THP-1 cells were incubated with pathogenic leptospires at MOI of 100 for 1 and 8 hours. Infected cells were treated with anti-*Leptospira* antibody, DAPI, and anti-LAMP2a antibody, which stained pathogenic leptospires, nucleus, and lysosome, respectively before visualization under confocal microscopy to determine co-localization of pathogenic *Leptospira* with lysosome in RAW264.7 cells (Figure 16A) and THP-1 cells (Figure 16B). Pathogenic *Leptospira* were found to reside in the cytoplasm of RAW264.7 and THP-1 cells. Co-localization of pathogenic *Leptospira* with lysosomes in the RAW264.7 cells was observed at 1 hour and 8 hours p.i. but was not detected in THP-1 cells at 8 hour p.i. (Figure 16A, B). This result suggests that pathogenic *Leptospira* were associated with lysosomes, possibly autophagolysosome, in murine macrophages at least up to 8 hours of infection. However, leptospires may be able to dissociate or escape from lysosome-containing compartment including autophagolysosome before 8 hours of infection.

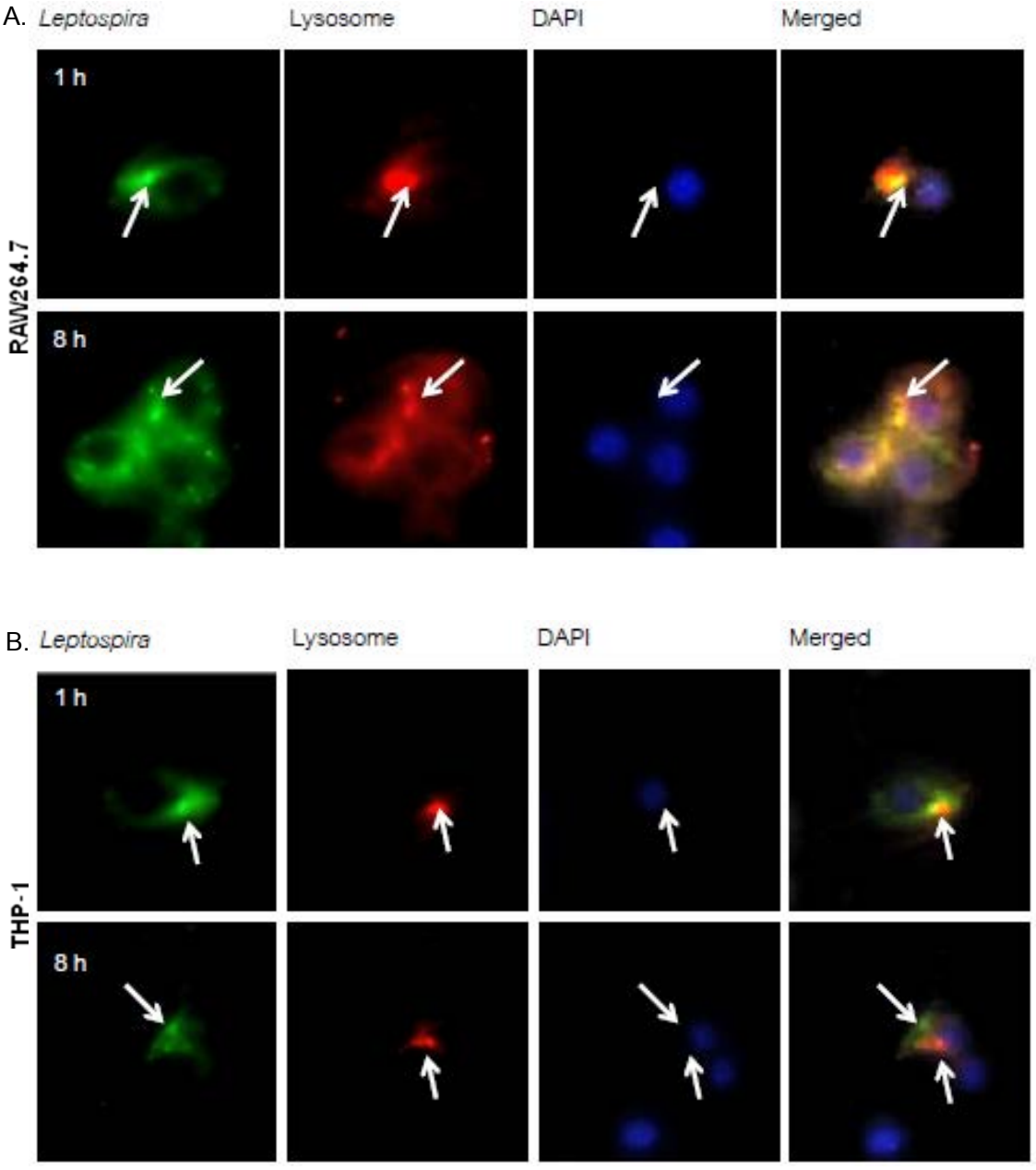
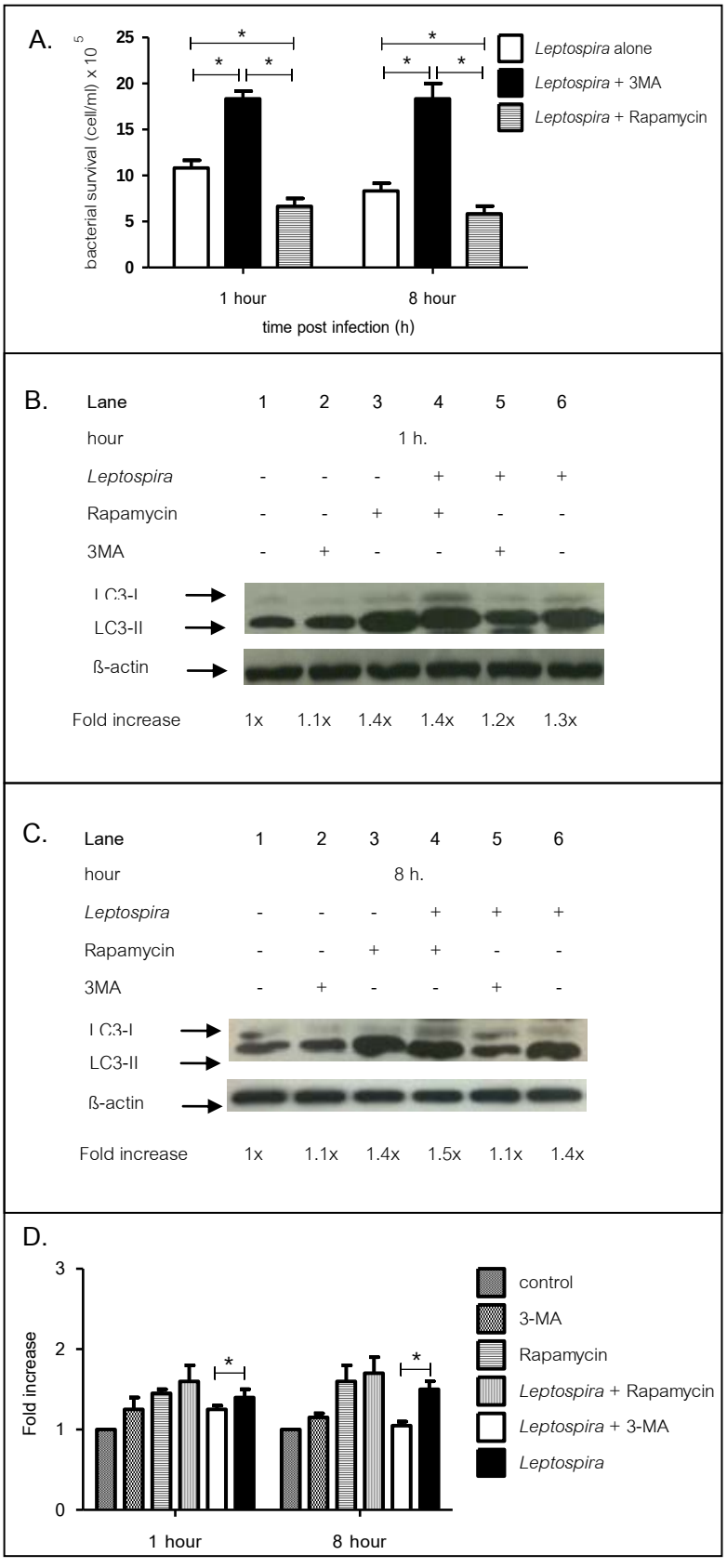

A. Leptospira
Lysosome
DAPI
Merged
1 h
8 h
RAW264.7
B. Leptospira
Lysosome
DAPI
Merged
1 h
8 h
THP-1

Figure 16. Co-localization of pathogenic *Leptospira* in the cytoplasm of RAW264.7 (A) and THP-1 cells (B). RAW264.7 and THP-1 cells were infected with pathogenic *Leptospira* at MOI of 100. The extracellular leptospires were killed by gentamycin. At 1 hour and 8 hours post infection, the infected RAW264.7 and THP-1 cells were stained with anti-*Leptospira* and anti-LAMP2a antibodies and DAPI. The arrow indicates the location of lysosome and pathogenic *Leptospira* in RAW264.7 and THP-1 cells.

Viability of pathogenic *Leptospira* in murine and human macrophages

3-methyladenine (3-MA), an autophagy inhibitor, and rapamycin, an autophagy stimulator, were used to evaluate their effect on autophagy formation and viability of pathogenic *Leptospira* in macrophages. In RAW264.7 cells, more viable pathogenic *Leptospira* were observed after treatment with 3-MA at 1 and 8 hours p.i. (Figure 17A-D). On the contrary, rapamycin reduced the survival of pathogenic *Leptospira* after treatment at 1 and 8 hours p.i. However, in the human macrophage cell line (THP-1), the clearance of pathogenic *Leptospira* was not affected by 3-MA treatment, but was enhanced by rapamycin (Figure 17E-H). The observation suggests that autophagy process seems to participate in the killing of pathogenic *Leptospira* in murine macrophages but is not the major mechanism in the clearance of pathogenic *Leptospira* in human macrophages. In addition, pathogenic leptospires were able to survive better in human macrophages than in murine macrophages.


A.
bacterial survival (cell/ml) x 10^5
25
20
15
10
5
0
1 hour
8 hour
time post infection (h)
Leptospira alone
Leptospira + 3MA
Leptospira + Rapamycin
B.
Lane 1 2 3 4 5 6
hour 1 h.
Leptospira - - - + + +
Rapamycin - - + + - -
3MA - + - - + -
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.1x 1.4x 1.4x 1.2x 1.3x
C.
Lane 1 2 3 4 5 6
hour 8 h.
Leptospira - - - + + +
Rapamycin - - + + - -
3MA - + - - + -
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.1x 1.4x 1.5x 1.1x 1.4x
D.
Fold increase
3
2
1
0
1 hour
8 hour
control
3-MA
Rapamycin
Leptospira + Rapamycin
Leptospira + 3-MA
Leptospira

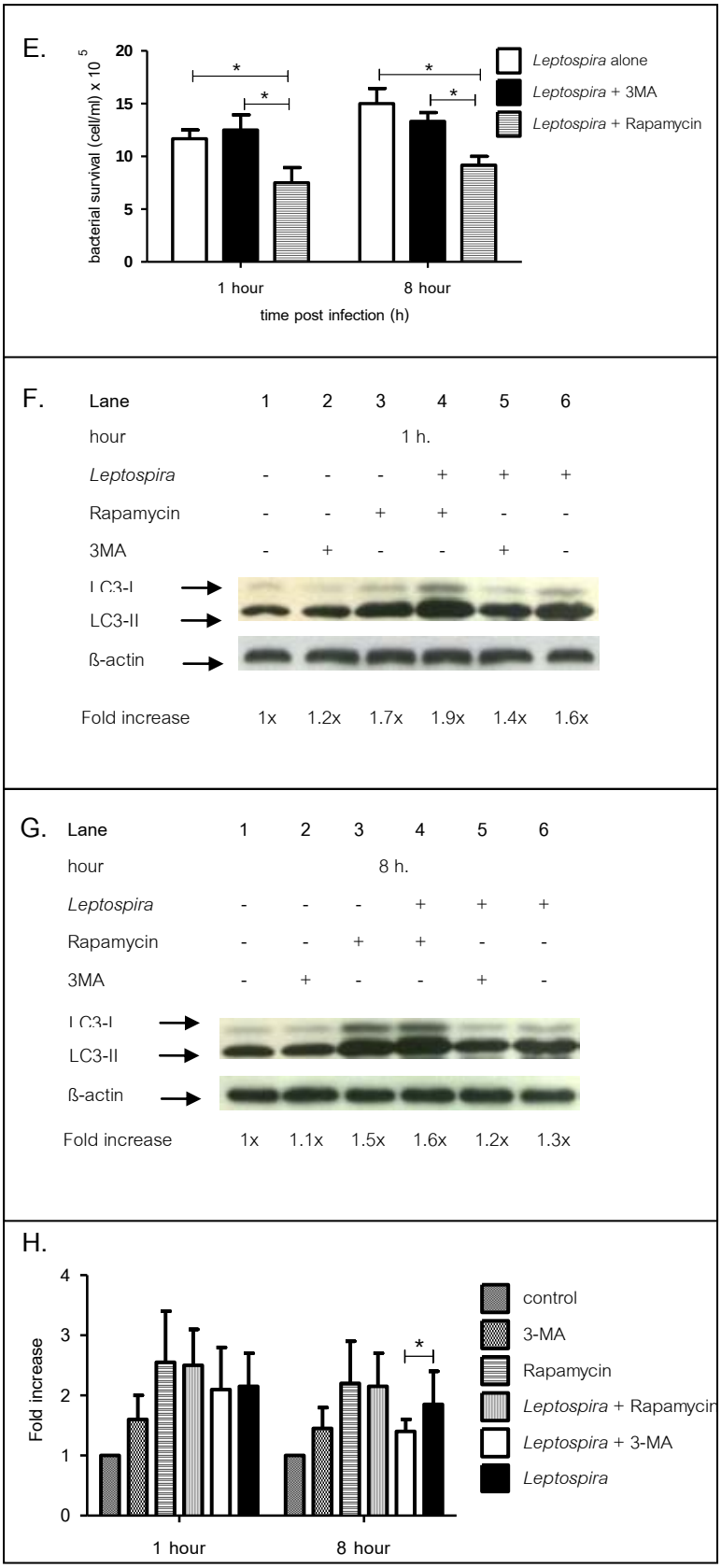

E.
bacterial survival (cell/ml) x 10 5
20
15
10
5
0
1 hour
8 hour
time post infection (h)
Leptospira alone
Leptospira + 3MA
Leptospira + Rapamycin
F.
Lane 1 2 3 4 5 6
hour 1 h.
Leptospira - - - + + +
Rapamycin - - + + - -
3MA - + - - + -
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.2x 1.7x 1.9x 1.4x 1.6x
G.
Lane 1 2 3 4 5 6
hour 8 h.
Leptospira - - - + + +
Rapamycin - - + + - -
3MA - + - - + -
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.1x 1.5x 1.6x 1.2x 1.3x
H.
Fold increase
4
3
2
1
0
1 hour
8 hour
control
3-MA
Rapamycin
Leptospira + Rapamycin
Leptospira + 3-MA
Leptospira

Figure 17. The effects of 3-MA and rapamycin on the viability of pathogenic *Leptospira* in RAW264.7 cells (A-D) and THP-1 cells (E-H). RAW264.7 and THP-1 cells were infected with pathogenic *Leptospira* at MOI of 100 for 1 hour. After infection and gentamycin treatment, infected RAW264.7 and THP-1 cells were then treated with *Leptospira* alone, 5 mM 3-MA or 200 nM rapamycin with complete media. The internal pathogenic *Leptospira* was quantitated after lysis of the macrophages at 1 and 8 hours post infection and followed their growth in EMJH by counting viable leptospires every 2 weeks. The recovered viable pathogenic *Leptospira* were reported as bacteria survival (cell/ml) at 4 week culture. * P value < 0.05.

Toll-like receptor-related autophagy in murine macrophages

Toll-like receptor signaling was determined whether it was associated with autophagosome formation. Since 3-MA had the effect on autophagy formation only in murine macrophages in earlier study (Figure 17), only RAW264.7 cells were used in this experiment. Anti-TLR4 antibody was used to block TLR4, before cells were infected with pathogenic *Leptospira* and then autophagy process was determined by detection of LC3-II. Previous incubation with anti-TLR4 was shown to reduce LC3-II at 1 and 8 hour p.i. (Figure 18A and 18 B, lane 4), when compared with untreated cells (Figure 18A and 18B, lane 6). In addition, anti-rabbit IgG, a mock control, had no effect on LC3-II (Figure 18A and 18B, lane 5). These finding suggests that TLR4 may contribute to the occurrence of autophagy after pathogenic *Leptospira* infection in murine macrophages.

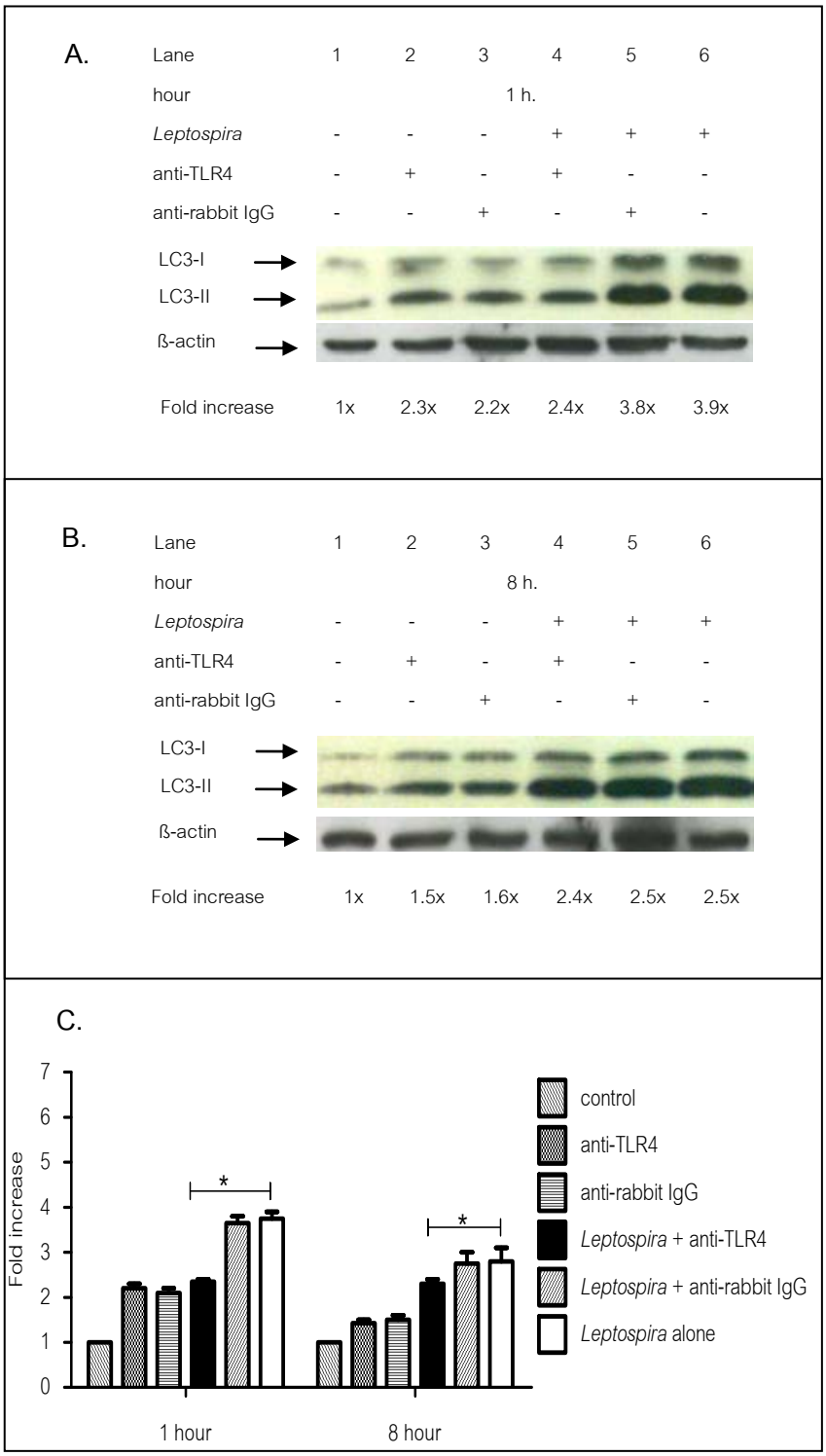

A.
Lane 1 2 3 4 5 6
hour 1 h.
Leptospira - - - + + +
anti-TLR4 - + - + - -
anti-rabbit IgG - - + - + -
LC3-I
LC3-II
ß-actin
Fold increase 1x 2.3x 2.2x 2.4x 3.8x 3.9x
B.
Lane 1 2 3 4 5 6
hour 8 h.
Leptospira - - - + + +
anti-TLR4 - + - + - -
anti-rabbit IgG - - + - + -
LC3-I
LC3-II
ß-actin
Fold increase 1x 1.5x 1.6x 2.4x 2.5x 2.5x
C.
Fold increase
0 1 2 3 4 5 6 7
*
*
1 hour
8 hour
control
anti-TLR4
anti-rabbit IgG
Leptospira + anti-TLR4
Leptospira + anti-rabbit IgG
Leptospira alone

Figure 18 TLR4-induced LC3-II conversion in pathogenic *Leptospira*-infected RAW264.7 cells (A-B). RAW264.7 were incubated with 5 μg/ml of rabbit anti-TLR 4 antibody, or 5 μg/ml of rabbit IgG (control) for 1 hour, and then infected with pathogenic *Leptospira* at MOI of 100 for 1 hour. The extracellular bacteria were killed with gentamycin. At 1 hour or 8 hours post infection, infected cells were collected for analyses by Western blot to detect autophagic markers. The result was reported as fold increase of LC3-II intensity in comparison with uninfected cell control (lane 1) as shown in the bottom line (A and B) and bar graphs (C). *P value < 0.05

CHAPTER VI
DISCUSSIONS

Autophagy has been found to be a crucial component of innate cellular immune responses against not only intracellular but also extracellular microorganisms [1-3]. After phagocytosis of microorganism, autophagosomes are formed to degrade the ingested bacterium by the lysosomal killing mechanism. However, intracellular bacteria including *Legionella pneumophila*, *Listeria monocytogenes*, and *Mycobacterium* have developed different mechanisms to evade the autophagic cellular surveillance in macrophages [4-6]. Although the double membrane-bound compartments bearing autophagic markers were induced, its maturation into autolysosomes was shown to be arrested or delayed. Evasion of the autophagy by microorganism favors its own replication and survival in the host [7, 8].

Pathogenic leptospires have been considered to be extracellular pathogens. However, previous studies showed that pathogenic leptospires may temporary reside within host cells such as Vero cells [9]. In this study pathogenic *Leptospira* was shown to induce LC3, which is an essential component and a well characterized marker of the autophagy system, in human and murine macrophages after brief contact, and subsequently entered the host cells. This finding suggested that pathogenic *Leptospira* infection may induce autophagy in both murine and human macrophages with different kinetic patterns. Different effects of leptospires on the level of LC3-II in either cell type may depend on various factors such as multiplicity of infection (MOI), time period of infection, and number of infected pathogenic *Leptospira* allowed into the cells. In addition, baseline level of LC3 was distinct between murine and human macrophages.

The entry into host cells, as for the pathogens such as *Shigella*, and *Salmonella*, is an essential step during infection [10]. Most intracellular bacteria enter into the host cells through a pathway in which phagocytic vesicles are formed [11, 12]. After that, the phagosomes fuse with lysosomes and the bacteria are killed by lysosomal hydrolases [11, 12]. Many intracellular bacteria, including *Salmonella, Shigella*, and *L. monocytogenes*, were shown to escape from the phagosomes into the cytosol, where they then replicated and survived [13]. In the present study, leptospires were shown by TEM to be located with double-membrane bound vesicles, a characteristic of autophagosome, in murine macrophages, whereas the leptospires were distributed in the single-membrane bound vacuoles, a characteristic of autophagolysosome, and/or in the cytosol of human macrophages (Figure 15B and C). This finding may be associated with better survival of leptospires in human macrophages than in murine macrophages. It is possible that in human macrophages pathogenic *Leptospira* are able to resist killing in autophagolysosome or escape out of the autophagosome into the cytosol prior to fusion with lysosomes. In addition, study on co-localization of pathogenic leptospires and lysosome under confocal microscopy found that in contrast to pathogenic *Leptospira* in murine macrophages, leptospires in human macrophages were dissociated from lysosomes at 8 hours p.i. (Figure 16). In addition, 3-MA treatment had lower effect on the clearance of pathogenic *Leptospira* in human macrophages than that in murine macrophages (Figure 17) possibly due to ability of leptospires to evade autophagosome in human macrophages. These findings support the idea that pathogenic leptospires may evade from autophagosome in human macrophages before fusion with lysosomes leading to their better survival in human macrophages than those in murine macrophages. Therefore, the fate of intracellular leptospires is different in murine and human macrophages, which may be helpful in understanding the difference in severity of leptospirosis between humans and mice.

In *E. coli*, LPS has been shown to specifically induce autophagy by signaling through TLR4 [14], and recent data indicate that signaling via any of the TLRs can induce autophagy [15, 16]. In the present study, we found that in RAW264.7 cells pathogenic *Leptospira* may induce autophagosome formation via TLR4 since anti-TLR4 was observed to reduce LC3-II level in murine macrophages at 1 and 8 hour p.i. by Western blot analysis. These data suggest that pathogenic *Leptospira* infection may induce autophagy in murine macrophages via TLR4. .

CHAPTER VII
SUMMARY

The aim of this study is to determine autophagy induction by pathogenic *Leptospira* in infected human and murine macrophages. Pathogenic *Leptospira* were shown to be able to induce autophagy in both murine macrophages and human macrophages. LC3-II was induced in murine and human macrophages with different kinetic patterns depending on multiplicity of infection and time period of infection. Double-membrane autophagosomes were observed under transmission electron microscope in the murine macrophages after infected with pathogenic *Leptospira*. However, leptospires were associated with single membrane compartment or evaded into the cytosol in human macrophages. This finding was consistent with the result shown in co-localization study by confocal microscopy that pathogenic leptospires was dissociated from lysosomes at 8 hours post infection in human macrophages. The survival of pathogenic *Leptospira* after an autophagy inhibitor, 3-MA, treatment in murine macrophages suggests that autophagosome formation seems to participate in the clearance of pathogenic *Leptospira* in murine macrophages. However, autophagosome was not a major mechanism in the clearance of pathogenic *Leptospira* in human macrophages. In addition, TLR4 may contribute to induction of autophagy after pathogenic *Leptospira* infection in murine macrophages. It is possible that pathogenic leptospires are able to evade into the cytosol of human macrophages prior to fusion with lysosomes leading to better survival in the cells. This finding may explain the difference in pathogenicity of leptospires in different hosts.

Further investigations are required to confirm the evasion of pathogenic leptospires from autophagosomes in human macrophages and identify their virulence factors responsible for this mechanism.

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APPENDICES

APPENDIX A
BUFFER AND REAGENTS

Reagent for EMJH media

1. Albumin fatty acid supplement stock solution

$CaCl_2$+ $MgCl_2$.6 H_2O	0.076	g	Store at -20 ºC
$ZnSO_4$.7H_2O	0.04	g	Store at -20 ºC
$CuSO_4$. 5H_2O	0.03	g	Store at 4 ºC
Vit B12	0.002	g	Store at -20 ºC
Tween 80	1	g	Store at -20 ºC
Glycerol	1	g	Store at -20 ºC

Dissolve each reagent separately in 10 ml of distilled water.

2. Albumin fatty acid supplement solution, ready to use (50ml)

BSA	5	g
$CaCl_2$+ $MgCl_2$.6 H_2O	750	µl
ZnSO4.7H2O	500	µl
$CuSO_4$. 5H2O	50	µl
Vit B12	500	µl
Tween 80	6.25	ml
Glycerol	500	µl
$FeSO_4$	0.025	g
Sodium pyruvate	0.02	g

Dissolve in distilled water and adjust pH 7.4-7.6 with HCl(conc.). Adjust volume with distilled water to make 50 ml. Sterilize the solution by filtration. Store at -20 ºC.

3. Basal Media

Bacto Leptospira Media Base EMJH dehydrated 0.23 g

Dissolve in distilled water and adjust volume with distilled water to make 90 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

4. EMJH media

Basal media	90	ml
Albumin fatty acid supplement solution	10	ml

Mix the solution and store at 4 ºC.

Reagent for SDS- Polyacrylamide Gel Ectrophoresis (SDS-PAGE)

1. 1 M Tris-HCl pH 8.8

Tris base	12.11	g

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

2. 0.5 M Tris-HCl pH 6.8

Tris base	6.055	g

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc). Adjust volume with distilled water to make 100 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

3. 2X Laemmli buffer (SDS-dye) 10 ml

1M Tris-HCl pH 6.8	1	ml(final concentration 100 mM)
10% SDS	4	ml(4%v/v)
99.5% glycerol	2.01	ml (20% v/v)
HPLC water	2.989	ml
Bromphenol blue	0.001	g

Mix the reagent vigorously, and store at -20 ºC.

4. 4X Tris HCl/SDS pH 8.8 (100 ml)

This base	18.21	g
SDS	0.4	g

Dissolve in distilled water and adjust pH to 8.8 with HCl (conc). Stored at 4 ºC.

5. Running buffer

This base	15.1	g
Glycine	72	g
SDS	5.0	g

Dissolve in distilled water and adjust volume to 10 ml. Store at room temperature.

6. 6X sample buffer with DTT

4X Tris-HCl pH 8.8	7	ml
Glycerol	3	ml
SDS	1	g
DTT	0.93	g
Bromphenol Blue	1.2	mg

Dissolve in distilled water and adjust volume to 10 ml. Store at room temperature.

7. 10% Ammonium Persulfate (APS)

APS	1	g
Distilled water	10	ml

Mix the solution and store at -20 ºC

8. 10% Sodium laury sacosine (SDS)

Sodium laury sacosine (SDS)	1	g
Distilled water	10	ml

Mix the solution and store at -20 ºC.

9. 30% Acrymide/0.8 % Bisacrylamide

Acrylamide	30	g
Bis-acrylamide	0.8	g

Dissolve in distilled water and adjust volume to 10 ml. Sterilize the solution by filtration. Store in dark at room temperature.

10. 12% SDS-PAGE

Separating gel (15ml)

Distilled water	4	ml
1.5 M Tris-Hcl pH 8.8	3.75	ml
40% Acrylamide and Bis-acrylamide solution	6	ml
10% SDS	0.15	ml
10% APS	75	µl
TEMED	7.5	µl

Stacking gel

Distilled water	2.7	ml
1.5 M Tris-HCl pH 6.8	0.5	ml
40% Acrylamide and Bis-acrylamide solution	0.67	ml
10% SDS	40	µl
10% APS	40	µl
TEMED	4.0	µl

Reagent for Western blot

1. TBS

1 M Tris base pH 7.5	20	ml
NaCl	29.22	g

Dissolve in distilled water and adjust volume to 1,000 ml. Sterilize the solution by autoclaving at 121 ºC for 15 minute.

2. TBS-0.1% (v/v) Tween

TBS	500	ml
Tween-20	500	µl

Mix the solution and store at room temperature.

3. Blotting buffer

Tris base	2.42	g
Glycine	11.24	g
Distilled water	800	ml

Dissolve in distilled water and add 200 ml methanol. Store at room temperature.

4. Blocking solution

PBST	200	ml
Non-fat dry milk	3	%

Mix the solution and store at 4 Cº.

5. 5X Running buffer

This-base	15.2	g
Glycine	94	g
SDS	5	g
Deionized water	1000	ml

Mix the solution and store at room temperature.

6. Transfer buffer for Western blot

This-base	5.08	g
Glycine	2.9	g
SDS	0.37	g
Deionized water	800	ml
Absolute methanol	200	ml

7. ECL substrate of HRP

Coumaric acid (90 mM) was diddolved in DMSO in total volume 10 ml. Then, the solution aliquots were keep at -20 ºC.

Luminol (250mM) was diddolved in DMSO in total volume 10 ml. Then, the solution aliquots were keep at -20 ºC.

Solution A

100 mM Tris-HCl pH 8.5	4	ml
(stored at 4 ºC)		

90 mM coumaric acid	17.6	µl
250 mM luminal	40	µl
Solution B		
100 mM Tris-HCl pH 8.5	4	ml
(stored at 4 ºC)		
30% H_2O_2	2.4	ml

Reagent for cell culture

1. Complete RPMI 1640 100 ml

RPMI 1640	90	%
FBS	10	%
Penicillin	100	U/ml
Streptomycin	0.4	mg/ml
Sodium pyruvate	1	%
HEPES	1	%

2. Complete DMEM 100 ml

DMEM	90	%
FBS	10	%
Penicillin	100	U/ml
Streptomycin	0.4	mg/ml
Sodium pyruvate	1	%
HEPES	1	%

3. Freezing media 10 ml

Complete media	90	%
DMSO	10	%

4. FBS inactivatation

Before using FBS, FBS must be inactivated at 56 ºC for 30 minutes using water bath.

5. RIPA buffer for protein extraction 10 ml

50mM Tris-HCl, pH 7.4	1	ml
150 mM NaCl	1.5	ml
1.0% NP-40	100	µl
0.5% $C_{24}H_{39}O_4Na$	1	ml
0.1% SDS	100	µl

Adjusted volume to 10 ml using deionized water.

6. 1XPBS pH 7.4

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
$KH_2 PO_4$	0.24	g
Deionized water	1000	ml

autoclaved at 121ºC and pressure 15 psi for 15 min.

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